Variants mining of Kappa casein (K-CN) and Prolactin (PRL) genes among four indigenous cattle breeds in Nigeria

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Abstract
The study determined the allele and genotype frequencies of genetic variants in two genes associated with milk production traits in four indigenous cattle breeds (N'dama, White Fulani, Muturu and Keteku) in Nigeria. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) technique was used to detect genetic polymorphisms in two milk genes (kappa casein (K-CN) and Prolactin (PRL)). Three genetic variants (AA, AB and BB) of *K-CN* gene were detected in White Fulani and Muturu cattle breeds among the four breeds considered in this study. While the *PRL* gene was found monomorphic in all the sampled animals as they were all genotyped as AA homozygous *PRL* genotype. The detection of genetic polymorphism in White Fulani and Muturu cattle breeds revealed the following allele frequencies of kappa-casein gene A and B estimated as 0.69, 0.31 for White Fulani; 0.56 and 0.44 for Muturu respectively. The detection of both alleles *A* and *B* as observed in these animals established a high degree of genetic variability among the four cattle breeds for *K-CN* locus. The milk genes are polymorphic in their expression and there were genetic diversities in the cattle population understudy. Also, a higher frequency of A allele in the animal sample population which has been associated with unfavorable milk production and protein yield revealed the need to breed for increased B allele among the Nigerian indigenous cattle through genomic selection. Overall, the results of this study showed that breeding to improve milk production among indigenous cattle breeds are feasible through genomic selection.

Keywords: Genes, Cattle, PCR-RFLP, frequencies, polymorphism

1 INTRODUCTION

Indigenous cattle in Nigeria serve as the backbone of relevant and sustainable cattle production because of better adaptation to survive and reproduce under harsh environments though with low performance as compared to high performing exotic counterpart breeds. However, most Nigerian indigenous cattle are better meat producer but poor or low milk producer (Ezekwe, 2001).

In the past, animal breeders have made effective efforts to improve the production performance of livestock species by altering their genome through the selection of superior parents for the next generation. This involved the direct use of phenotypic records without the knowledge of the molecular information. The outcome may improve the production while ignoring some reproductive performance of the animal. Sometimes, attempts were made through cross breeding of the indigenous animals with the exotic ones which has not been without difficulties like loss of distinctive qualities such as disease resistance, heat tolerance, ability to survive and reproduce under stress and low feed input thus posing threat to the indigenous breed. Whereas, the advent of molecular techniques has made breed characterization a possibility by determining genetic relationships among animals based on differences in their DNA. Therefore, there is need to adopt methods of selection that are based on genomic studies. (Karp et al., 1996, André, 2012).

The molecular DNA markers have been deployed for breed characterization in both plants and animals. For example, marker-assisted selection have been practiced in dairy cattle whereby some genes were identified as potential candidates which are associated with performance traits in dairy cattle (Marson et al., 2005, Hassen et al., 2007). Selection process can take in to account the variations in animal's performance that is related to the genetic polymorphism.

Reinhardt et al. (2012) stated that milk is an important source of essential nutrients for both lactating calves and human food. Studies have shown that ruminant's milk protein genes are highly polymorphic with a large number of unusual polymorphism (Nilsen et al., 2009). As far back as 1955, Aschaffenburg and Drewry have discovered variants A and B for β lactoglobulin in cattle which has become a worldwide interest in genetic polymorphisms in milk proteins. These variants are believed to be controlled by the codominant autosomal genes which are according to Mendelian inheritance. This study was corroborated with a review by Caroli et al. (2009) who also identified variants in milk protein.

Milk genetic variants are different by few amino acid substitutions or deletions within the polypeptide chain (Eigel et al. 1984, Caroli et al., 2004 and D'Alessandro et al. 2011). Milk protein genetic variability in cattle is necessary at both the DNA and protein levels for evolutionary and biodiversity analyses (Caroli et al., 2004).

The frequencies of genetic variants of milk proteins in different cattle breeds and the possible relationships with the milk production traits such as milk composition, milk quality and quantity have been widely studied due to their possible use for milk protein characterization as a mean to genomic selection (Jeichitra et al., 2003; Caroli et al., 2004; and Yasemin and Cengiz, 2006).

On the other hand, there is no available comprehensive study of allelic variants in Nigerian indigenous cattle breed up till date. A study to identify different allelic variants in

milk genes, allele frequencies and their effects in indigenous breed performance is important for improvement in Nigerian cattle breeds. Genotyping dairy animal at the molecular level is not limited to cows alone. There is possibility to genotype males, non-lactating females and embryos at the DNA level because milk protein typing does not require the gene products (Caroli et al., 2004).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based analysis is a popular technique for genotyping. Besides being valuable for the determination of intraspecies variation, the PCR-RFLP technique is very popular for species identification and differentiation(Rojas et al., 2009). For example, Hassen et al (2007) used PCR-RFLP to assess genetic variability within and among five indigenous Ethiopian cattle breeds. The analysis revealed that within breed genetic variation was much higher than that between breeds. The PCR-RFLP technique is quick, less expensive and requires less expertise which makes it to be easily adopted for molecular characterization.

The current study was designed to utilize PCR-RFLP technique to identify genetic variants in two milk genes (kappa casein (K-CN) and Prolactin (PRL) with a view to determining the allele and genotype frequencies of milk gene variants among four indigenous cattle breeds in Nigeria and to make possible recommendation for genomic selection in place of the current tradition selection methods for dairy cattle improvement in Nigeria.

2 MATERIALS AND METHODS

2.1 Animal Sampling

A total of 300 animals comprising 84 White Fulani, 78 Keteku, 62 Muturu and 76 N'dama indigenous cattle were randomly selected and genotyped. The animals were sampled from the Teaching and Research Farm, Federal University of Technology Akure (FUTA), Federal College of Agriculture, Akure (FECA) and Institute of Agricultural Research and Training (IAR&T), Ibadan.

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2.2 Genomic DNA extraction

Blood samples were collected from 300 animals for genomic DNA isolation through the jugular vein in vacutainers containing ethylene diamine tetra acetic acid (EDTA) and kept on ice to maintain low temperature in order to prevent cell lysis. Subsequently the blood samples were transported to the laboratory and stored at 4°C prior to genomic DNA isolation. Genomic DNA was extracted from whole blood using standard procedures as recommended by the manufacturer (Qiagen Inc., Valencia, CA) and the DNA was stored at 4 °C until ready for use.

2.3 Amplification of genomic DNA by Polymerase chain reaction (PCR)

Specific primer pairs (from Table 1) were used for each reaction. A final volume of 25 µl reaction mixture was prepared with 2µl of genomic DNA (50–80 ng), 12.0 µl Taq DNA polymerase master mix, 60ng of each primer, and 10 µl of nuclease free water. PCR-reactions were carried out in 0.2 ml of PCR reaction tubes using a programmable thermal cycler (BioRad, C1000 Touch™) with cycling conditions as, initial denaturation at 94◦C for 3min, denaturation at 94◦C for 30 sec, annealing at 60◦C for 35sec, and extension at 72◦C for 3min were carried out for 40 cycles followed by final extension at 72◦C for 10 min. After 40 amplification cycles, the amplified products were verified by electrophoresis on 1.5% w/v agarose gel in 1×TAE buffer with 100bp ladder for 50mins. The gels were stained with Ethidium Bromide and visualized under UV light by the gel documentation system (Enduro, Inc).

2.4 Variants identification through RFLP and agarose gel electrophoresis

In order to identify genetic variants among the sampled animals, 20 µl of PCR products were digested with 10 units of the restriction enzyme (Invitrogen, USA) which is specific for each gene (Table 1) in a final reaction volume 25 µl. The reaction mixture was incubated at 37°C in heating blocks for 5hours and fragments were separated in agarose gel. Briefly, after restriction digestion the fragments were analyzed and separated through electrophoresis in 3% agarose gel which was stained with ethidium bromide and a 100-bp ladder was used as the molecular marker. Thereafter, the gel was visualized by the UV gel documentation system (Enduro, Inc).

Table 1 Primer sequences information and restriction enzymes for kappa casein and prolactin genes

Gene symbol	5′-3′ sequence	Annealing Temperature	PCR amplicon size (bp)	GC(%)	Restriction enzyme
PRL	F: CGAGTCCTTATGAGCTTGATTCTT R: GCCTTCCAGAAGTC GTTTGTTTTC	59.00 60.82	294	55.00 52.38	*Rsal*
K-CN	F: ATA GCC AAA TAT ATC CCA ATT CAG T R: TTT ATT AAT AAG TCC ATG AAT CTT	58.00 59.97	530	60.00 52.38	*HindIII*

Table 2: Polymorphism at the K-casein locus and allele distribution in four indigenous cattle breeds in Nigeria

Breed	Genotype	Frequency	Allele frequency	x^2
N'dama	AA	100.00	A -1.00	0.72ns
	BB	-	B -	
	AB	-		
White Fulani	AA	44.12	A-0.69	2.42ns
	BB	5.88	B -0.31	
	AB	50.00		
Muturu	AA	52.00	A-0.56	0.04ns
	BB	40.10	B-0.44	
	AB	7.90		
Keteku	AA	100.00	A -1.0	0.12ns
	BB	-	B -	
	AB	-		

ns = not significant (P>0.05)

2.5 Statistical Analysis

The estimate of gene and genotypic frequencies within the population were determined by direct gene counting which was evaluated by a chi-square test to know if the population was in Hardy-Weinberg equilibrium (Soysal, 1998).

3 RESULTS AND DISCUSSION

The knowledge of molecular genetic technologies has helped in identifiying specific DNA markers which are associated with production traits in farm animals. Hence, candidate genes can be selected for analysis based on the knowledge of their relationship with productivity of a given trait. In the present study, genetic variants in kappa casein (K-CN) and Prolactin (PRL) genes among four indigenous cattle breeds in Nigeria were assessed by PCR-RFLP technique. This technique allows for the detection of changes in the nucleotide sequence of a PCR product that is due to single base substitution. The results of this technique showed good evidence for molecular markers linked to genetic variabilities among the indigenous cattle breeds.

3.1 PCR-RFLP polymorphic detection

The amplified products from each of the two milk genes gave a specific expected band size across the 300 animals with 530-bp and 294-bp for kappa casein (K-CN) and Prolactin (PRL) genes respectively (Fig. 1-2). Upon digestion of the amplified products with the appropriate restriction enzyme according to table 1, PRL gene results in one undigested fragment at 294-bp for GG genotype.

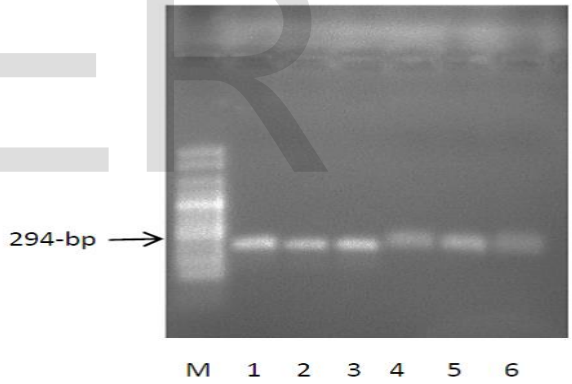

Fig. 1 Gel electrophoresis of PCR products representing amplification of PRL gene in four indigenous cattle breeds in Nigeria. Lane 1: 50-bp ladder marker. Lanes: 2–10: 294-bp PCR products amplified from sampled DNA

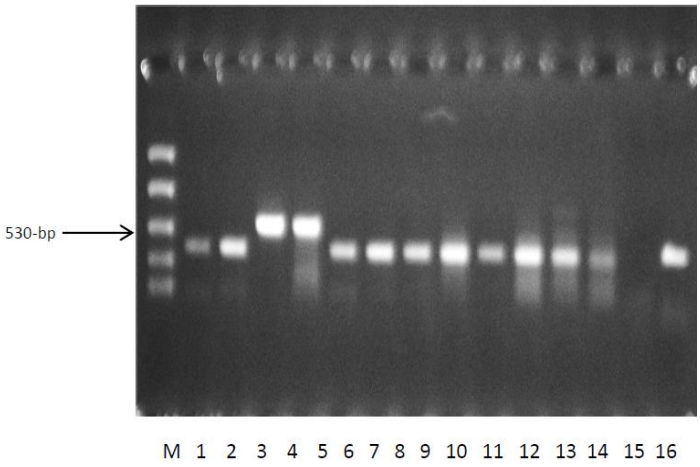

Fig. 2 Gel electrophoresis of amplified PCR products of K-CN gene in four indigenous cattle in Nigeria. Lane M: 100-bp ladder marker. Lanes 1–16: 530-bp PCR products amplified from sampled DNA.

All investigated animals in this study were genotyped as GG homozygous genotype (Fig.3). Prolactin gene is 10-kb long and was mapped to chromosome 23 in bovine. It consists of five exons and four introns. Based on the sequence analysis, the transition of G into A at position 8398 of PRL gene creates a restriction site for RsaI endonuclease (Mitra *et al*., 1995).

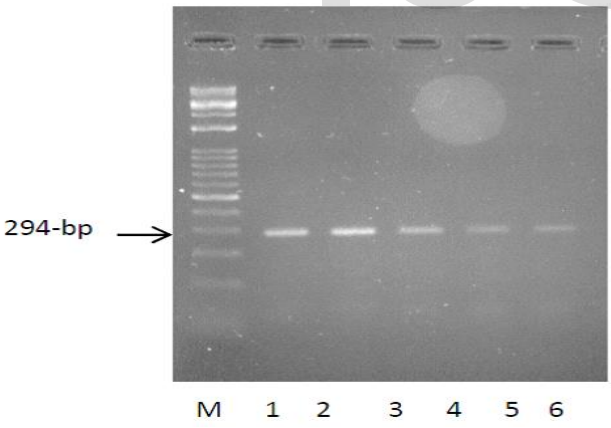

Fig. 3: The electrophoretic pattern obtained after digestion of PCR amplified buffalo PRL products with RsaI. Lane 1: 100-bp ladder marker. Lanes 2–10: Homozygous GG genotypes showed one undigested fragment at 294-b

The digestion of the 294-bp PCR amplified fragments with this restriction enzyme ought to result in two restriction fragments at 162-bp and 132-bp for AA genotype, one undigested fragment at 294-bp for GG genotype and three fragments at 294-bp, 162-bp and 132-bp for AG heterozygous genotype (Lewin *et al*., 1992). Contrariwise, the four breeds of indigenous cattle investigated in this research were genotyped as GG homozygous genotype when all tested amplified fragments were digested with RsaI endonuclease and gave one undigested fragment at 294-bp. Prolactin gene (PRL) plays an important regulatory function in mammary gland development, milk secretion, and expression of milk protein genes. Prolactin's biological activity consists of various roles in the reproduction, lactation and a number of homeostatic biological functions including immune functions. Furthermore, various studies have showed that prolactin has important functions like the development of mammary gland affecting milk yield and composition especially dairy cattle (Brym *et al*., 2005; Kumari *et al*., 2008). It is also one of the most multifunctional hormones in the body hence; the PRL gene serves as a potential genetic marker and seems to be an excellent candidate for linkage analysis of quantitative trait loci (QTL) affecting milk production traits in dairy cattle. The high frequencies of allele G observed from this study corroborate similar findings in the reports on different cattle breeds ranging from 0.61 in Brown Swiss breed (Mitra *et al*., 1995) to 0.95 in Holstein breed (Chrenek *et al*., 1998). Previous study by Mehmannavaz *et al*. (2009) further showed that the frequencies of A and G alleles were 0.069 and 0.931, respectively in Iranian Holstein bulls. Their study also confirm that the allelic substitution effect was significant for milk and protein yield ($p < 0.05$) suggesting that the G allele was unfavorable for milk and protein yield. This might establish the fact that most Nigerian indigenous cattle breeds are not good milk producers hence the need for genetic improvement of the local breed through genomic selection.

3.2 Kappa-casein (K-CN) gene polymorphisms

The digestion of amplified fragments of K-CN gene by *Hind*III endonuclease at 530-bp was able to identify three different genotypes which were differentiated as follows: AA with undigested one fragment at 530-bp, BB with two digested fragments at 370-bp and 160-bp and AB with three fragments at 530-bp, 370-bp and 160- bp. All N'dama and Keteku cattle breeds investigated in the present study are genotyped as AA with one undigested fragment at 530-bp (Fig.4). White Fulani and Muturu animals showed the three genotypes AA, BB and AB with three fragments at 530-bp, 370-bp and 160- bp (Fig. 4).

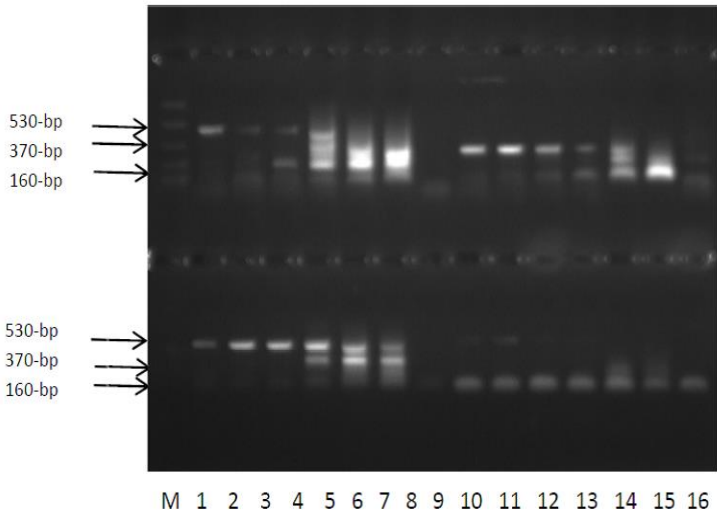

Fig. 4 The electrophoretic pattern obtained after digestion of PCR amplified cattle K-CN products with *Hind*III. Lane M: 100- bp ladder marker. Lane 10-16: Undigested fragment at 530-bp. Lanes 1-3 and10–13: Homozygous BB genotypes showed two restricted fragments at 370- and 160-bp.

Genetic variants of bovine kappa-casein (K-CN) gene are associated with protein content of milk and have a significant influence on rennet clotting time, firmness and cheese yield of milk. Milk protein polymorphisms have received considerable research interest because of their potential use as an aid to genetic selection and to genetic characterization of bovine breeds (Del Lama and Zago, 1996; Golijow *et al.*, 1996 and 1999; Kemenes *et al.*, 1999). Kappa-casein (K-CN) gene is located on bovine chromosome 6q31 and the overall length of the K-CN gene is close to 13- kb. Out of known kappa-casein genetic variants, the A and B are the most common in the majority of cattle breeds (Histor *et al*., 2013). The k-casein variants A and B differ in amino acid 136 and 148. In position 136, Thr (ACC) is changed for Ile (ATC) in position 148, and Asp (GTA) is changed for Ala (GCT) (Lin *et al.*, 1992, Strzalkowska *et al.*, 2002, Tinaev, 2003).

Studies have reported that some of these bovine protein variants, particularly certain k-casein, are associated with lactation performance and have a major influence on milk composition and its processing properties (Marziali and Ng-Kwai- Hang, 1986; Aleandri *et al.*, 1990; Sabour *et al*., 1996; Romonasova, 1999; Kastonina *et al.*, 2004; and Edriss *et al*., 2008).

The PCR–RFLP techniques in this study demonstrated that some of the animals were monomorphic while others are polymorphic for the kappa-casein gene. Both allele A and B were observed in these animals which was similar to the result of Rachagani and Gupta (2008) who analyzed the allelic variants of the K-CN gene in Sahiwal and Tharparkar cattle breeds. They identified that genotype BB of the K-CN gene had more influence on the milk yield, solids-not-fat yield and protein yield in the Sahiwal cattle. The relation between K-CN polymorphisms and milk performance traits in Holstein-Friesian heifer cows was reported in Poland by Beata *et al*. (2008). In contrast to the previously mentioned results, the authors reported that the AA genotype of K-CN gene were characterized by the highest milk, fat and protein yield, while the lowest fat and protein contents were observed in milk of cows with the BB genotype. This association between AA genotype with higher milk production agreed with the results of Curi *et al*. (2005)

3.3 Allele distribution of K-CN gene

Although, K-CN gene has been extensively studied the present study showed a high degree of genetic variability among the four cattle breeds for K-CN locus. The genotypic frequencies K-CN among the indigenous cattle sample population are as follows: White Fulani cattle breed had 44.12, 50.00 and 5.88 for AA, AB and BB, respectively while, Muturu cattle breed had 50.00, 41.30, and 8.70 for AA, AB and BB, accordingly. N'dama and Keteku animals were genotyped as homozygous AA genotypes. Overall, allele B was more frequent in the entire population of animals in this study. Muturu cattle breed had the highest allele frequency for k-casein B with a value of 0.44 followed by White Fulani breed with a value of 0.31 as compared to other breeds in this study while N'dama and Keteku breeds had the highest allele frequency for k-casein A with a value of 1.0 (Table 2). The genotypic frequencies of k-casein AA and AB are significantly different in the four breeds examined in this study. There is no significant departure from Hardy-Weinberg equilibrium ($p<0.05$) in the four breeds. Studies of genetic characterization of cattle breeds have shown that the B allele of K-CN occurs at higher frequencies in breeds originating from *Bos taurus* than in those of *Bos indicus* origin (Backer and Manwell, 1980; Golijow *et al.*, 1996; Del lama e Zago, 1996; Kemenes *et al.*, 1999). Those finding aligned with the result from this study as Muturu (*Bos taurus*) also had the highest B allele among the cattle breeds in this study although with a closer range with White Fulani (*Bos indicus*).

Although, previous studies have shown that genotype BB of the K-CN gene had more influence on the milk yield, solids-not-fat yield and protein yield. Hence, White Fulani and Muturu cattle breeds could be good milking animals upon selection for improvement. According to Marziali and Ng-Kwai-Hang (2006) cheese production can be increased by 10 percent if milk is from cow of the BB genotype of K-CN when compared with milk from AA animals. Therefore, it has been proposed to increase the frequency of K-CN B

within breeding programs, preferring sires with the favorable kappa-casein genotypes.

4 CONCLUSION

The present study used PCR–RFLP technique to detect the genetic polymorphism in two different milk genes among four indigenous breeds of cattle in Nigeria. This study detected genetic polymorphism in PRL gene with a higher frequency of G allele in the animal sample population which has been unfavorable for milk and protein yield. Hence the need to breed for increased A allele among the Nigerian indigenous cattle through genomic selection. Also, The PCR–RFLP techniques used in this study demonstrated that White Fulani and Muturu cattle breeds were polymorphic while N'dama and Keteku were monomorphic for the kappa-casein gene. Both allele A and B as observed in these animals established a high degree of genetic variability among the four cattle breeds for K-CN locus. A high frequency of A allele as discovered in this study strengthens the fact that most of the Nigerian indigenous cattle are more of meat producer rather than milk producer. This may be due to poorly developed breeding program, genotype-environment interaction and polymorphism in traits.

5 RECOMMENDATION

For future improvement of the indigenous breed of cattle, a high throughput sequencing technology like genotyping by sequencing (GBS) is thus recommended for genotyping of larger population of animals.

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