Morphological and Molecular Characterization of *Amblyomma variegatum* (Acari: Ixodidae) Ticks from Nigeria

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**SUMMARY**

The association of most tick-borne pathogens with specific tick species has made it imperative that proper identification and characterization of such tick vectors is necessary for the purpose of developing effective tick and tick-borne control strategies. This study was undertaken to identify and characterize *Amblyomma* species ticks collected from cattle in Plateau State, North-Central, Nigeria. They were morphologically identified using diagnostic characters. Further confirmation and characterization was done genetically using a 460bp-long partial fragment of the 16S rRNA gene amplified by polymerase chain reaction (PCR). The amplified fragment was cloned and sequenced for the phylogenetic dendogram. All the examined ticks were identified as *A. variegatum* which was confirmed by 16S rRNA gene sequences analysis, and phylogenetic inferences showed a 99% similarity and grouping to *A. variegatum* of African origin. However, the *A. variegatum* sequences from Nigeria were clustered into 2 groups, but formed a distinct clade from the *A. variegatum* sequence from Ethiopia. This study was able to conclusively identify and characterize *A. variegatum* ticks from the study areas by utilizing morphology and molecular genotyping based on sequencing of the 16S rRNA gene.

**Key words:** *Amblyomma variegatum*, Morphology, 16S rRNA, PCR, Sequencing.

**INTRODUCTION**

The genus *Amblyomma* has 130 valid species most of which are known to transmit a wide variety of bacterial, fungal, protozoan, rickettsial, and viral agents in man, companion and domestic animals. Guglielmone *et al.*, 2010). Besides their manifested abilities as vectors, they also inflict notable dermatitis and predispose their hosts to other arthropod-borne infestations such as myiasis. (Molia *et al.*, 2008). Most of the *Amblyomma* spp ticks so far studied are known to accidentally parasitize humans and potentially act as vectors of zoonoses (Marrelli *et al.*, 2007), and consequently are of significant medical and veterinary importance. Of the above number of *Amblyomma* species in the world, six species (*A. gemma*, *A. lepidum*, *A. maculatum*, *A. nuttalli*, *A. splendidum* and *A. variegatum*) have been reported in
Nigeria (Nuttal et al., 1926; Mohammed, 1974; Talabi et al., 2011; Tonjura et al., 2012; Eyo et al., 2014). However, none of these studies described the morphological features of the different species mentioned, nor were they subjected to any molecular method for confirmation. Also, there are no reference specimen of these species (except for A. variegatum) in the archives for comparison and differential diagnosis. Thus, casting doubts on the authenticity of the mentioned species.

The importance of different stages and species of ticks in disease transmission and as reservoir of infectious pathogens in nature requires proper identification of ticks which is also necessary for field research on tick ecology, and for effective control measures, and veterinary intervention. Traditionally, tick identification is achieved using several developmental stages (larvae, nymphs and adults) based on the external anatomy and morphological features of the ticks. This method of identification could be laborious, difficult and sometimes impossible especially when using phenotypic characteristics which sometimes overlap between individual species or when dealing with the immature stages or females of some tick species (Nava et al., 2009). These difficulties have led to the use of other techniques either solely or in combination with the phenotypic characteristics in the identification and understanding of the relationship of ticks (Mangold et al., 1998). Advances in molecular biology techniques have made the identification of tick species using any of the tick stages or eggs, easy (Zahler et al., 1995). Such techniques include DNA sequencing of molecular markers such as internal-transcribed-spacers (ITS 1&2), 16S, 12S, and 18S rDNA genes (Norris et al., 1999; Labruna et al., 2007; Marrelli et al., 2007). Previous studies have demonstrated that these genes provide useful markers for the genetic identification and characterization studies, and the phylogenetic relationships of ticks and their pathogens at different taxonomic levels (Chao et al., 2009; Chitimia et al., 2010).

Molecular characterisation and Phylogenetic studies on ticks is imperative as such study has not been hitherto carried out in the study area. This preliminary study, thus focused on the use of morphological and molecular tools to identify and characterize A. variegatum ticks collected from cattle in the Guinea Savanna vegetation zone of Plateau state, Nigeria. We also inferred the phylogenetic position of sequences of A. variegatum species from our study areas with respect to other Amblyomma species from other parts of the world based on the analysis of mitochondrial 16S rRNA gene sequences of the ticks.

MATERIALS AND METHODS

Tick Collection and Preservation

Partially fed Amblyomma sp. ticks (n=200) were collected from 50 cattle owned by Fulani pastoralists in three Local Government Areas (LGA) of Plateau State, Nigeria. These local government areas include: (Barkin Ladi; 9°32’00”N–8°54’00”E, Mangu; 9°18’00”N–9°11’34”E, and Jos-South; 9°47’00”N–8°51’00”E) which have high concentration of pastoralists in the state (Majekodunmi et al., 2014). The vegetation of our study areas consists of short trees, grasses and plateau type mosaic vegetation, and lies within the Northern Guinea Savanna zone. The area records an average daily temperature range of 23 °C–27 °C and has a tropical climate with a moderate annual rainfall mean of about 1311.75 cm (Odumodu, 1983).

Ticks were carefully removed by grasping with a blunt curved forceps as close to the skin over the scutum and pulled outward with a steady even pressure, ensuring that the mouth parts were completely removed with the whole tick. Damaged ticks were removed and safely disposed while the intact ticks were cleaned by first sterilizing them in sodium hypochlorite and subsequently rinsing twice in distilled water then
preserved in 70% ethanol into which 5% glycerine was added. Morphological identification to species level was done with the aid of a Zeiss stereomicroscope at X60 magnification and Olympus microscope at ×100 magnification. Diagnostic characters of the different parts of the ticks (hypostome, dorsum, legs and spurs, including the shapes and positions of the anal groove, festoons, genital aperture and spiracles) were carefully observed and used in drawing conclusions according to the guidelines for tick identification as provided by Okello-Onen et al., (1999) and Walker et al., (2003). Ten (10) representative tick samples (5 females and 5 males) from the study areas were used for molecular studies.

DNA Extraction from Ticks
Total genomic DNA (50 ng/µL – 80 ng/µL) was isolated from the whole ticks. Briefly, ticks from the areas under study were individually dissected using separate sterile scalpel blades to remove the internal organs. DNA was extracted from all the organs using TriReagent (Sigma–Aldrich, Madrid, Spain) according to the manufacturer’s instructions. Isolated DNA were run on 1% agarose gel to evaluate their integrity. DNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Wilmington, DE, USA), and then stored at −20 °C until used for further analysis.

PCR Amplification, Cloning and Sequencing of the 16S rRNA Gene
Primer sets and conditions for Tick 16S rRNA gene (forward 16S+1 5’- CTG CTC AAT GAT TTT TTA AAT T-3’ and reverse 16S−1 5’-CCG GTC TGA ACT CAG ATC AAG TA-3’) designed by Black & Piesman, (1994) that target the 460bp fragment of 16S rRNA genes for ticks were used. DNA from A. americanum was used as positive control; whereas negative controls containing the reaction mix without DNA were always tested alongside and if there was a reaction the entire mixture were discarded. The amplicons from the above PCR where electrophoresed on 1.5% agarose gel, stained with SYBR Safe DNA gel stain (Invitrogen, Eugene, OR, USA) and size of the amplified fragments were checked by comparing the fragments to a standard molecular weight marker (1 kb MassRuler®, Fermentas). The amplified DNAs were purified using Purelink™ PCR purification kit (Invitrogen™, USA), then cloned into pGEM-T vector (Promega, Madison) and transformed into JM109 high-efficiency competent Escherichia coli cells. Plasmid DNA was purified with the QIAprep® Spin Miniprep kit (Qiagen, Valencia, CA) whilst a Pvu II enzyme kit (Fermentas®, Vilnius) was used to digest the plasmid DNA for size evaluation on 1% agarose prior to sequencing. At least two clones from each purified plasmid were submitted for sequence confirmation in an automatic sequencer (3730 DNA analyzer, Applied Biosystems®, Carlsbad, CA).

Sequence and Phylogenetic Studies
BLAST search for previously reported sequences that are identical to the sequences in this study was done using the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignments were carried out using Clustal W algorithm (Thompson et al., 1994). The output of the tree was constructed with the MEGA 4.0 program (Tamura et al., 2007). For pairwise comparisons, the following 16S rRNA sequences of Amblyomma species deposited in the GenBank were used; A. hebraeum (L34316), A. americanum (L34313), A. variegatum (L34315) and A. cajennense (L34317), while Ixodes scapularis (L34293) was used as out group. The distance matrix was calculated by use of Kimura-2 parameters, whilst 1000 bootstrap replicates were used to estimate the reliabilities of the nodes on the neighbour-joining tree. A
bootstrap value of 70% was considered significant evidence for phylogenetic grouping (Hills and Bulls, 1993).

**RESULTS**

All the 200 (100%) sampled ticks were morphologically identified as *A. variegatum* based on the very colourful scutum which covered the entire dorsum of the male and the anterior one third of the female. The punctations on the scutum were small to medium in size; the basis capituli were rectangular and had posterior margins slightly concave shaped. The ticks examined had long palps with 3 segments; the hypostome were long with dentitions. The festoons of the ticks were prominent and dark coloured, where as the marginale grooves were continous. The legs had brownish colours with white bands at the extremities, the coxae I had two unequal spurs, while coxae IV had a single stout and long spur (the spur on coxae IV of female ticks were short and not as prominent as in the male) The spiracles were large, triangular, with rounded edges and located behind coxae IV (Plate 1).

PCR analysis of the isolated DNA indicates amplification of 460bp. Blast results for our cloned sample sequences across the DDBJ/EMBL/GenBank databases shows that all the submitted samples had 99% similarity to mitochondrial *A. variegatum* 16S rRNA, Genbank accession numbers L34312 and L34315. All the seven sequences of *A. variegatum* obtained in this study were deposited in the GenBank under accession numbers: JF949794 - JF949801.

To access the phylogenetic relationships of *A. variegatum* ticks from Nigeria, a neighbour-joining tree was constructed using 16S rRNA nucleotide sequence alignments based on Kimura two-parameter model (Figure 1). All the *A. variegatum* tick sequences were clustered into one group with 100% bootstrap value. However, the Nigerian *A. variegatum* ticks were separated into two clusters. The first cluster with a bootstrap support value of 99% comprised three sequences (JF949794, JF949795 and JF949798) while the second cluster with 71% bootstrap value had one sequence (JF949799). Also, the high bootstrap value supports the close relationship between the *A. variegatum* ticks and *A. hebraeum* (L34316), both of which are of African origin.

**DISCUSSION**

Tick species belonging to the genus *Amblyomma* are known to be reservoirs and vectors of several disease pathogens that can cause considerable economic losses in livestock in Nigeria, as in most tropical and subtropical regions of the world (Ogo et al., 2012; Vesco et al., 2013). The notoriety of these ticks in terms of damage to the skin of animals and disease transmission has earned them the name “koti” (‘dangerous tick’) amongst the Fulani herdsmen of Nigeria, and are accorded much attention during acaricidal treatment and the routine hand de-ticking exercise (Bayer & Maina 1984).

The identification of ticks in Nigeria is usually achieved using morphological characteristics. However, when sampled ticks become damaged, or closely related species are available this method becomes inadequate and may require a more decisive molecular based method. Our inability to access previously reported species of *Amblyomma* in Nigeria limited this study as it did not afford us the opportunity to draw inferences. However, the presence of several diagnostic characteristic features on the samples studied phenotypically identified them as *A. variegatum*. Confirmation of the species using PCR analysis of the 16S rRNA gene marker produced 460bp fragments which were consistent with the range of fragment lengths of previously reported studies (Guglielmone et al., 2013) on ticks in the same genus using the 16S rRNA gene. This target gene is highly conserved, with large numbers of registered sequences in the
Plate 1: Adult male (A) and female (D) *A. variegatum* showing medium sized punctations (1) on their coloured scutum; B shows banded legs (2), two unequal spurs on coxae 1(3), and a single long spur on coxae IV (4) of a male tick; E, short spur on coxae IV (5) of a female tick and triangular shaped spiracles (6); C, F shows the long hypostome and genital aperture (7) and (8) respectively
GenBank, and is suitable for species identification and validation. In this study, we phylogenetically established the status of *A. variegatum* ticks from Plateau State, Nigeria, based on the partial sequence of 16S rRNA gene. Results of the phylogenetic tree showed a clustering of *A. variegatum* tick sequences into two groups with 99% and 71% bootstrap values. Based on the above values, the possibility of having a diverse population of *A. variegatum* ticks in our study area cannot be ruled out. However, a more detailed phylogenetic study of this tick species needs to be undertaken to confirm any intraspecies variation. This study provides the first genetic characterization of the mitochondrial 16S rRNA gene of *A. variegatum* ticks collected from Nigeria, and supports the use of both morphological and molecular methods in conjunction with bioinformatics tools for the identification of ticks in Nigeria.

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**REFERENCES**


