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Genetic polymorphism of myostatin (MSTN) in Nigerian sheep breeds

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ABSTRACT: Myostatin (MSTN) also known as growth differentiation factor 8 (GDF-8) has been implicated to play an important role in growth regulation, and it is a candidate gene in marker assisted selection (MAS). This study was carried out to identify the polymorphism of MSTN gene as a genetic marker for growth traits in Nigerian indigenous sheep. Genomic DNA (gDNA) was extracted from blood samples of Balami, Yankasa, Uda and West African Dwarf (WAD) breeds of sheep. Parts of 5'UTR, intron and exon1 (614bp) was amplified using a primer sequence designed by FastPCR-primer software. The amplicons were digested with restriction enzyme HaeIII and the fragments produced were stained with luminescent dye and run on gel electrophoresis. The genetic structure of the sampled population was investigated after analysis with POPGENE32 software. The HaeIII digested results showed that Myostatin has three polymorphs (AA, AB and BB), controlled by two alleles (A and B), with B having a higher allelic frequency (82.84%) and BB genotype has the highest frequency of 73%. The sampled population showed a deviation from Hardy-Weinberg equilibrium ($p<0.05$) while the F-statistics results of the Nigerian breeds of sheep showed the breeds are genetically identical (33.40%) within them. The genetic distance matrix established that Uda and Yankasa show the greatest distant (3.00%) while Uda and WAD are almost identical (99.85%). The four breeds of sheep studied showed polymorphism for Myostatin gene in the intron 1 and exon 1. Myostatin, therefore, could be considered a candidate gene for MAS.

Keywords: MAS, myostatin, PCR-RFLP, polymorphism, sheep.

INTRODUCTION

The general principles of animal improvement are well documented and there is no reason it should be different in Nigeria. There are however, some peculiarities of sheep production circumstances in Nigeria and the prominent among them is the availability of large number of stocks or genetic materials and the lack of information about them (FAO, 2007). Decisions on application of marker assisted selection (MAS) for genetic improvement of any breed needs to take into account available information on traits of economic value, specific adaptive features, presence of unique genes or phenotypes, local importance of a breed in production system and availability of resources where breeds are located (Filipo et al., 2015). There are four breeds in sheep in Nigeria that that are easily identified phenotypically (Adu and Ngere, 1979).

Plans for genetic improvement of these breeds should therefore start with proper characterization/ documentation of their existing genetic materials upon which further studies can be based to identify the association between the documented genetic materials (genetic markers) and quantitative trait loci (QTL) which of course will enhance indirect selection (MAS) and ultimately lead to genetic improvement of the Nigerian indigenous sheep. Growth traits of animals are regulated by many genes which are responsible for the economic value of the animal (Chen et al., 2012). The genes are, therefore, important to consider when designing breeding programs and identification of such genes is critical for establishing marker-assisted

selection (Li et al., 2006).

Myostatin (MSTN) gene, otherwise known as growth differentiation factor-8 gene is essentially expressed in skeletal muscle and has been shown to repress muscle growth (Bellinge et al., 2005). Myostatin is situated in chromosome 2 of sheep (Archibald et al., 2010). Loss of functional myostatin is known to cause the "double-muscled" phenotype in different species (McPherron and Lee, 1997; Grobet et al., 1998 in cattle, Broad et al., 2000 in sheep, Li et al., 2006 in goat). Mutations within the myostatin gene were attributed to muscular hypertrophy (mh) allele in double muscle breeds (Kambadur et al., 1997). Such a major effect on a single gene on processing yields opened a potential channel for improving processing yields of animals using knock out technology (Kocabas et al., 2002). Kemieniec, Pomeranian, Medras and Mecheri breeds of sheep have all been reported to be polymorphic for Myostatin and their heterozygotes show higher body weight (Amiya et al., 2017).

The majority of the studies done in characterizing Nigerian breeds of sheep are morphological (Agaviezor et al., 2012; Asamoah-Boaheng and Sam, 2016). This makes molecular markers scarce. The paucity of which makes breeding program targeted at genetic improvement of indigenous breeds of sheep unattainable. Therefore, examining the Myostatin gene and its variants in Nigerian breeds of sheep is important to finding animals that possess desirable genotypes of this gene for future selection programs, especially in MAS for economic traits. Thus, the objective of this study was to identify the polymorphism of MSTN gene as a genetic marker for growth traits in Nigerian indigenous sheep.

MATERIALS AND METHODS

A total of 400 blood samples (5 mls per sample) comprising 100 healthy adult individuals per breed (Balami = 100, Uda = 100, yankasa = 100 and West African Dwarf = 100) were collected from the jugular vein of the animals. The research animals were sourced from Akinyele sheep and goat market in Ibadan, Oyo State, Nigeria. The market is located at longitude 3.9470ºE and latitude 7.5503ºN. The blood collected using 5 mls syringes were emptied into vacutainer tubes containing EDTA as an anticoagulant and transported in ice boxes to Ervena Laboratories Limited, Bodija, Ibadan where the laboratory activities were carried out.

DNA extraction

DNA was extracted using the DNA extraction kit manufactured by Jena Biosciences according to the manufacturer's protocol. The concentration of the DNA was estimated using agarose gel electrophoresis. 42% (169) samples from all breeds yielded positive results for further analysis.

Table 1. HaeIII digestion mixture composition.

HaeIII restriction digestion mix component	Volume (µl)
PCR product	10.0
10XNE buffer	1.5
HaeIII enzyme (10units/µl)	0.3
Nuclease free water	3.2
Total	15.0

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The part of 5'UTR, exon1 and part of intron1 of the MSTN gene was amplified using primer sequence, designed by FastPCR-Primer designing (FastPCR software for PCR in silico PCR and oligonucleotide assembly and analysis) software designed by Inqaba West Africa Ltd.

Thirty (30) cycles of amplification were carried out in 0.2 ml microfuge tubes using thermal cyclers and PCR was performed with a total reaction mixture of 37 µl using 8 µl master mix (Amplicon), 1.6 µl each of forward and reverse primers (10 pmol/µl), 3 µl template DNA (50 ng/µl) and 25.8 µl of nuclease-free water (NFW).

The amplification reaction was carried out with a program of 5 min denaturation at 95°C for 35sec annealing at 60°C for 30 sec and with a final extension for 5 min at 72°C. The primer sequences set used are:

Forward primer: $^{5'}$ACTGGTGTGGCAATTTTGTCT $^{3'}$
Reverse primer: $^{3'}$TCCTTACGTACAAGCCAGCAG$^{5'}$

Digestion

HaeIII restriction enzyme was used for digestion at 37°C for 2 hrs (Table 1). The enzyme was activated by increasing the incubation temperature to 80°C for 20 mins. The digestion products were checked at 2% agarose gel with 50 bp DNA ladder in a gel documentation system. The gel was treated with luminescent dye to make the DNA visible.

RESULTS AND DISCUSSION

Polymorphism

In this study, the polymorphism of part of 5'UTR, exon1 and part of intron1 of MSTN gene was examined. Figures 1 to 4 shows the gel bands from the four breeds of sheep studied. MSTN have been implicated in myogenesis by surpressing proliferation, differentiation and protein synthesis in mammals (Lee and McPheron, 2001; Taylor et al., 2001; Ríos et al., 2002). Polymorphism is essential for making genetic progress in animal breeding and MSTN has revealed from this study to be a viable candidate for MAS. This is because all the four breeds of Nigeria sheep

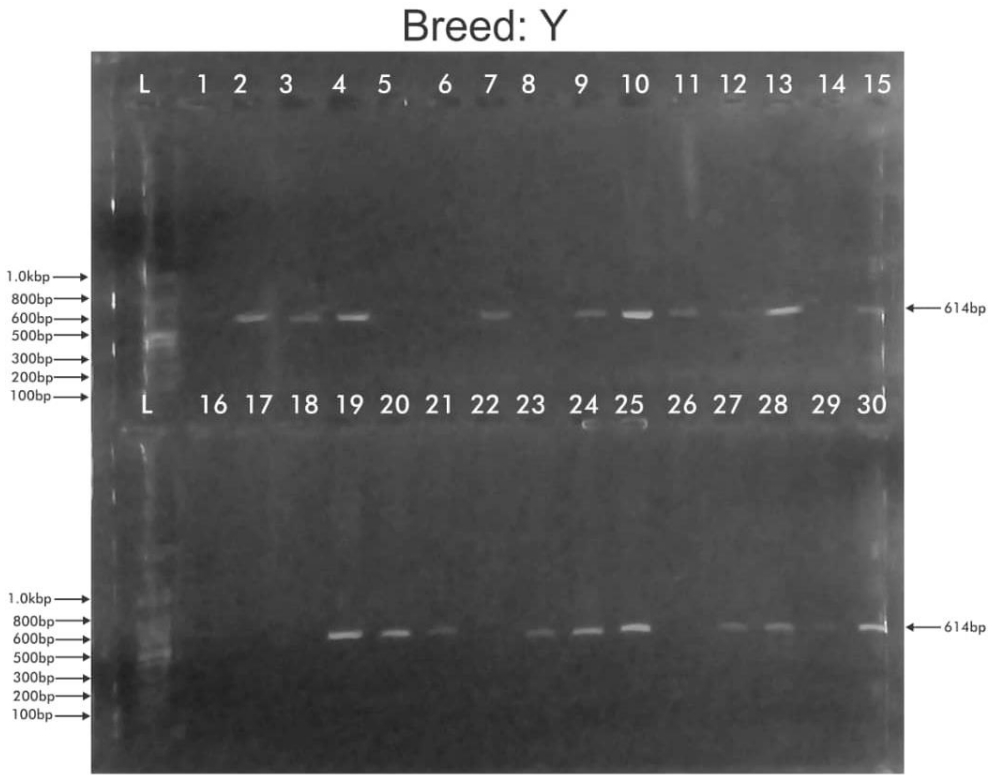


Figure 1. PCR amplicon of part of 5'UTR, exon 1 and part of intron 1 of *MSTN* gene of Yankasa breed of sheep.

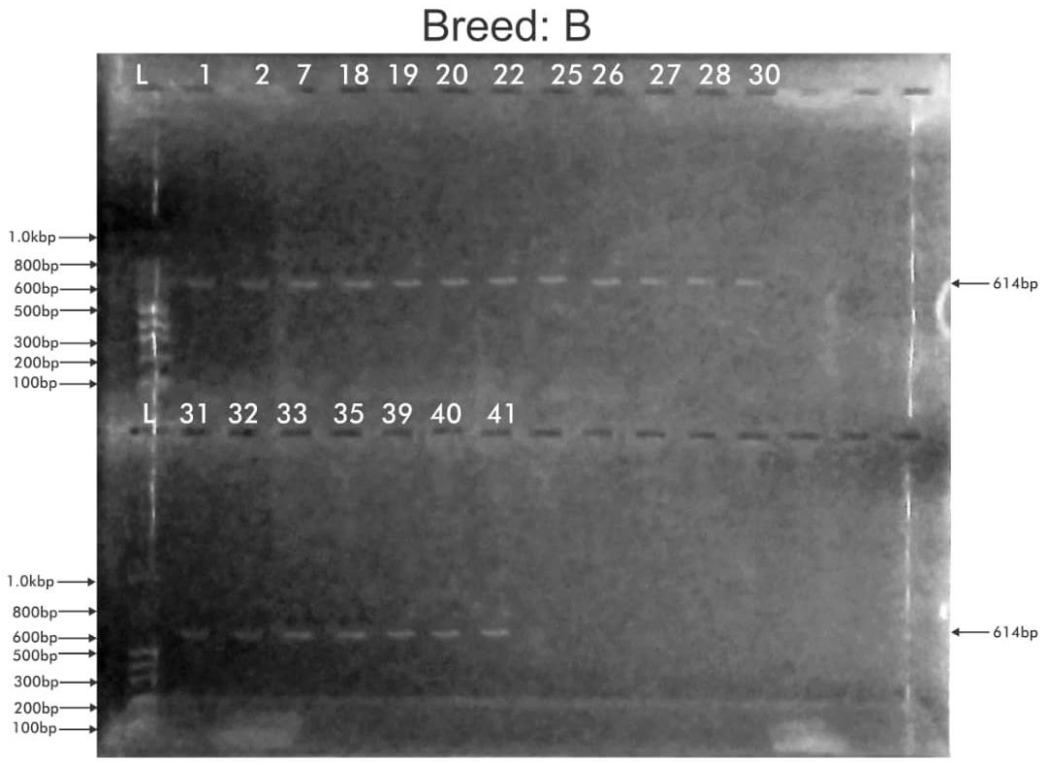


Figure 2. PCR amplicon of part of 5'UTR, exon 1 and part of intron 1 of *MSTN* gene of Balami breed of sheep.

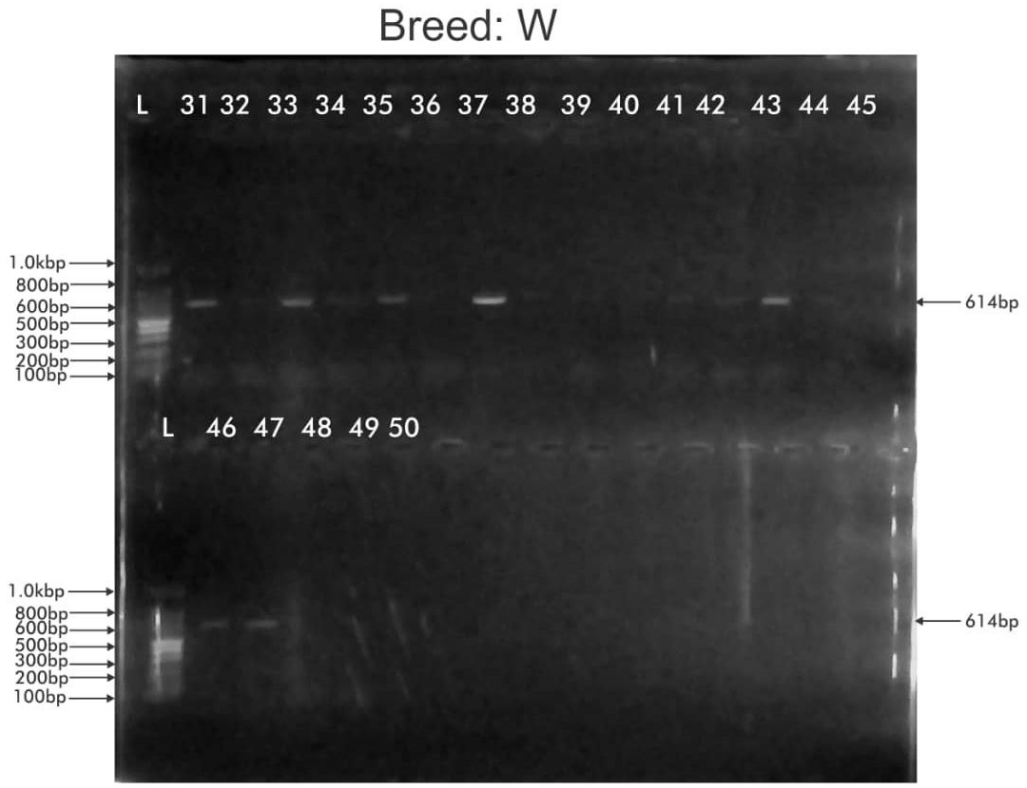
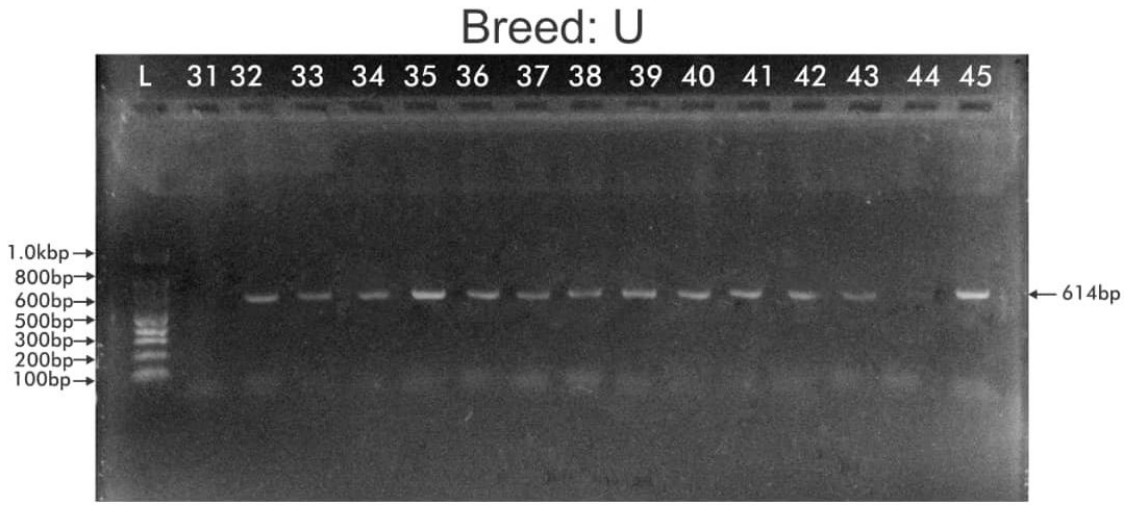


Figure 3. PCR amplicon of part of 5'UTR, exon 1 and part of intron 1 of *MSTN* gene of West African Dwarf breed of sheep.



Fiure 4. PCR amplicon of part of 5'UTR, exon 1 and part of intron 1 of *MSTN* gene of Uda breed of sheep.

studied showed polymorphism for Myostatin locus. Awobajo et al. (2015) and Fijabi et al. (2019) obtained similar polymorphisms in goats and turkey domiciled in Nigeria. Meanwhile, in horse, a single nucleotide polymorphism (SNP) of MSTN (g.66493737 C>T) has been associated with body composition of race horse (Hill et al., 2010; Tozaki et al., 2010; Tozaki et al., 2011), and later revealed to be associated with proportions of muscular fibre types (Peterson et al., 2013). In cattle, dogs, mice and humans, different variants of MSTN has been associated with muscle hypertrophy phenotypes, different growth rates and different body weights (Grobet et al.,

1997; Schuelke et al., 2004; Mosher et al., 2007). Therefore, selection for growth rate, body weight and meat quality which are of economic importance in sheep production can be improved.

Allelic and genotypic frequencies

The relative abundance and distribution of alleles in a population gives us insight into the genetic structure of that population. The genetic structure of the sampled Nigerian breeds of sheep revealed two alleles that were observed in the four breeds, 1.47 alleles in Balami, 1.65 in Yankasa, 1.21 in Uda and 1.31 in WAD are the effective number of alleles and has equal allelic frequencies that would be able to produce the same level of heterozygosity as observed in the studied population (Table 4). Two alleles - A and B with frequencies 17.16% and 82.84% respectively were observed across the studied population (Table 2). Minor allele in Myanmar native horses was associated with higher proportion of Type 2B muscular fibre and lower proportion of Type 1 muscular fibre (Okuda et al., 2016). Further studies to associated the allelic frequencies of MSTN in these breeds of sheep studied to production performance will most likely open another window of improvement. Overall, there is a deviation from Hardy-Weinberg equilibrium, and since there has been no artificial selection program targeted at increasing the B allele, the higher frequency observed could be attributed to random drift. Random drift, which alongside mutation, gene flow, sexual selection, natural selection, population bottleneck etc. must be absent for Hardy-Weinberg equilibrium to be achieved, does not mean that there is no cause for the drift, or that it happened by chance, rather the drift could be as a result of allele B having more adaptive superiority over allele A (Ayala, 1982). However, carrying out this research with a higher sample population will go a long way to expose if this drift is a result of allele B having more adaptive superiority over allele A or not because the larger sample size will reduce the effect of random drift on allelic frequency. If confirmed that the adaptive superiority of allele B is the reason for the drift, it is therefore necessary that artificial selection targeted at conserving A allele is carried out, because allele A is heading to extinction, especially in Uda where it has 9.76%.

Three (3) genotypes (AA, AB and BB) were observed across the breeds, this agrees with the findings by Amiya et al. (2017) and Magdalena et al. (2019) that also reported three genotypes in Kemieniec, Pomeranian, Medras and Mecheri breeds of sheep. These studies also reported higher body weight in heterozygote animals when compared to homozygote animals. According to Table 3, 124 of the animals appeared to be homozygote B (B,B), 13 are homozygote A (A,A) and 32 are heterozygote (A,B). A study on polymorphism of MSTN in goat breeds and their effect on growth and performance revealed that goats that are heterozygote performed better than those that are homozygote (Zhang et al, 2011). This also necessitates further elucidation of efforts on Nigeria breeds of sheep to establish the effect of the various genotypes reported in this study.The higher number of observed homozygote B is due to the high B allele frequency across the studied population.

The null hypothesis is rejected in the Yankasa, Uda and WAD breeds, since the chi-square test for Hardy-Weinberg equilibrium showed that there is 95% or more probability, that the difference between the observed and expected (HWE) genotype frequency is real and 5% or less than they are due to chance. Studies on genetic variation is crucial when establishing rational breeding strategies for economic animal species (Maudet et al., 2002), studies have been done on farm animals such as water buffalo (Baker et al., 1997; Moioli et al., 2001; Kumar et al., 2006), goat (Baker et al., 2001; Muadet et al., 2002; De Arauyo et al., 2006) and cattle (MacHugh et al., 1997). Studies revealed high level of variation in Turkish breeds where HWE was also rejected (Gutierrez-Gil et al., 2006). Deviation from HWE has alson been reported by Luikart et al. (1999), Laval et al. (2000), Baker et al. (2001), Hasssen et al. (2003) and Elfawal et al. (2006). The null hypothesis is rejected, the studied population is, therefore, not in equilibrium. Hardy-Weinberg equilibrium relies on the assumptions that mating must be random, natural selection must be absent, the population must be large, there must be no gene flow or migration, there must be no mutation and the locus must autosomal. The deviation from Hardy-Weinberg equilibrium recorded in this study can be explained by the fact that these breeds most times migrate, cross each other, and this gives rise to gene flow that makes genetic divergence of sub-populations from each other very difficult. There could also be a case of natural selection where animals choose mates that are similar to them phenotypically or genotypically (assortative mating), thereby causing a drift in the population (Georgieva et al., 2015).

Fixation index and F-statistics

The fixation index is a measure of population differentiation according to genetic structure (Holsinger and Weir, 2009). The results generated by this study revealed 33.40% fixation index (Table 5), indicating a considerable level of interbreeding between these breeds, and this has led to a considerable level of similarity among these breeds at the Myostatin locus. This result could explain the direct low count of heterozygosity observed among the breeds and the deviation from HWE detected in the studied locus.

The study revealed that at the Myostatin loci, Balami breed showed 16.67% similarity, while WAD, Yankasa and Uda showed 27.30, 37.88 and 44.59% similarities respectively (Table 5). The Uda breed thrives only in the

Table 2. Allelic frequencies of MSTN gene.

Allele	Balami	Yankasa	Uda	WAD	Overall
A	0.2000	0.2683	0.0976	0.1404	0.1716
B	0.8000	0.7317	0.9024	0.8596	0.8284

Table 3. Genotypic frequency and Chi-square test for Hardy-Weinberg equilibrium.

Breeds	Genotype	Obs. (O)	Exp. (E)	$(O-E)^2/E$	2*O*Ln (O/E)	X^2	Px	G^2	Pg
	A, A	2	1.1186	0.6944	2.3241				
Balami	B, A	8	9.7627	0.3183	-3.1860	1.0533	0.3047	0.9408	0.3221
	B, B	20	19.1186	0.0406	1.8027				
	A, A	6	2.8519	3.4752	8.9255				
Yankasa	B, A	10	16.263	2.4327	-9.7671	6.3614	0.0117	5.8879	0.0152
	B, B	25	21.8519	0.4535	6.7295				
	A, A	2	0.3457	7.9171	7.0216				
Uda	B, A	4	7.3086	1.4978	-4.8221	9.4970	0.0021	5.5889	0.0181
	B, B	35	33.3457	0.0821	3.3894				
	A, A	3	1.0619	3.5369	6.2311				
WAD	B, A	10	13.8761	1.0827	-6.5517	4.7090	0.0300	3.6434	0.0563
	B, B	44	42.0619	0.0893	3.9641				
	A, A	13	4.9050	13.3594	25.3418				
Overall	B, A	32	48.1899	5.4392	-26.2025	19.3639	0.0000	15.8819	0.0000
	B, B	124	115.9050	0.5654	16.7426				

Degree of freedom = 1, X^2 = Chi-square test is significant for Hardy-Weinberg equilibrium when ($p<0.05$), Px = Probability for chi-square test, G^2 = Likelihood ratio test for Hardy-Weinberg equilibrium, Pg = Probability for likelihood ratio.

Table 4. Statistics of the allele population of each of the breeds.

Breed	Sample size	na*	ne*	I*
Balami	60.0000	2.0000	1.4706	0.5004
Yankasa	82.0000	2.0000	1.6464	0.5816
Uda	82.0000	2.0000	1.2137	0.3197
WAD	114.0000	2.0000	1.3181	0.4056
MSTN	338.0000	2.0000	1.3972	0.4584

*na = Observed number of alleles, *ne = Effective number of alleles, *I = Shannon's information index.

Table 5. Fixation index.

Allele	Balami	Yankasa	Uda	WAD	Between breeds
A	0.1667	0.3788	0.4459	0.2730	0.3340
B	0.1667	0.3788	0.4459	0.2730	0.3340

Northern part of Nigeria, the level of cross-breeding with other breeds is considerably lower than it is in the other breeds. This lower level of cross-breeding is the possible reason for the high level of similarity within the Uda breed.

The F-statistics is a measure of genetic structure, it describes the statistically expected level of heterozygosity in a population; more specifically the expected degree of a reduction in heterozygosity when compared to Hardy-

Table 6. F-statistics and gene flow.

Locus	Sample size	FIS	FIT	FST	Nm*
MSTN	338	0.3066	0.3263	0.0284	8.5523

Nm = Estimated gene flow.

Table 7. Heterozygosity statistics.

Breed	S/S	Obs. Hom.	Obs. Het.	Exp. Hom*	Exp. Het*	Nei*	Ave. Het.
Balami	60.0000	0.7333	0.2667	0.6746	0.3254	0.3200	0.2825
Yankasa	82.0000	0.7561	0.2439	0.6025	0.3975	0.3926	0.2825
Uda	82.0000	0.9024	0.0926	0.8217	0.1783	0.1761	0.2825
WAD	114.0000	0.8246	0.1754	0.7566	0.2433	0.2413	0.2825
MSTN	338	0.8107	0.1893	0.7149	0.2851	0.2843	0.2825

S/S = Sample Size, Obs. Hom. = Observed Homozygosity, Obs. Het. = Observed Heterozygosity, Exp.Hom = Expected homozygosity, Exp. Het. = Expected Heterozygosity, Ave. Het. = Average Heterozygosity. *Number of polymorphism loci = 1, *Percentage of polymorphic loci = 100.00%.

Table 8. Measure of genetic identity and distance.

Population	Balami	Yankasa	Uda	WAD
Balami	*****	0.9943	0.9906	0.9965
Yankasa	0.0057	****	0.9704	0.9821
Uda	0.0095	0.0300	*****	0.9985
WAD	0.0035	0.0181	0.0015	*****

Nei's genetic identity (above diagonal), Nei's genetic distance (below diagonal).

Weinberg expectations (Nei and Li, 1977). The F-statistics results of the Nigerian breeds of sheep showed the breeds are genetically identical within them. FIS reveals the proportion of the variance in a breed contained in an individual, high FIS values The mean FIS, i.e the fraction of the total divergence contained in a subpopulation relative to the total genetic variance value of the studied population is 0.3066 (Table 6), indicating a reasonable level of inbreeding within the population studied. FST is used to determine how much variation is within the studied population, it looks at the proportion of genetic variation that is within the population in relation to that is between populations (Nei and Li, 1977). The result revealed that the four breeds are 28.40% similar. The uncontrolled mating within these breeds could be responsible for this level of similarity.

Genetic distance

The genetic matrix (Table 8) revealed the relationship between the four breeds for the MSTN locus. It shows that Uda and WAD are very similar (99.85%), this may be due to interbreeding. Given the size of Uda and its ability to survive in the Southern part of Nigeria unlike Balami, and the disease-resistant ability of WAD, crossing these two breeds makes economic sense, there is therefore a very high chance that local farmers might have been crossing these breeds, therefore, these breeds have a very chance of sharing so much similarity. This result however counters the report of th morphological study conducted on these breeds which revealed that WAD and Uda breeds has the longest distance between them while Balami and Uda are the closest (Yunusua et al., 2013). Studies on other loci would go a long way to expose if this similarity is peculiar to MSTN loci or if this discrepancy is due the fact that morphological data are less reliable when compared with molecular data.

Furthermore, the phylogenetic relationship between the four Nigeria breed of sheep for the MSTN locus (Figure 1) shows that two nodes are stemming from the root. The first node carries Balami and Yankasa, indicating they are more homologous to the ancestors of sheep in Nigeria than Uda and WAD (Table 8), this also counters the report of Yunusua et al. (2013). This genetic variation in myostatin in the studied populations is advantageous as it is an important force in evolution, it provides raw materials for natural selection to increase the frequency of alleles already present in the population. It also enables some individuals to adapt to the environment while maintaining the survival of the population. Given the variation that exists between Balami and WAD for the myostatin locus,

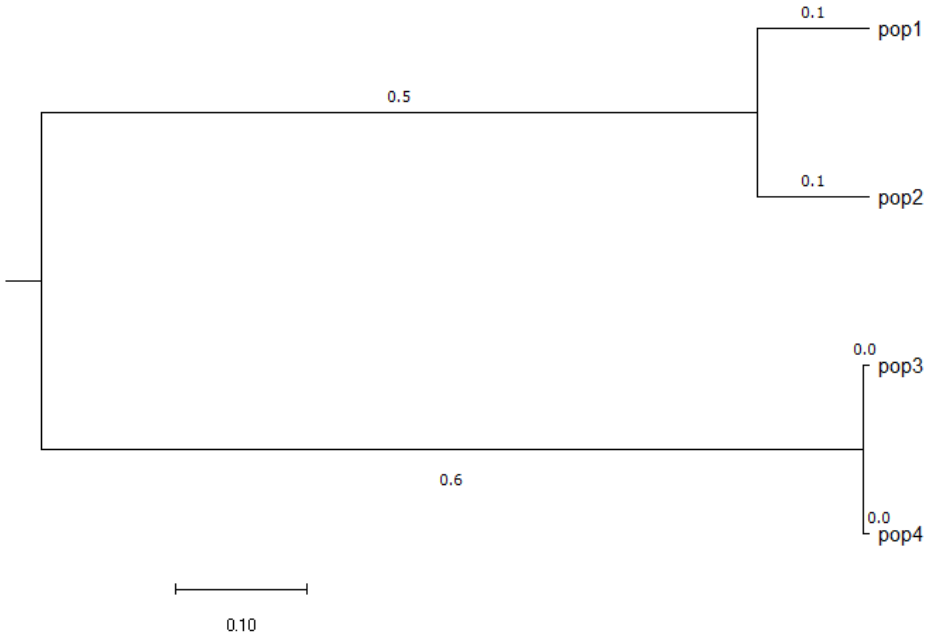


Figure 5. Phylogenetic tree showing the relationship between the breeds at the MSTN locus**.** Pop1 = Balami, Pop2 = Yankasa, Pop3 = Uda and Pop4 = WAD.

a cross between the two will give rise to better growth rate and muscling.

Although, the pattern of result revealed in this study points to the direction of improvements in Nigeria indigenous sheep, it has some limitations. The purity of the breed studied cannot be accounted as there is no reserve around where pure breeds can be accessed. Association studies would be instrumental to phenotypically (body weight or muscles of sacrificed sheep) correlate the polymorphism detected from this study. Studies of this nature are sample size sensitive,thus larger sample size would be proposed for the next step in characterization. Actualization of this was impacted with financial limitation. To work with larger sample size and associate the variants of MSTN identified to production traits, thereby making the study more comprehensive and reliable but were constraind by finance.

Conclusion

This study was carried out to investigate the polymorphism of myostatin gene in the four breeds of Nigerian indigenous sheep. From the results obtained, it can be concluded that myostatin is polymorphic in each of the four breeds of sheep studied, and thus, could be considered as a candidate gene for Marker Assisted Selection (MAS) in Nigerian breeds of sheep. Two alleles (A and B) and three genotypes (AA, AB and BB) were detected in the myostatin gene. The results of this study indicated that allele B is the dominant allele in the myostatin gene of the Nigerian indigenous breeds of sheep.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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