Estimating the Age of Toll-Like Receptor 5 Variants in N'Dama and Other Taurine Cattle

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ABSTRACT

This study was conducted to assess the age of allele and linkage disequilibrium among the polymorphism detected in Toll-like receptor 5 of N'Dama and other taurine cattle. The results showed mutations that evolved around 10800 and 8256 generations ago and almost around the period of domestication of cattle before human selection for different traits of interest.

Segregating and relatively new mutations (993A/G, and 2127C/T) emerged in N'Dama TLR5 at about 5121 generations ago. A great deal of non-random association existed between SNPs located on exonic position 470 and those on 1131, 1324, 2524, and 2542. Also, there was significant association between SNPs located on exonic position 1131 and those on 1324, 2524, and 2542. SNPs on 1975 and 2208 were also significantly associated, likewise, in the taurine cattle.

From the present study those SNPs from the same breed have common LD (such as SNPs 470, 1131, 2524, and 2542), move through generations together and hence could be expected to have common economically important traits. The pattern of LD in N'Dama cattle compared to that of other taurine cattle is different and may explain some of the differential ability of N'Dama cattle to better withstand harsh and disease endemic conditions compared to other taurine cattle.

(Keywords: allele, age, linkage disequilibrium, Toll-like receptor 5, taurine cattle, genetic analysis)

INTRODUCTION

The origin and fate of mutations which often lead to speciation is an ultimate factor in the evolution of a species (Hannah *et al.,* 2014). The variation within a species is usually a result of mutation which occurs at allelic level over a period of time. To understand how different breeds, exist within a species and the physiological variations within breeds, geneticists have developed several ways to study the time spent by group of animals within breed or species before diversion.

Determination of the age of an allele is one of the possible ways used by the early population geneticists to know the common time a group of animals spent together before segregation (Kimura, 1973). Age of allele also known as mutation age refers to the time since the allele first evolved by mutation. Several approaches have been used to estimate the allele age and they include methods based on the frequency of the allele in a population and the genetic variation that occurs within different copies of the allele, also known as intra-allelic variation. These methods can be used independently to estimate the allele age, but the combined used of both increases the accuracy of the estimation and provide more information for selection purpose.

A method described by Montgomery and Bruce (2000) often refers to 'Dating' involves the use of stems from the extensive DNA sequencing and marker typing being done to map and clone alleles that cause genetic diseases (Montgomery and Bruce, 2000). A more recent method that is being used to estimate the age of an allele was proposed by Albers and Mcvean

(2018) and is a non-parametric method, using probabilistic, coalescent-based models of mutation and recombination. This new method specifically infers the time to the most recent common ancestor between hundreds or thousands of chromosomal sequence (hyplotype) pairs. This information is then combined using a composite likelihood approach to obtain an estimate of the time of mutation at a single locus (Albers and Mcvean, 2018).

Taurine cattle are European cattle that originated in the Near East (Bollongino *et al.,* 2012). Both taurine and indicine cattle (zebu) are descended from aucrochs (Wilson, 2005) however, the entire modern stock of taurine cattle from genetic research suggests that they might have arisen from as few as 80 aurochs tamed in the upper reaches of Mesopotamia about 10,500 years ago near the villages of Cayonu in southeastern Turkey and Dja'de el Mughara in northern Iraq (Bollongino, *et al.,* 2012). Domestication and then selection by human accounted for great deal of genetic variation and highly differentiated phenotypes within breed of cattle (Bradley *et al.,* 1998; Helmer *et al.,* 2005). This is because the most fitted breed to human environment were domesticated among which selection for preferred traits were done by man.

The origin of new mutation and genetic diversity is one of the key concepts of population genetics. Even though, great resemblance exists among breeds, large genetic variation probably brought about by recurrent mutation at allelic level can also be observed. Single nucleotide polymorphisms (SNPs) and microsatellite analyses have been used to assess population structures and genetic diversity in order to gain insight into origin, history and adaptation of cattle (Magretha *et al.,* 2018).

Several authors have identified different mutations in the coding region of TLR5 of different cattle breeds (Seabury *et al*., 2007; Areal *et al*., 2011; Smith *et al.,* 2012). They observed that there was direct evidence that bovine (bo)TLR5 is functional and maybe under positive or adaptive evolution. Toll-like receptor 5 (TLR5) is a crucial determinant of pathogen-host interaction and essential for immune homeostasis (Abreu *et al.,* 2005). Bacterial flagellins of diverse bacteria are the molecular stimuli that ligate and activate TLR5 in various vertebrates (Adersen-Nissen *et al.,* 2007). Recognition of bacteria by TLR5 contributes to non-infectious disease. Vijay- Kumar (2010) reported that TLR5 mediates various functions such as shaping the microbiota and immune balance as well as contributing to metabolic tolerance in the intestinal tract of vertebrates. Studies by Gewirtz (2004), Adersen-Nissen *et al.* (2007) and Galkin, (2008) showed that some bacterial species avoid TLR5 recognition by changing their flagellin protein primary sequence and by structural diversification. These they do to enhance their activities as pathogens or colonizers or symbionts.

Recognition of bacterial flagellins by different vertebrate TLR5 is a species-specific. Adersen-Nissen *et al.* (2007) and Keestra *et al.* (2008) found out that species-specific single nucleotide variations in the TLR5 gene exist and a single nucleotide polymorphism (SNP) in the extra-cellular domain (ECD) of TLR5 in mice, chickens and humans is associated with a species-specific response to flagellin. Several studies had shown that alterations in the sequence within the TLR5 gene may affect its functions as immune gene. For instance, a study by Hawn *et al.* (2003) showed that a stop codon polymorphism in human TLR5 present in approximately 25% of the human population is associated with an increased susceptibility to Legionnaire's disease (Hawn *et al.,* 2003) while Blohmke *et al.* (2010) reported that it reduced inflammatory damage in cystic fibrosis (Blohmke *et al.,* 2010).

N'Dama cattle is a unique breed of taurine cattle that has stronger resistant to trypanosomes (Mattioli *et al.,* 2000) than other taurine breeds. This major attribute accounted for its strong adaptability to the humid and cold climate of tropical environment and hence overall increase in their population and expansion of their geographical range (Rege and Tawa, 1999). Therefore, this present study aims to estimate the age of TLR5 variants in N'Dama and other taurine cattle.

MATERIALS AND METHODS

Sampling, DNA Extraction, and Purification

One milliliter of blood was collected using sterilized needle and syringe from sixty unrelated N'Dama cattle sampled from Institute of Agricultural Research and Training (IAR and

T) Moore plantation Apata, Ibadan, Fasola farms in Oyo and the Cattle Production Venture (CPV) of the Federal University of Agriculture Abeokuta, Nigeria. The blood was transferred into EDTA bottle where sample was taken and dropped on FTA classic card and allowed to air-dry.

Genomic DNA was extracted from air-dried blood samples preserved on FTA classic cards using Whatman Biosciences extraction kits with the recommended manufacturer protocol. The extracted DNA was quantified for concentration and purity. Electrophoresis was carried out in 1% agarose gel to determine the potential DNA degradation after which the samples were kept at -20˚C for further analyses. All procedures were approved by the Animal Experimentation local ethics board at Federal University of Agriculture, Abeokuta, Nigeria.

To be able to assess the mutations in other taurine breed based on TLR5 gene, taurine sequences (Table 1) were downloaded from the National Centre for Biotechnology Information (NCBI) and included in the analysis.

Polymerase Chain Reaction (PCR) and DNA Sequencing of Toll –Like Receptor 5

The target region from the extracted DNA was amplified using a published primer sets based on cattle TLR5 (Table 2) with the amplification product length of 2577 bp exonic region. The PCR mixture consisted of 1 disc of cattle DNA on FTA card as template, 0.25µl of 10 µM of forward and reverse primers, 3.2 µl dNTP mixture, 0.2 µl Taq polymerase (Promega, USA), 2.0 µl 10X Buffer and ddH20 to a final volume of 20 µl.

Table 1: TLR5 Sequences of Taurine Breeds with their Accession Number from NCBI.

s/no	Accession number	Cattle Name	Cattle Name
1	EU006638.1	Holstein	*Bos taurus*
2	JQ805137.1	Angus	*Bos taurus*
3	JQ805136.1	Maine Anjou	*Bos taurus*
4	JQ805135.1	Texas Longhorn	*Bos taurus*
5	JQ805134.1	Hereford	*Bos taurus*
6	JQ805131.1	Red Angus	*Bos taurus*
7	JQ805129.1	Holstein	*Bos taurus*
8	JQ805127.1	Charolais	*Bos taurus*
9	JQ805126.1	Brown Swiss	*Bos taurus*
10	EU006640.1	Limousin	*Bos taurus*
11	JQ805133.1	Hereford	*Bos taurus*
12	JQ805132.1	Texas Longhorn	*Bos taurus*
13	JQ805131.1	Red Angus	*Bos taurus*
14	JQ805130.1	Maine Anjou	*Bos taurus*
16	JQ805129.1	Holstein	*Bos taurus*
17	JQ805128.1	Holstein	*Bos taurus*
18	JQ805127.1	Charolais	*Bos taurus*
19	JQ805126.1	Brown Swiss	*Bos taurus*
20	EU006639.1	Angus	*Bos taurus*
21	EU006640.1	Limousin	*Bos taurus*
22	JQ805125.1	Angus	*Bos taurus*
23	DQ335128.1	Holstein	*Bos taurus*

Table 2: Cattle Toll-like receptor 5 sequence primers

Sequence Primer	Primer	Type Direction	Sequence
TLR5 Primer Set 1	Forward	5' > 3'	GCTCAGTGCCTTGAGCTTAGA
	Reverse	5' > 3'	TCAAGGAATTCAGTTCCCG
TLR5 Primer Set 2	Forward	5' > 3'	CCGATGCTGTATTAAAAGATGG
	Reverse	5' > 3'	TTCAGCTCCTGGAGTGTCTC
TLR5 Primer Set 3	Forward	5' > 3'	CCAGGAGCTCGATGATACAG
	Reverse	5' > 3'	GGGCATGGTTTTGGTGAC
TLR5 Primer Set 4	Forward	5' > 3'	TTCCTTCTCCAGGTACCTCATC
	Reverse	5' > 3'	AAAGACTGTAAATGGAAACCCC
TLR5 Primer Set 5	Forward	5' > 3'	ATCACAATAGCTGGGTCTCCA
	Reverse	5' > 3'	CAGGCCACCTCAAGTACTGC
TLR5 Primer Set 6	Forward	5' > 3'	CCCAGAGTCTGCTGTTCAAG
	Reverse	5' > 3'	GGCTTGCGATAAGTGGAAAC

Smith *et al*., 2012

After initial denaturation of 95^{0}C for 3 min and final denaturation of 95^{0}C for 1 min, the samples were subjected to 35 cycles of 60^{0}C annealing temperature, 72^{0}C for 90 sec initial elongation followed by 75^{0}C final elongation step for 5 min on Agilent Surecycler 8800. The PCR products were run on 1.2% agarose gel with ethidium bromide (0.05µL/ml) using 200 bp size standard ladder. After passing 100v for 5 minutes, the gel was viewed under UV light and photographed using Alpha Mega® 2200version 5.5 gel documentation system. The PCR product were purified using commercial kit (QIAquick® PCR purification kit, Canada). The purified product were subjected to sequencing in a 20 µL reaction mixture comprising approximately 20 ng of purified PCR products, 3.2 pmol of primer and 8 µl of BigDye® terminator cycle sequencing kit (Big Dye Terminator Ready Reaction Mix (mixture of dNTPs, ddNTPs, buffer, enzyme and MgCl2)), 8 µL of deionized water, 2 µL template DNA using a ABI 3730×1 (Applied Biosystems) capillary DNA analyzer with 25 cycles at 96^{0}C for 10s, 50^{0}C, for 5 s, and 60^{0}C for 4 min followed by a rapid thermal ramp to 4^{0}C after the last cycle and holding until the purification of the sequencing product. This was carried out at genome Quebec facility situated in the McGill University Campus, Quebec, Canada.

DATA ANALYSIS

Sequence viewing, trimming and editing were carried out using BioEdit software (Hall 1999) on 2577-bp long only exon of TLR5 used for the analysis. Sequence alignment with the reference sequence excluding all gaps was carried out using Codon code aligner (www.codoncode.com/aligner/down loade.htm) and CLUSTAL W software (Thompson *et al.,* 1994) implemented in MEGA 7 (Tamura *et al*. 2011). Polymorphism within the sequence of TLR5 of N'Dama and other downloaded taurine cattle populations with their allele frequencies were determined using MEGA 7 (Tamura *et al*. 2011) and Codon code aligner (www.codoncode.com/aligner/down loade.htm). Hereford cattle sequence (accession number JQ805134.1) was used as the reference sequence. The age of allele was calculated using the method described by (Montgomery and Bruce, 2000) by using the formulae below:

Age of allele = (-4Np/1-p)ln(p) for major allele

Age of allele = (-4Nq/1-q)ln(q) for minor allele

Where N= effective population size for cattle p = Frequency of the Major allele q = Frequency of the minor allele

For all the SNPs, the value of N for cattle was taken as 4000 according to Emily and David (2014).

Estimation of linkage disequilibrium was done on the SNPs identified using DnaSP V6 (Librado and Rozas, 2009) while Fischer test (r^2) was used to determine the significant difference. The Federal University of Agriculture, Abeokuta Animal Care and Use Committee approved the sampling procedures including the number of animals sampled. The samples involved no endangered or protected animal species while blood sample collection was carried out by veterinarian with no tranquilizer nor short-acting anesthetics used on manually restrained animals.

RESULTS AND DISCUSSION

Age of Alleles

The results of single nucleotide polymorphisms, the allele frequencies, and age of the allele based on TLR5 sequences of taurine cattle used in this study were presented in Tables 3 and 4. The result showed that 11 polymorphic sites were identified in TLR5 sequences of 23 taurine cattle downloaded from NCBI (Table 3) while 4 polymorphic sites were identified from TLR5 sequences of 28 N'Dama samples that passed quality test and used in this study (Table 4). All the mutations identified from TLR5 sequences of taurine cattle were ambiguous where T changed to Y (C/T) for heterozygous individuals or G changed to R or S (where Y is T/C, R is G/A and S is G/C)'(Table 3). Out of the 4 SNPs identified from TLR5 sequences of N'Dama breed, 2 SNPs (1761T/C and 2460G/A) were fixed while the other two (993A/G and 2127C/T) are still segregating (Table 4). With respect to the age of allele, major alleles were older than the minor alleles for all the SNPs identified.

Estimating the time at which a certain allele appeared allows researchers to infer patterns of animal migration, disease, and natural selection. In this study, it was discovered that all the SNPs identified in TLR5 sequences of Texas Longhorn (Table 3), are recent mutations which emerged around 2146 generations ago while TLR5 variants in Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou, Texas Longhorn showed mutations that evolved about 10800 and 8256 generations ago and almost around the period of domestication of cattle (Bollongino, *et al*., 2012) before human selection for different trait of interest. This also suggest that those mutations are ancestral and have moved across generations. The occurrence of these polymorphisms in this immune gene in cattle with most of them non-synonymous and having occurred for these generations supported the fact that they may be functional, under selection or adaptive evolution (Smith *et al*., 2012; Seabury *et al*., 2010).

Segregating and relatively new mutations (993A/G, and 2127C/T) emerged in N'Dama TLR5 at about 5121 generations ago. These mutations were only found in N'Dama breed but not in other taurine breeds. N'Dama cattle is an African indigenous taurine found in central and West Africa with ability to resist various diseases especially trypanosomiasis and adapt to different climatic changes which are largely as a result of natural and artificial selection. This has led to an overall population increase and expansion of their geographical range (Rege and Tawa, 1999; Mattioli *et al.,* 2000; Mwai *et al.,* 2015). This may suggest the reason while N'Dama cattle population is more than other taurine cattle in West African countries. Their exposure to diverse agro ecological conditions with various disease and climatic conditions may have led to the accumulation of new variations in this population.

Table 3: Polymorphism and Age of allele of Taurine Toll-Like Receptor 5.

S/N	Alleles	Exonic position	Major allele frequency	Minor allele frequency	Age of major allele	Age of minor allele	Breeds
1	T/Y	470	0.96	0.04	15676	2146	Texas Longhorn
2	C/Y	1131	0.96	0.04	15676	2146	Texas Longhorn
3	A/R	1132	0.96	0.04	15676	2146	Texas Longhorn
4	T/Y	1324	0.96	0.04	15676	2146	Texas Longhorn
5	T/Y/C	1761	0.52	0.48	11335	10840	Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou, Texas Longhorn
6	C/Y	1938	0.96	0.04	15676	2146	Charolais
7	G/R	1975	0.96	0.04	15676	2146	Texas Longhorn
8	G/S	2208	0.96	0.04	15676	2146	Texas Longhorn
9	G/R/A	2460	0.70	0.30	13316	8256	Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou,
10	G/R	2524	0.96	0.04	15676	2146	Texas Longhorn
11	G/R	2542	0.96	0.04	15676	2146	Texas Longhorn

Age of allele is estimated as number of generations.

Table 4: Polymorphism and Age of Allele at N'Dama Toll-Like Receptor 5.

S/N	Alleles	Exonic position	Major Allele frequency	Minor Allele frequency	Age of Allele major	Age of Allele minor
1	G/A	993	0.86	0.14	14824	5121
2	T/C	1761	1	0.0	---	---
3	C/T	2127	0.86	0.14	14824	5121
4	G/A	2460	1	0.0	---	---

Age of allele is estimated as number of generations.

Several studies had shown that new mutations in the sequence within the TLR5 gene may have detrimental or beneficial effects. For instance, Hawn *et al.* (2003) found out that a stop codon polymorphism in human TLR5 present in approximately 25% of the human population is associated with an increased susceptibility to Legionnaire's disease (Hawn *et al.,* 2003) while Blohmke *et al.* (2010) reported that it reduced inflammatory damage in cystic fibrosis (Blohmke *et al.,* 2010).

It was also reported that TLR5-deficient mice have an increased susceptibility to urinary tract infections yet lack pulmonary inflammatory responses (Andersen-Nissen *et al.,* 2007; Feuillet *et al.,* 2006) and exhibit improved survival in experimental melioidosis (West *et al.,* 2013).

Fisher (r^2) Estimate of Linkage Disequilibrium among SNPS of Taurine Breeds

Linkage disequilibrium (LD) levels within or among different populations of breeds or species, often used to determine the level of genetic diversity and historical change in different population size. It gives an idea of the existence of non-random association between alleles or markers and hence determines whether two alleles shared common ancestor (Porto-Neto *et al.,* 2014). The average values of LD based on the Fisher (r^2) criterion, of N'Dama and other taurine TLR5 genes used in this study were presented in Tables 5 and 6, respectively.

There was no recombination observed among the SNPs identified in this study. Ardlie (2002) reported that LD is more possible in the absence of recombination, hence diversity arises through mutation. The LD levels were considerably different among the taurine cattle and N'Dama cattle based on the sequences of TLR5 gene. The values of LD estimated from the different pairing of 11 SNPs identified in taurine TLR5 gene (Table 5) showed the degree of non-random association among the SNPs.

A great deal of non-random association existed between SNPs located on exonic position 470 and those on 1131, 1324, 2524 and 2542. Also, there was significant association between SNPs located on exonic position 1131 and those on 1324, 2524 and 2542. SNPs on 1975 and 2208 were also significantly associated, likewise, there was significant association between SNPs on 2460 and 2542. On the other hand, four polymorphisms detected in N'Dama cattle TLR5 (Table 6) when paired for linkage disequilibrium estimation revealed that only SNPs located on exonic positions 1761 and 2460 were significantly associated. The LD estimate can be used to explore the diversity between cattle breeds with different evolutionary history (Karim *et al.,* 2015) and also the possible association between alleles or markers.

From the present study those SNPs from the same breed have common LD (such as SNPs 470, 1131, 2524 and 2542), move through generations together and hence could be expected to have common economic important traits. Diego *et al.* (2018) gave similar report when they worked on linkage disequilibrium levels and allele frequency distribution in Blanco Orejinegro and Romosinuano Creole cattle using medium density SNPchip data. The above authors found out that breeds with similar LD levels shared common SNPs and QTLs of economic importance. The pattern of LD in N'Dama cattle compared to that of other taurine cattle is different. This could suggest that different evolutionary processes are being encountered by the gene in the two populations. This is possible as N'Dama cattle are subjected to harsh tropical condition of central and West Africa characterized with endemic diseases and adverse weather conditions. Hannah *et al.* (2014) reported that TLR5 exhibits pleiotropy and the functional status of TLR5 may be critical for the susceptibility, outcome, and the host range of infection. Also, Jinying *et al.* (2006) ascertained that knowledge about differences in LD patterns between disease and general populations is crucial for association studies of complex diseases (Jinying *et al.,* 2006).

Table 5: Fisher (r^2) Estimate of Linkage Disequilibrium among Polymorphism of Taurine cattle.

	Site 2									
Site 1	**1131**	**1132**	**1324**	**1761**	**1938**	**1975**	**2208**	**2460**	**2524**	**2542**
470	1.000*	0.0004^{NS}	1.000*	0.021^{NS}	0.0004^{NS}	0.0004^{NS}	0.0004^{NS}	0.0064^{NS}	1.000*	1.000*
1131		0.0004^{NS}	1.000*	0.0213^{NS}	0.0004^{NS}	0.0004^{NS}	0.0004^{NS}	0.0064^{NS}	1.000*	1.000*
1132			0.0004^{NS}	0.0218^{NS}	0.0004^{NS}	1.000*	1.000*	0.0064^{NS}	0.0004^{NS}	0.0004*
1324				0.0213^{NS}	0.0004^{NS}	0.0004^{NS}	0.0004^{NS}	0.0064^{NS}	1.000*	1.000*
1761					0.0213^{NS}	0.0213^{NS}	0.0213^{NS}	0.2970^{NS}	0.0213^{NS}	0.0213^{NS}
1938						0.0004^{NS}	0.0004^{NS}	0.0718^{NS}	0.0004^{NS}	0.0004^{NS}
1975							1.000*	0.0064^{NS}	0.0004^{NS}	0.0004^{NS}
2208								0.0064^{NS}	0.0004^{NS}	0.0004^{NS}
2460									0.0064^{NS}	1.000*

Table 6: Fisher (r^2) Estimate of Linkage Disequilibrium among Polymorphism of Taurine Breeds.

	Site 2		
Site 1	**1761**	**2127**	**2460**
993	0.0058^{NS}	0.0256^{NS}	0.0058^{NS}
1761		0.0058^{NS}	1.000^{*}
2127			0.0058^{NS}

CONCLUSION

Our analysis of the age of TLR5 variants and LD estimates showed that new mutations emerged in N'Dama TLR5 and there was no association between these mutations and those from other taurine cattle. The pattern of LD in N'Dama cattle compared to that of other taurine cattle is different. This may explain differential ability of N'Dama cattle to withstand better harsh and disease endemic conditions compared to other taurine as different evolutionary processes are being encountered by the gene in the two populations.

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