NIGERIAN VETERINARY JOURNAL

ISSN 0331-3026

Nig. Vet. J., September 2017 Vol 38 (3): 260-267.

ORIGINAL ARTICLE

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

Ogo, N. I.[1]; Okubanjo, O. O. [2]; Inuwa, H. M. [3] and Agbede, R. I. S.[4]

[1]National Veterinary Research Institute, Vom, Plateau State. [2]Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria, Nigeria. [3]Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria. [4]Department of Veterinary Parasitology and Entomology, University of Abuja, FCT, Nigeria. *Corresponding author: Email: ogoendy@yahoo.com; Tel No:+234 8034521514

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´´) 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 Savannah 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% boostrap value. However, the Nigerian *A. variegatum* ticks were separated into two clusters. The first cluster with a boostrap support value of 99% comprised three sequences (JF949794, JF949795 and JF949798) while the second cluster with 71% boostrap value had one sequence (JF949799). Also, the high boostrap 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

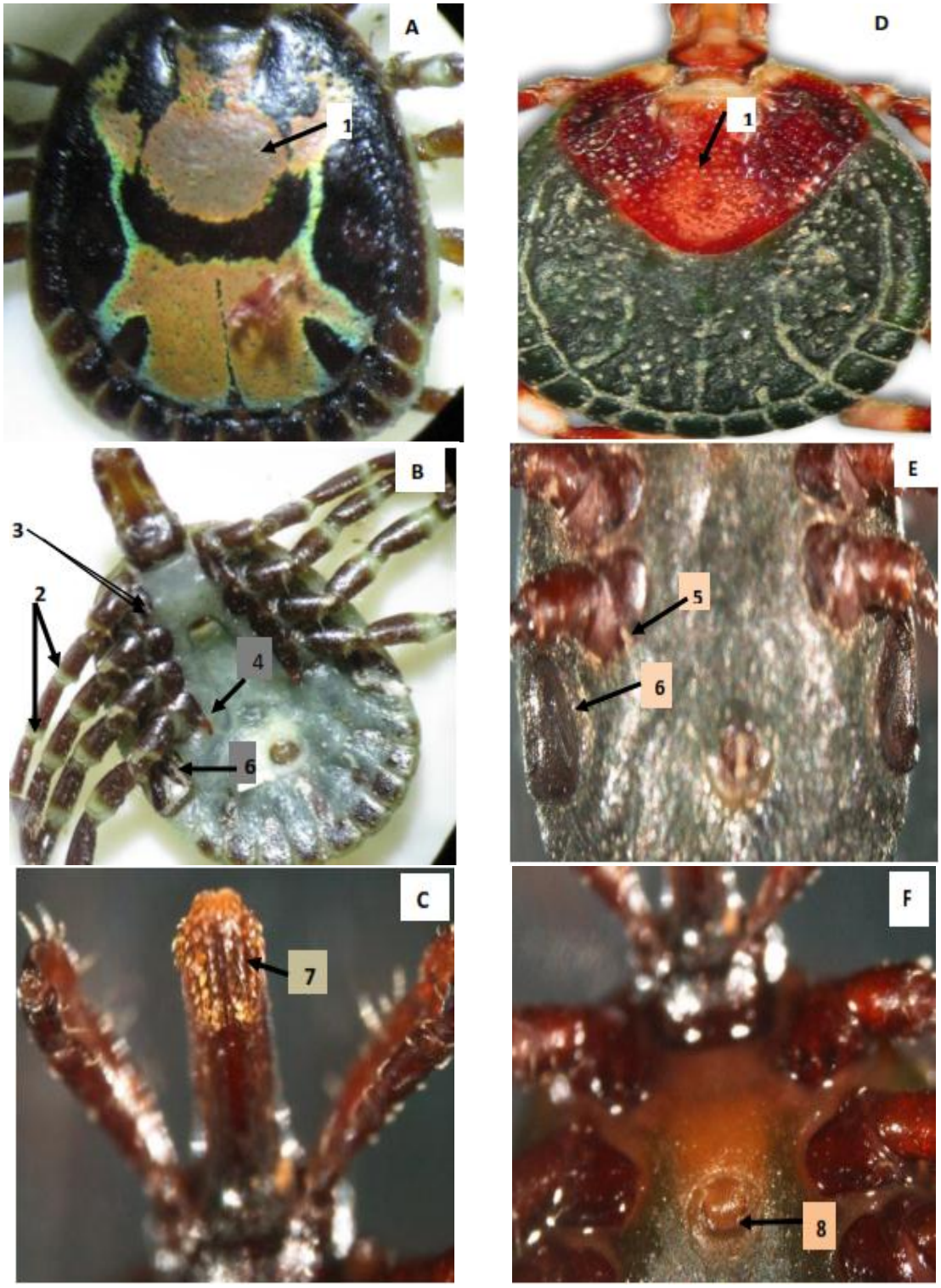

Plate 1: Adult male (A) and female (D) *A. variegatum* showing medium sized punctations (1) on their coloured scutum; B shows banded legs (2), two unequal spurs on coxae 1(3), and a single long spur on coxae IV (4) of a male tick; E, short spur on coxae IV (5) of a female tick and triangular shaped spiracles (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% boostrap 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.

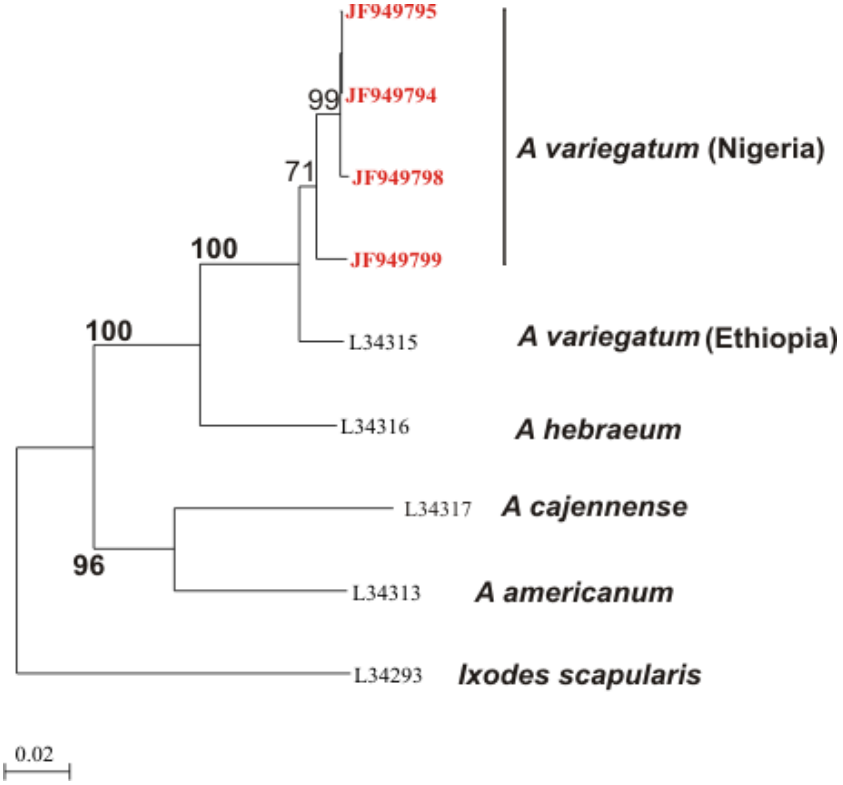

Figure 1: Phylogenetic relationships of 16S rRNA gene sequences of *A. variegatum* ticks from Nigeria and 5 *Amblyomma* species based on neighbour-joining reconstruction using the Kimura two-parameter distance. Bootstrap values are shown above the nodes. The scale bar indicates the nucleotide substitution per site. *A. variegatum* sequences detected in this study (JF949795, JF949794, JF949798 and JF949799) are highlighted in red. *Ixodes scapularis* (GenBank number L34293) was used as outgroup species

ACKNOWLEDGEMENT

Authors are grateful to the management of National Veterinary Research Institute, Vom, Plateau State, Nigeria for their permission to conduct this study.

REFERENCES

BAYER, W., and MAINA, J.A. (1984). Seasonal pattern of tick load in Bunaji cattle in the subhumid zone of Nigeria. *Veterinary Parasitology*. **15**: 301-307.

BLACK, W.C., and PIESMAN, J. (1994). Phylogeny of hard ticks and soft-ticks taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proceedings of the National Academy Science*. **91**: 10034-10038.

CHAO, L., WU, W. and SHIH, C. (2009). Molecular analysis of *Ixodes granulatus*, a possible vector tick for *Borrelia burgdorferi* sensu lato in Taiwan. *Experimental & Applied Acarology*. **48**: 329–344.

CHITIMIA, L., LIN, R.Q., COSOROABA, I., WU, X.Y., SONG, H.Q., YUAN, Z.G., and ZHU, X.Q. (2010). Genetic characterization of ticks from Southwestern Romania by

sequences of mitochondrial cox1 and nad5 genes. *Experimental & Applied Acarology*. **52**(3):305-11.

EYO, J.E., EKEH, F.N., IVOKE, N., ATAMA, C.I., ONAH, I.E., EZENWAJI, N.E., and IKELE, C.B. (2014). Survey of Tick Infestation of Cattle at Four Selected Grazing Sites in the Tropics. *Global Veterinaria*. **12**: 479-486.

GUGLIELMONE, A.A., NAVA, S., MASTROPAOLO, M. and MANGOLD, A.J. (2013). Distribution and genetic variation of *Amblyomma triste* (Acari:Ixodidae) in Argentina. *Ticks & Tick Borne Diseases*. 386-390.

GUGLIELMONE, A.A., ROBINS, R.G., APANASKECH, D.A., PETNEY, T.N., ESTRADA-PENA, A., HORAK, I.G., SHAO, R. and BARKER, S.C. (2010). The argasidae, ixodidae and nuttalliellidae (Acari: Ixodida) of the world: A list of valid species names. *Zootaxa*. **2528**: 1-28.

HILLS, D.M., and BULL, J.J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis, *Systemic Biology*. **42**: 182–192.

LABRUNA, M.B., SANFILIPPO, L.F., DEMETRIO, C., MENEZES, A.C., PINTER, A., GUGLIELMONE, A.A. and SILVEIRA, L.F. (2007). Ticks collected on birds in the state of Sao Paoulo, Brazil. *Experimental & Applied Acarology*. **43**: 147-160.

MAJEKODUNMI, A.O., FAJINMI, A., DONGKUM, C., SHAW, A.P.M., and WELBURN S.C (2014). Pastoral livelihoods of the Fulani on the Jos Plateau of Nigeria. *Pastoralism: Research, Policy and Practice* 2014, 4:20

MANGOLD, A.J., BARGUES, M.D., and MAS-COMA, S. (1998). Mitochondrial 16S rRNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among Metastriata. (Acari: Ixodidae). *Parasitology Research*. **84**: 478–484.

MARRELLI, M.T., SOUZA, L.F., MARQUES, R.C., LABRUNA, M.B., MATIOLI, S.R., TONON, A.P., RIBOLLA, P.E.M., MARINOTTI, O. and SCHUMAKER, T.T.S. (2007). Taxonomic and Phylogenetic Relationships between Neotropical Species of Ticks from Genus *Amblyomma* (Acari: Ixodidae) Inferred from Second Internal Transcribed Spacer Sequences of rDNA. *Journal of Medical Entomology*. **44**: 222-228.

MOHAMMED, A.N. (1974). The seasonal incidence of ixodid ticks of cattle in Northern Nigeria and in the Netherlands, with particular reference to their role in the transmission of bovine piroplasms. Ph.D Dissertation, Ahmadu Bello University, Zaria: Nigeria.

MOLIA, S., FREBLING, M., VACHIERY, N., PINARELLO, V., PETITCLERC, M., ROUSTEAU, A., MARTINEZ, D. and LEFRANCOIS, T. (2008). *Amblyomma variegatum* in cattle in Marie Galante, French Antilles: Prevalence, control measures, and infection by *Ehrlichia ruminantium*. *Veterinary Parasitology*. **153**: 338-46.

NAVA, S., GUGLIELMONE, A.A. and MANGOLD, A.J. (2009). An overview of systematics and evolution of ticks. *Frontiers in Biosciences.* **14**: 2857-2877.

NORRIS, D.E., KLOMPEN, J.S.H. and BLACK, W.C. (1999). Comparison of the mitochondrial 12S and 16S ribosomal DNA genes in resolving phylogenetic relationships among

hard ticks (Acari: Ixodidae). *Annals of Entomological Society of America*. **92**: 117–129.

NUTTALL, G.H., WARBURTON, C. and ROBINSON, L.E. (1926). Ticks: A monograph of the Ixodoidea. Part IV. The genus *Amblyomma*. Cambridge University Press; London.

ODUMODU, L.O. (1983). Rainfall distribution, variability and probability in Plateau State, Nigeria. *International Journal of Climatology*. **3**: 385-393.

OGO, I.N., FERNÁNDEZ DE MERA, I.G., GALINDO, R.C., OKUBANJO, O.O., INUWA, H.M., AGBEDE, R.I.S., TORINA, A., ALONGI, A., VICENTE, J., GORTÁZAR, C., and DE LA FUENTE, J. (2012). Molecular identification of tick-borne pathogens in Nigerian ticks. *Veterinary Parasitology*. **187**: 572–577.

OKELLO-ONEN, J., HASSAN, S.M. and ESSUMAN, S. (1999). Taxonomy of African ticks. An identification Manual. ICIPE Science Press, Nairobi.

TALABI, A.O., OYEKUNLE, M.A., OYEWUSI, I.K., OTESILE, E.B., ADELEKE, G.A. and OLUSANYA, T.P. (2011). Haemo and Ecto-Parasites of Cattle in the Trans-Boundary Areas of Ogun State, Nigeria**.** *Bulletin of Animal Health and Production in Africa*. **59**: 68406.

TAMURA, K., DUDLEY, J., NEI, M. and KUMAR, S. (2007). MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.1. *Molecular Biology & Evolution.* **24**: 1596-1599.

THOMPSON, J.D., HIGGINS, D.G. and GIBSON, T.J. (1994). CLUSTAL, W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. **22**: 4673–4680.

TONGJURA, J.D.C., AMUGA, G.A., OMBUGADU, R.J., AZAMU, Y. and MAFUIYA, H.B. (2012). Ectoparasites infesting livestock in three local government areas (LGAs) of Nasarawa state, Nigeria. *Science World Journal*. **7**: 15-17.

VESCO, U., KNAP, N., LABRUNA, M.B., AVSIC-ŽUPANC, T., ESTRADA-PEÑA, A., GUGLIELMONE, A. A., BECHARA, G. H., GUEYE, A., LAKOS, A., and GRINDATTO, A. (2013). An integrated database on ticks and tick-borne zoonoses in the tropics and subtropics with special reference to developing and emerging countries. *Experimental & Applied Acarology*. **54**: 65-83.

WALKER, A.R., BOUATTOUR, A., CAMICAS, J.L., ESTRADA-PENA, A., HORAK, I.G., LATIF, A.A., PEGRAM, R.G. and PRESTON, P.M. (2003). Ticks of Domestic Animals in Africa: A Guide to Identification of Species. Bioscience Reports, Edinburgh, United Kingdom.

ZAHLER, M., GOTHE, R., and RINDER, H. (1995). Diagnostic DNA amplification from individual tick eggs, larvae and nymphs. *Experimental & Applied Acarology*. **19**: 731-736.