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Genetic Variation and Relationship of N'Dama Cattle Toll-like Receptor 5 with Other Bovine Species

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ABSTRACT

Toll-like receptor 5 is involved in innate immune responses that are initiated by host pattern recognition receptors (PRRs), which recognize molecular structures of conserved pathogen-associated molecular patterns (PAMPs) expressed by microorganisms especially bacteria. In this study, we sequenced 2577 bp bovine TLR5 in N'Dama cattle and discovered four synonymous mutations with one (C2127T) being shared between the N'Dama and the wild cattle. Sequences of other bovine species including *Bos taurus*, *Bos indicus* and *Bos javanicus* from public domain revealed higher number of non-synonymous mutations 19, 7 and 6 in wild cattle, *Bos indicus* subspecies and the *Bos taurus* respectively with a higher ratio of total number of non-synonymous mutations to that of synonymous mutations suggesting that the gene is evolving under adaptive evolution. The results of genetic diversity revealed a combination of high haplotype diversity and low nucleotide diversity which is an evidence of past and rapid demographic expansion from a small effective population size. Haplotype reconstructions, median-joining networks and phylogenetic analysis revealed haplotype sharing among *Bos taurus*, *Bos indicus* and their hybrid suggesting retention of conserved ancestral variation that predates subspecies divergence in this immune gene. There is no haplotype sharing between the wild and the domestic cattle, but a close relationship of the wild cattle clade with one of the N'Dama cluster suggesting

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little exchange of genetic material between these two groups of cattle. This results will facilitate effort towards understanding the relationship between mutations in different bovine species and their involvement in differential susceptibility and or tolerance to various diseases.

Keywords: Bovine phylogeny, genetic diversity, network analysis, Toll-like receptor 5

INTRODUCTION

Livestock has historically constituted one of Africa's major economic resources in terms of the livelihoods of its populations and this is because livestock has largely resisted to transfer from the traditional sector to modern production methods, especially in West-Central Africa. Indeed, throughout most of this region, the majority of the livestock, especially cattle is still being managed under traditional system of rearing (Blench & MacDonald, 2006). Cattle domestication (*Bos taurus* and *Bos indicus*) around 11,000 years ago from wild aurochsen (*Bos primigenius*) represents a major development in the Neolithic transition and was an important step in human history, leading to extensive modifications of the diet, the behavior, and the socioeconomic structure of many populations (Beja-Pereira et al., 2006; Bellwood, 2004; Clutton-Brock, 1999; Edwards et al., 2004). Recent mitochondrial DNA (mtDNA) studies indicated that *B. taurus* was introduced into Europe and Africa, where they interbred with local wild animals (Beja-Pereira et al., 2006). Hybridization of domestic bovine (*Bos indicus*) with their wild species (*Bos javanicus*) or banteng occurs worldwide either spontaneously or through organized cross breeding which usually lead to hybrid vigour or combination of desired attributes of the parental species (Nijman et al., 2003). The Indonesian Madura zebu breed is reputed to be hybrid of domestic and wild cattle as it carries mitochondrial DNA of either zebu or banteng origin. The banteng mitochondrial type was found in all animals of isle of Bali, Indonesia and the introgression was also found in 35% of the animals from a Malaysian Bali-cattle population (Nijman et al., 2003). In Africa, introgression of the Indian zebu in taurine breeds occurs, and has been hypothesized to improve the tolerance of the cattle (*Bos taurus*) to hot and dry environments (Bradley et al., 1994, 1996; Epstein, 1971; Hanotte et al., 2000; Loftus et al., 1994). The N'Dama taurine cattle breed from West Africa is trypanotolerant and have resistance to tick and tick-borne diseases as well as resistance to *Haemonchus contortus*. The cattle also possesses important attributes such as heat tolerance, adaptation to harsh environments and ability to survive on poor quality feeds and has been involved in various breeding progammes to exploit its adaptive potentials (Claxton & Leperre, 1991; Murray et al., 1991). The mammalian Toll-like receptors (TLRs) play an important role in the recognition of invading pathogens and the modulation of innate immune responses (Aderem & Ulevitch, 2000). Several studies have shown that mutations in the TLRs may reduce the ability of the protein to recognize PAMP, and hence

interfere with innate immune activation (Lin et al. 2012; Medmedev, 2013; Pandey & Agrawal, 2006). Characterizing genetic variation in these loci may be useful for guiding genetic selections for disease resistances. Toll-like receptor 5 (TLR5) is known to bind bacterial flagellin while these gene in *E. coli*, primates and other mammals have shown evidences of adaptive positive selection which suggest that interspecies competition between host and pathogen is likely to be driving the co-evolution of pathogen and host (Areal et al., 2011; Smith et al., 2012; Takeda & Akira, 2001). Genes that modulate innate immunity have often been considered as candidate loci for improving host resistance to disease in agricultural species (Plain et al., 2010). The ultimate goal of bovine genomic study is the identification of genetic variation that modulates corresponding variation in economically important production traits, differential susceptibility to disease, and favorable host response to vaccines, which is expected to enable the improvement of these phenotypes via informed genomic selections (VanRaden et al., 2009). The bovine genome sequence and first-generation HapMap projects (Elsik et al., 2009; Tellam, 2009) have directly enabled genome-assisted selective breeding (VanRaden et al., 2009), nascent investigations of non-traditional traits such as marker assisted vaccinations (as diagnostics for enhanced vaccine design or animal response), the development of a new class of anti-infectives known as innate immunologicals (Rosenthal, 2006), and the elucidation of loci that have evolved under strong selection, thus providing important computational evidences for genomic regions which may underlie economically important traits. Indiscriminate hybridization especially in natural population may have had a significant impact on the formation of domestic breeds and can also affect the genetic integrity and diversity of domestic and wild species. Evaluation and monitoring the species composition of these animals may be essential for future preservation of genetic diversity. Therefore the current study is to assess the relationship of N'Dama cattle with other bovine species using TLR5 sequence. To evaluate the naturally occurring variation and haplotype structure of Toll-like receptor 5 in bovine species and determine whether there is variation and haplotype sharing between the wild and the domestic cattle.

MATERIALS AND METHODS

Sampling, DNA Extraction and Purification

Genomic DNA was extracted from air-dried blood samples preserved on FTA classic cards (Whatman Biosciences) with the recommended manufacturer protocol, from 60 unrelated N'Dama cattle samples that were sampled from three different locations; 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 extracted DNA was quantified for concentration and purity, A260/280

ratio between 1.8 and 2.0 were assessed using a Nanodrop 1000 spectrophotometer in congruent with protocol reported by Desjardins and Conklin (2010). The potential genomic DNA degradation was determined using 1% agarose gel electrophoresis. After quantification 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 assess the relationship of N'Dama cattle with other bovine populations based on TLR5 gene, sequences of the gene of *Bos indicus* (n = 5), *Bos javanicus* (n = 1), crossbred (n = 3) and other *taurine* (n = 20) TLR5 were downloaded from the National Centre for Biotechnology Information (NCBI) and included in the analysis (Supplementary Table ST1). The downloaded sequences have not been previously analyzed for relationship among bovine species.

Table ST1

Sequence of cattle TLR5 retrieved from GENBANK and their accession numbers

Accession number	Cattle name	Cattle type
JQ805131.1	Red Angus	*Bos taurus*
JQ805126.1	Brown swiss	*Bos taurus*
JQ805132.1	Texas Longhorn	*Bos taurus*
JQ805135.1	Texas Longhorn	*Bos taurus*
JQ805125.1	Angus	*Bos taurus*
JQ805137.1	Angus	*Bos taurus*
EU006639.1	Angus	*Bos taurus*
JQ805128.1	Holstein	*Bos taurus*
JQ805129.1	Holstein	*Bos taurus*
EU006638.1	Holstein	*Bos taurus*
DQ335128.1	Holstein	*Bos taurus*
EU006640.1	Limousin	*Bos taurus*
JQ805127.1	Charolais	*Bos taurus*
EU006637.1	Charolais	*Bos taurus*
JQ805133.1	Hereford	*Bos taurus*
JQ805134.1	Hereford	*Bos taurus*
JQ805130	Maine Anjou	*Bos taurus*
JQ805136.1	Maine Anjou	*Bos taurus*
AY634631.2		*Bos taurus*
NM_001040501.1		*Bos taurus*
XM_019976811.1	Nelore	*Bos indicus*
EU006636.1	Brahman	*Bos indicus*
GQ248711.1	Sahiwal	*Bos indicus*
GQ866979.1		*Bos indicus*
EU006641.1	Nelore	*Bos indicus*
EU006643.1	Romagnola	*Bos taurus x Bos indicus*

Table ST1 *(Continued)*

Accession number	Cattle name	Cattle type
EU006642.1	Piedmontese	*Bos taurus x Bos indicus*
EU006635.1 JQ811841.1	Braford	Bos taurus x Bos indicus Bos javanicus

Toll–like Receptor 5 PCR Assay and DNA Sequencing

Published primer sets based on cattle TLR5 (Table 1) was used to amplify the target region from the extracted DNA 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 ddH_20 to a final volume of 20 µl. After initial denaturation of 95°C for 3 min and final denaturation of 95°C for 1 min, the samples were subjected to 35 cycles of 60°C annealing temperature, 72°C for 90 sec initial elongation and followed by 75°C final elongation step for 5 min on Agilent Surecycler 8800. The PCR products were run on 1.2% agarose gels with ethidium bromide (0.05µL/ml) using 200 bp size standard ladder. After passing 100 v for 5 mins, the gels were viewed under UV light and photographed using Alpha mega® 2200version 5.5 gel documentation system. The PCR products were purified using a commercial kit (QIAquick®PCR purification kit, Canada). The purified products 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 $MgCl_2$), 8 µL of deionized water, 2 µL template DNA using a ABI 3730×1 (Applied Biosystems) capillary DNA analyzer with 25 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min followed by a rapid thermal ramp to 4°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.

Table 1

Cattle Toll-like receptor 5 sequence primers (Smith et al., 2012)

Sequence Primer	Primer Type	Direction	Sequence
TLR5 Primer Set 1	Forward	5' > 3'	GCTCAGTGCCTTGAGCTTAGA
	Reverse	3' > 5'	TCAAGGAATTCAGTTCCCG
TLR5 Primer Set 2	Forward	5' > 3'	CCGATGCTGTATTAAAAGATGG
	Reverse	3' > 5'	TTCAGCTCCTGGAGTGTCTC

Table 1 *(Continued)*

Sequence Primer	Primer Type	Direction	Sequence
TLR5 Primer Set 3	Forward	5' > 3'	CCAGGAGCTCGATGATACAG
	Reverse	3' > 5'	GGGCATGGTTTTGGTGAC
TLR5 Primer Set 4	Forward	5' > 3'	TTCCTTCTCCAGGTACCTCATC
	Reverse	3' > 5'	AAAGACTGTAAATGGAAACCCC
TLR5 Primer Set 5	Forward	5' > 3'	ATCACAATAGCTGGGTCTCCA
	Reverse	3' > 5'	CAGGCCACCTCAAGTACTGC
TLR5 Primer Set 6	Forward	5' > 3'	CCCAGAGTCTGCTGTTCAAG
	Reverse	3' > 5'	GGCTTGCGATAAGTGGAAAC

Data Analysis

Cattle TLR5 that had only one exon which was 2577-bp long was used for the analysis. Other sequences that were not complete were removed during quality check and preliminary analysis. Sequences viewing, trimming and editing were carried out using BioEdit software (Hall, 1999), sequence alignment with the reference sequence excluding all gaps was carried out using CLC Genomics Workbench 7 (http://www.clcbio.com/blog/clc-genomics-workbench-7-5/) and CLUSTAL W software (Thompson et al., 1994) implemented in MEGA 7 (Tamura et al., 2011). Polymorphism within the sequences of TLR5 of N'Dama and other downloaded 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. Genetic diversity indices in terms of number of haplotypes, haplotype diversity, nucleotide diversity and average number of nucleotide differences were estimated using DnaSP V6 (Librado & Rozas, 2009). For phylogenetic and median-joining network analyses, a neighbor-joining with distance matrices generated according to the Kimura 2p model was used using MEGA7 (Tamura et al., 2011) and NETWORK 5.0.0.3 (Bandelt et al., 1999; Fluxus Technology [www.fluxus-engineering.com]) softwares were used to determine the evolutionary relationship between the N'Dama TLR5 gene and other downloaded taurine, zebu and the cattle hybrid populations. The reliability of the inferred phylogenetic tree was evaluated using bootstrap analysis of 1000 replications.

The Federal University of Agriculture, Abeokuta Animal Care and Use Committee approved the sampling procedures including the number of animals sampled. The license number for our sampling procedures was unaabACUC F003/1015. 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

Identification of Single Nucleotide Polymorphism within the Tlr5 Sequence of N'Dama, Other Taurine, Zebu, Crossbred and Banteng

Because of the length of the gene (2577 bp) and the use of four primer pairs, analysis of the N'Dama sequences showed only 28 samples that passed quality test and were complete out of the 60 animals sampled. These complete samples were used for the downstream analysis. Screening for polymorphism within the sequences of the N'Dama cattle used in the course of this study revealed four polymorphisms with two of them being fixed (T1761C, G2460A) in N'Dama population and were also detected in other taurine and zebu cattle, but not in banteng (Table 2). The two other polymorphisms were specific (G993A, C2127T) to our samples and were however still segregating as their frequencies in the population were less than 100. All the four polymorphisms are synonymous as seen in Table 2.

Table 2

Single nucleotide polymorphisms (SNPs) detected in NDAMA cattle TLR5

S/N	Alleles	Exonic position	Observed frequency	Amino acid position	Amino acid	Effect on protein function
1	G/A	993	14.29	331	A/A	Synonymous
2	T/C	1761	100	587	L/L	Synonymous
3	C/T	2127	14.29	709	N/N	Synonymous
4	G/A	2460	100	820	E/E	Synonymous

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

From the 20 sequences of TLR5 of taurine cattle available at NCBI, 11 polymorphisms were observed with four of these polymorphisms synonymous while the remaining seven were non-synonymous (Table 3).

The two synonymous polymorphisms at position 1761 and 2460 were shared with our N'Dama samples while seven of the polymorphism were being shared with the zebu cattle with four specific to taurine cattle as shown in Table 3.

Eleven polymorphisms were detected within this region of zebu cattle that were available in the public domain. Five of these polymorphisms were synonymous while the other six were non-synonymous (Table 4). Eight of these polymorphisms were shared between the zebu and taurine cattles while three were specific to the zebu including two non-synonymous (A541R, C2037S) and one synonymous (A504R) as shown in Table 4.

Only two synonymous polymorphisms that were shared between both taurine and

Table 3

Single nucleotide polymorphisms (SNPs) detected in taurine cattle TLR5

S/N	Alleles	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breeds
1	C/Y	1131	377	D/D	Synonymous	Texas longhorn
2	T/Y/C	1761	587	L/L	Synonymous	Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou, Texas longhorn
3	C/Y	1938	646	V/V	Synonymous	Charolais
4	G/R/A	2460	820	E/E	Synonymous	Holstein, Angus, Charolais, N'Dama, Brown Swiss, Maine Anjou
5	T/Y	470	157	I/T	Non-Synonymous	Texas longhorn
6	A/R	1132	378	K/E	Non-Synonymous	Texas longhorn
7	T/Y	1324	442	Y/H	Non-Synonymous	Texas longhorn
8	G/R	1975	658	E/K	Non-Synonymous	Texas longhorn
9	G/S	2208	736	P/?	Non-Synonymous	Texas longhorn
10	G/R	2524	842	E/K	Non-Synonymous	Texas longhorn
11	G/R	2542	848	A/T	Non-Synonymous	Texas longhorn

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

Table 4

Single nucleotide polymorphisms (SNPs) detected in zebu cattle TLR5

S/N	Allele	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breed
1	A/R	504	168	E/E	Synonymous	Sahiwal
2	C/Y	1131	377	D/D	Synonymous	Nelore, Brahman, Sahiwal, other indicus
3	T/Y/C	1761	587	L/L	Synonymous	Nelore, Brahman, other indicus
4	G/C/S	2208	736	P/P	Synonymous	Nelore, Brahman, other indicus

Table 4 *(Continued)*

S/N	Allele	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breed
5	G/A/R	2460	820	E/E	Synonymous	Nelore, Brahman, other indicus
6	A/R	541	181	K/E	Non-Synonymous	Sahiwal, Nelore
7	A/R	1132	378	K/E	Non-Synonymous	Nelore, Brahman, Sahiwal, other indicus
8	T/Y/C	1324	442	Y/H	Non-Synonymous	Nelore, Brahman, other indicus
9	G/R	1975	659	A/T	Non-Synonymous	Nelore, Brahman, Sahiwal, other indicus
10	C/S	2037	679	F/L	Non-Synonymous	Nelore, Sahiwal
11	G/R	2524	842	E/K	Non-Synonymous	Nelore, Brahman, Sahiwal, other indicus

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

zebu cattles were detected in the sequence of crossbred cattle (Table 5). Twenty seven polymorphisms were observed within this region of banteng (*Bos javanicus)* sequence. Eight of these polymorphisms were synonymous while 19 were non-synonymous. *Bos javanicus* shared one non-synonymous polymorphism (A541R) with *Bos indicus,* one non- synonymous mutation (G2542R) together with taurine and two other polymorphisms, one synonymous (n.2208) and one non-synonymous (n.1975) with the taurine and zebu populations. *Bos javanicus* also shared one synonymous mutation (C2127T) with our locally adapted N'Dama cattle population (Table 6).

Table 5

*Single nucleotide polymorphisms (SNPs) detected in sequence of TLR5 of the crossbred (*Bos indicus *X* Bos taurus*) cattle*

S/N	Alleles	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breeds
1	T/Y/C	1761	587	L/L	Synonymous	Braford, Romangnola, Piedmontese
2	G/A/R	2460	820	E/E	Synonymous	Braford

The exonic position is based on Hereford cattle sequence with accession number JQ805134.1

Table 6

Single nucleotide polymorphisms (SNPs) detected in banteng sequence of TLR5

S/N	Alleles	Exonic position	Amino acid position	Amino acid	Effect on protein function	Breeds
1	G/R	633	211	R/R	Synonymous	Bos javanicus
2	C/Y	1005	335	L/L	Synonymous	*Bos javanicus*
3	C/Y	1512	504	L/L	Synonymous	*Bos javanicus*
4	T/A	1983	661	T/T	Synonymous	*Bos javanicus*
5	G/A	2070	690	T/T	Synonymous	*Bos javanicus*
6	C/T	2127	709	N/N	Synonymous	*Bos javanicus*
7	G/C/S	2208	736	P/P	Synonymous	*Bos javanicus*
8	T/Y	2241	747	A/A	Synonymous	*Bos javanicus*
9	G/R	190	64	A/T	Non- Synonymous	*Bos javanicus*
10	A/R	322	108	N/D	Non- Synonymous	*Bos javanicus*
11	G/R	397	133	D/N	Non- Synonymous	*Bos javanicus*
12	G/R	500	167	R/Q	Non- Synonymous	*Bos javanicus*
13	A/R	541	181	K/E	Non- Synonymous	*Bos javanicus*
14	G/R	785	262	R/H	Non- Synonymous	*Bos javanicus*
15	G/A	1558	520	G/R	Non- Synonymous	*Bos javanicus*
16	C/T	1844	615	S/F	Non- Synonymous	*Bos javanicus*
17	G/A	1852	618	G/R	Non- Synonymous	*Bos javanicus*
18	A/G	1882	628	S/G	Non- Synonymous	*Bos javanicus*
19	T/C	1884	628	S/G	Non- Synonymous	*Bos javanicus*
20	T/C	1934	645	L/S	Non- Synonymous	*Bos javanicus*
21	G/A	1935	645	L/S	Non- Synonymous	*Bos javanicus*
22	A/G	1966	656	I/V	Non- Synonymous	*Bos javanicus*
23	C/T	1970	657	T/I	Non- Synonymous	*Bos javanicus*
24	G/A/R	1975	659	A/T	Non- Synonymous	*Bos javanicus*
25	A/M	2115	705	E/D	Non- Synonymous	*Bos javanicus*
26	C/A	2158	720	H/N	Non- Synonymous	*Bos javanicus*
27	G/R	2542	847	A/T	Non- Synonymous	Bos javanicus

Bovine Haplotype Networks, Haplotype Sharing and Genetic Diversity

The network analysis revealed 19 haplotypes that differed from each other by a small number of mutations within the sequence of TLR5 of domesticated and wild bovine with an overall high diversity of 0.7675 and low nucleotide diversity of 0.00093 (Table 7), among which two were specific to the wild cattle forming an haplogroup

with 27 polymorphic site, high hd, low pi, and high average number of nucleotide differences. The other haplogroup was for the domesticated bovine and which formed a star. The median-joining networks (Table ST1, ST2 and Figure 1) of TLR5 in the bovine species revealed no haplotype sharing between the wild and the domesticated species and predicted low level of haplotype sharing between the *Bos taurus* and *Bos indicus*. The recent formation of hybrid of *Bos taurus* and *Bos indicus* predicted haplotypes which fell essentially within the network node that was dominated by both *Bos taurus* and *Bos indicus*. Some of the predicted haplotypes of the hybrid cattle were shared only with *Bos taurus* cattle while the other stood out as a unique network node. Our N'Dama which is an African taurine had two unique haplotypes and shared one other haplotype with the other taurine breeds used in the study. The least haplotype, nucleotide diversity and average number of nucleotide differences were detected in the N'Dama cattle. In total, 19 haplotypes were discovered in the study with 8 found in *Bos taurus* among which one haplotype (Hap_1) was shared with *Bos indicus* and the hybrid while another one was shared with the hybrid only (Table ST3). In the *Bos indicus*, nine haplotypes were discovered with only being shared with the *Bos taurus* and the hybrid and which could be referred to as the domesticated haplotype while the other eight were not shared but were specific to the sub species. The zebu also had high haplotype diversity (0.909±0.047) with the highest number of haplotypes. The hybrid had only 3 haplotypes with only one being specific or found in that population alone, while one is shared between the hybrid and the *Bos taurus* and the other one found among the hybrid, the taurine and the zebu. The result of the genetic diversity revealed that the *Bos taurine* possessed the least diversity while the highest was observed in the *Bos javanicus* or the wild cattle (Table ST3).

Table 7

Genetic diversity of Toll-like receptor 5 in bovine species

Breed	N	S	h	hd	π	K
N'Dama	40	4	4	0.502±0.098	0.00024±0.00022	0.63±0.22
Taurine	23	11	4	0.686±0.040	0.00047±0.00028	1.21±0.34
Zebu	10	11	14	0.909±0.047	0.0015±0.00035	3.86±0.47
Bos indicus X *Bos taurus*	3	2	3	0.679±0.122	0.00030±0.00021	0.79±0.19
Bos javanicus	1	27	3	0.833±0.222	0.0062±0.0011	16.00±3.43
Mean	66	38	19	0.7675±0.027	0.00093±0.00044	2.41±0.75

N = number of sample, Number of polymorphic site = S, haplotype = h, haplotype diversity = hd, Nucleotide diversity = π, Average number of nucleotide differences = K

Table ST2

Toll-like receptor 5 haplotypes detected in the bovine species

	0190	0322	0397	0470	0500	0504	0541	0633	0785	0993	1005	1131	1132	1324	1512	1558	1761	1844	1852	1882	1884	1934	1935	1938	1966	1970	1975	1983	2037	2070	2115	2127	2158	2208	2460	2524	2542	2524
Haplotypes	G	A	G	T	G	A	A	G	G	G	C	C	A	T	C	G	T	C	G	A	T	T	G	C	A	C	G	T	C	G	A	C	C	G	T	G	G	G
Hap_1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_2	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	A	.	.
Hap_5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_6	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.
Hap_7	.	.	.	C	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.
Hap_8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_9	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
Hap_10	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	A	A	.
Hap_11	.	.	.											C													A							C	.	A	.	.
Hap_12	.	.	.									T	G				C																				A	.
Hap_13	.	.	.			G						T	.	C													.	.	G	.	.	.	.	C	.	.	A	.
Hap_14	.	.	.	.	.	.	G	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.
Hap_15	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	A	.	.
Hap_16	.	.	.	.	.	.	.	.	.	.	.	T	.	C	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	A	.
Hap_17	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap_18	A	G	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	T	A	G	C	C	A	.	G	T	A	A	.	A	.	T	A	C	.	.	.	A
Hap_19	.	.	A	.	.	.	G	A	A	.	T	.	.	.	T	A	.	T	A	G	C	C	A	.	G	T	A	A	.	A	C	T	A	C	C	.	.	.

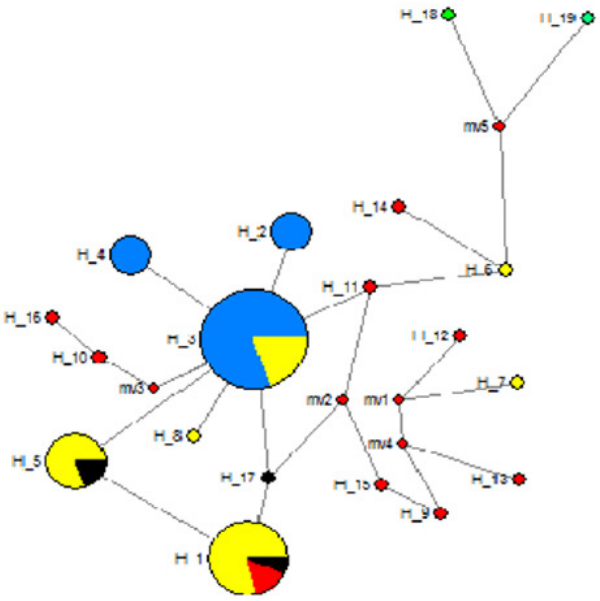

Figure 1. Toll-like receptor 5 haplotype network of bovine species constructed using median-joining method (Bandelt et al. 1999). The circles represent different haplotypes, and the areas of the circles are proportional to the frequency of each haplotype. The different species are distinguished by colour: Domesticated species: N'Dama (Blue), *Bos taurine* (Yellow), *Bos indicus* (Red), crossbred (Black), Wild Species: *Bos javanicus* (Green)

Table ST3

TLR5 haplotypes shared within and between the bovine species

Haplotype	Frequency	Species			
		Bos taurus	Bos indicus	Cross	Bos javanicus
Hap_1	28	22	4	2	-
Hap_2	8	8(N'Dama)	-	-	-
Hap_3	50	40(N'Dama) 10 (other taurine)	-	-	-
Hap_4	8	8(N'Dama)	-	-	-
Hap_5	16	13	-	3	-
Hap_6	1	1	-	-	-
Hap_7	1	1	-	-	-
Hap_8	1	1	-	-	-
Hap_9	1	-	1	-	-
Hap_10	1	-	1	-	-
Hap_11	1	-	1	-	-
Hap_12	1	-	1	-	-

Table ST3 *(Continued)*

Haplotype	Frequency	Species			
		Bos taurus	Bos indicus	Cross	Bos javanicus
Hap_13	1	-	1	-	-
Hap_14	1	-	1	-	-
Hap_15	1	-	1	-	-
Hap_16	1	-	1	-	-
Hap_17	1	-	-	1	-
Hap_18	1	-	-	-	1
Hap_19	1	-	-	-	1

Phylogenetic Relationships

Phylogenetic analyses were performed to estimate the genealogical relationships between the sequences and haplotypes that were detected in the study (Figures 2 and 3). Using the sequences, four clades were discovered with the major one being shared among *Bos taurus*, *Bos indicus* and their hybrid, two of clades were specific to our N'Dama cattle population while the last one belonged to the wild cattle. Although the topology of the consensus tree using the haplotypes (Figure 3) showed polytomies in the domesticated cattle in which the relationships could not be fully resolved to dichotomies, in general, we observed a clade that contained only the wild cattle which formed an haplogroup in the network (Figure 1), the other clade was regarded as the domesticated cattle haplogroup (Figure 1) with three subclades named C1, C2 and C3 (Figure 3). In this polytomy, subclades C3 comprises the *Bos taurus*, *Bos indicus* and their hybrid which indicate some levels of haplotype sharing between them. Subclade C2 is majorly dominated by *Bos taurus* while C1 was dominated by *Bos indicus*. Finally the three subclades correspond to expansions of haplotypes shared among the subspecies of cattle. The phylogenetic analyses further confirmed the close relationship between these two species.

DISCUSSION

Molecular information is crucial for preserving genetic diversity as well as preventing undesirable loss of alleles. The genomes of modern cattle basically reflect the history of animal movements by migratory farmers out of the ancient centers of the cattle domestication. Different cattle breeds therefore are expected to show genetic diversity consistent with their history of migration following domestication. TLR5 is among the evolutionary conserved pattern recognition receptors involved in the activation of the immune system in response to various pathogens and the innate defense against infection while polymorphisms within it have been shown to evolve differently under different evolutionary forces (Alcade & Edward, 2011; Seabury et al., 2007; Smith et al., 2012).

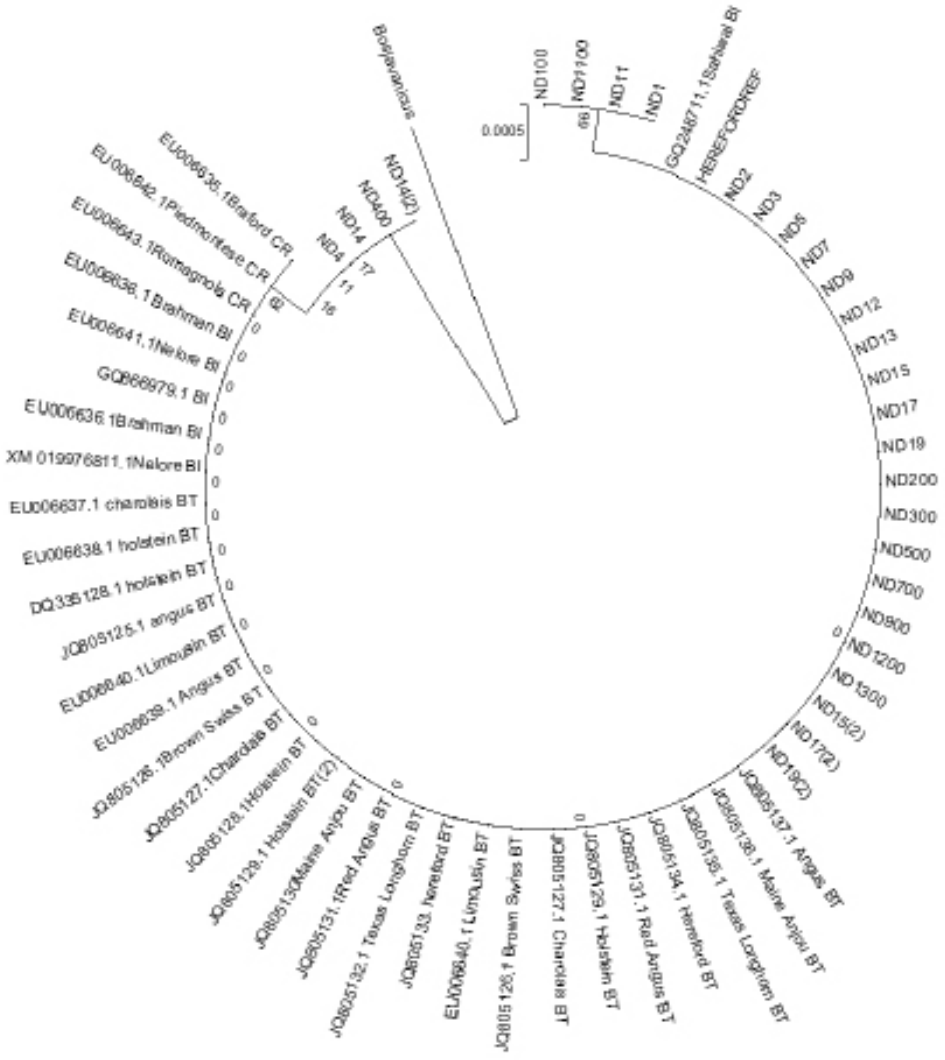

Figure 2. Neighbor-joining tree reconstructed from the sequence of Toll-like receptor 5 of our samples and other bovine species used in this study. The framework was based on Kimura 2p distances as grouped by the neighbor-joining method. The percentage bootstrap value is represented by the numbers at the node after 1000 replications

In this study four polymorphisms were detected in the TLR5 sequence of N'Dama cattle. The polymorphism rate in the animal is low suggesting that the gene is conserved. Although the polymorphism detected are synonymous, they may however be evolutionary relevant as evidence has suggested that supposed silent mutations have effect on protein expression, conformation and function of immune genes (Sauna & Kimchi-Sarfaty, 2011). On the basis of nucleotide sequence variation, two of the

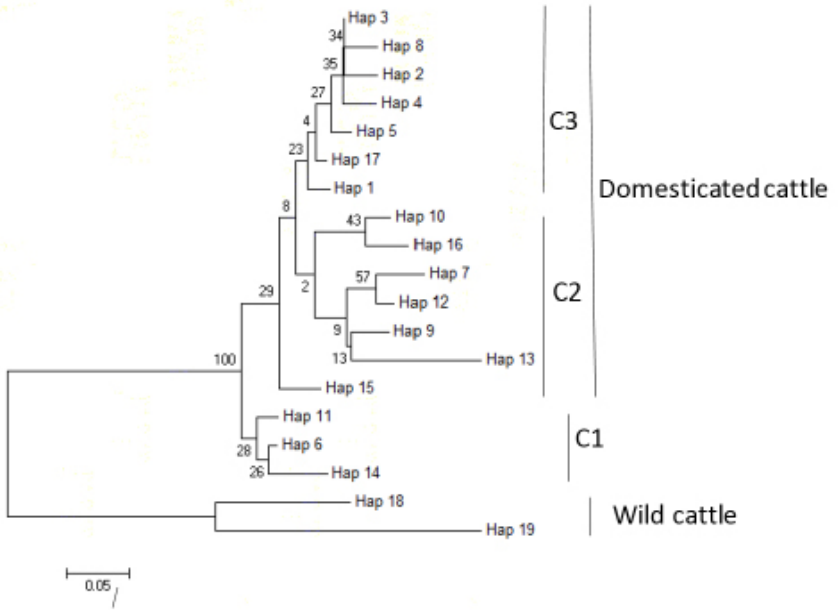

Figure 3. Neighbor-joining tree reconstructed from the 19 haplotypes identified in the TLR5 sequences of N'Dama and other bovine species in this study. The framework was based on p-distances as grouped by the neighbor-joining method. The percentage bootstrap value is represented by the numbers at the node after 1000 replications

mutations are fixed and are being shared between the sub-species of domestic cattle. These suggest that these two mutations must have been ancestral and in existence before the divergence of the *Bos taurus* and *Bos indicus* cattle some 250, 000 years ago (250 Kyr) (Bradley et al., 1996). Unlike other, the domesticated cattle, the N'Dama cattle shared one synonymous mutation with the wild cattle which supported an evidence of genetic introgression of wild cattle into the domestic cattle. It has been reported that hybridization between wild and zebu cattle sub-species occurs worldwide either spontaneously or by organized crossing and it is possible, especially if closely related species share an overlapping habitat or through human intervention during captive breeding (Nijman et al., 2003). The banteng (wild) mitochondrial type has been reported to be found in Bali cattle of Indonesia, while Madura animals also carry mitochondrial DNA of either zebu or banteng origin and which were confirmed using AFLP, SFLP and microsatellite markers (Nijman et al., 2003). In the sequence of TLR5 of bovine species used in this study, there is higher ratio of non-synonymous to synonymous mutations especially in the wild cattle which suggests adaptive evolution in this immune gene (Smith et al., 2012). Previous studies had found evidence of adaptive evolution

in mammalian TLR5 and also revealed that TLR5 genes of domestic livestock had a concentration of single nucleotide polymorphisms which suggests a specific signature of adaptation (Areal et al., 2011; Seabury et al., 2007; 2010; Smith et al., 2012; Wlasiuk et al., 2009). Smith et al. (2012) using codon models of evolution, detected concentration of rapidly evolving codons within the TLR5 extracellular domain which was a site of interaction between host and the bacterial surface protein flagellin.

The analysis of sequences generated for this study and from the sequences from the NCBI GenBank database revealed a high level of haplotype and nucleotide diversity suggesting the cattle population had remained stable with an old evolutionary history (Grant, 1998; Grant & Bowen, 1998). Domesticated cattle through analysis of metrics of genetic diversity by group revealed that domesticated cattle had moderate nucleotide and haplotype diversity which might suggest that they originated from small number of founders while the higher diversity observed in the wild cattle might indicate a bottleneck which was followed by a period of rapid expansion (Grant, 1998; Grant & Bowen, 1998). The combination of high haplotype diversity and low nucleotide diversity in this study can be evidence of past and rapid demographic expansion from a small effective population size (Avise, 2000). Recent studies have provided evidences of population bottlenecks at the time of domestication and breed formation in modern cattle (Bovine HAPMAP Consortium et al., 2009, Villa-Angulo et al., 2009). Apart from the wild cattle with the highest number of non-synonymous mutations, *Bos indicus* subspecies was found to contain a higher proportion of the total number of non-synonymous mutations compared to the total number of synonymous mutations suggesting that the mutations are evolutionarily recent events following the divergence of *Bos indicus* and *Bos taurus* which is estimated to have diverged as early as 200,000 years ago (Loftus et al., 1994). Future studies with more samples across the breeds which will give additional polymorphisms and haplotypes will be necessary to ascertain evolutionary history of this immune genes.

We found nineteen haplotypes that differ from each other by a small number of mutations with few of these haplotypes being shared among the *Bos taurus*, *Bos indicus* and their hybrid while majority of the haplotypes are specific to each subspecies which corroborate the assertion that the 250 Kyr divergence between these two subspecies has allowed genetic drift and or selection to drive different haplotypes to a high frequency between the subspecies (Bradley et al., 1996; Seabury et al., 2010). Haplotype sharing between these two subspecies lineages may suggest retention of conserved ancestral variation that predates subspecies divergence, or that both lineages have evolutionarily converged on a relatively small number of innate immune haplotypes at these loci (Seabury et al., 2010). Haplogoup C3

combines the two subspecies with their hybrid and can be regarded as the ancestral haplotype from which the other two radiate. The existence of this haplogroup with the other peripheral haplogroups as a star is an indication of population expansion from a small number of founders (Grant, 1998; Grant & Bowen, 1998). On the other hand, our results indicated that the dominant haplogroup C3 was the main founding haplotype of domesticated TLR5 since these haplogroup was detected in individuals of the *Bos taurus, Bos indicus* and their crossbred. The occurrence of no haplotype sharing between the domesticated and the wild cattle suggests that the evolution of this gene in the two groups is independent of each other. The presence of two unique haplotypes in N'Dama which is an African taurine suggests that the N'Dama although a taurine cattle, has been subjected to different evolutionary processes than the other taurines in the African sub-continent and might have contributed to its special adaptive features.

The phylogenetic relationship corroborated the result of the network analysis and revealed little or no introgression of the genetic material of the wild cattle in to the domestic cattle except for the sharing of mutation between the taurine N'Dama and the wild cattle and their grouping close together using sequences of TLR5. For the domestic cattle, a single maternal origin was predicted for the two subspecies before the recent sub speciation around 200,000 years ago. Our results also revealed introgression and exchange of genetic materials among the breeds of *Bos taurus* and *Bos indicus*. Despite the fact that our N'Dama cattle samples were from different locations, they were grouped into one cluster while some samples formed two other smaller clades. The phylogenetic relationship of the *Bos indicus* had shown that the breed Romangnola, Piedmontese, Braford were descendant from European cattle and they were from the same lineage with Brahman cattle, while *Bos indicus*, N'Dama and Sahiwal were closely related and are of African descent (Beja-Pereira et al., 2006). *Bos taurus* phylogenetic relationship also showed the relationship between all their breeds, indicating their descendant from the same ancestral lineage despite acquiring different polymorphisms and are slightly different from the N'Dama cattle because of their different evolutionary history having been subjected to different environmental conditions with different evolutionary processes (Beja-Pereira et al., 2006; Hanotte, 2000).

CONCLUSION

Our analysis of genetic variation, nucleotide diversity, haplotype network and phylogenetic relationship of bovine Toll-like receptor 5 immune gene will provide additional information in the study of the relationship between the mutations within this gene and the differential susceptibility and or tolerance or resistant to various diseases caused by bacteria, fungi and protozoans in future studies. Based on the use of TLR5 in bovine species, highest number of non-synonymous mutations was observed

in the wild cattle, followed by *Bos indicus* subspecies and the *Bos taurus* with a higher ratio of total number of non-synonymous mutations to that of synonymous mutations supporting the fact that the gene is evolving under adaptive evolution. The occurrence of sub specific haplotype sharing between the *Bos taurus* and *Bos indicus* with their hybrid led credence to the evidence of interbreeding between these subspecies. The separate clustering of the wild cattle from the domestic one except for sharing of mutation with N'Dama support the fact that the domestic cattle descends from aurochs with little or no introgression from the wild cattle.

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