sciendo

DOI: 10.2478/fv-2021-0028

FOLIA VETERINARIA, 65, 3: 60—68, 2021

MOLECULAR AND BIOLOGICAL CHARACTERIZATION OF *HAEMAPHYSALIS LEACHI* (ACARI: IXODIDAE) IN NIGERIA WEST AFRICA

Kamani, J.[1], Kadir, R. A.[2], Mshelbwala, F. M.[3], Olubade, T.[1]
Shekaro, A.[1], Balami, P. U.[1], González-Miguel, J.[4]

[1]National Veterinary Research Institute (NVRI), PMB 01 Vom, Plateau State
[2]Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Ilorin
[3]Department of Veterinary Pathology, College of Veterinary Medicine
Federal University of Agriculture (FUNAAB), Abeokuta
Nigeria
[4]Laboratory of Parasitology, Institute of Natural Resources and Agrobiology of Salamanca
(IRNASA-CSIC), C/ Cordel de Merinas 40-52, 37008, Salamanca
Spain

mshelizakj@gmail.com

ABSTRACT

Ticks constitute a serious threat to the wellbeing of humans and other animals. The accurate identification of ticks is paramount in epidemiological investigations. Genetic markers have been identified and used to overcome the limitations of phenotypic identification of ticks. In this study, the cytochrome c oxidase 1 (*Cox1*) gene was amplified and sequenced for the identification of *Haemaphysalis leachi*, the putative vector of *Babesia rossi* in Nigeria. Amplification was successful in 34 out of 39 (87 %) ticks collected from dogs in three Nigerian states with sequence homology of 99 % to *H. leachi* in Genbank (GenBank: MN663156.1). Maximum Likelihood phylogenetic analysis showed significant grouping of *H. leachi* sequences in independent monophyletic subclade with a bootstrap value of 100 %. Genetic distance analysis of *H. leachi* identified in this study indicated a very low level of intraspecific diversity (0.016 %) compared to 0.150—0.190 % interspecific distance to other *Haemaphysalis* species. The number of eggs laid by engorged female ticks maintained in the laboratory ranged from 885 to 2190 and was proportional to the ticks' initial weight. The mean value of other biological parameters; female engorgement weight, pre-oviposition period, oviposition period, total egg mass, egg size, efficiency rates of female ticks in converting their food reservoir to eggs and incubation period are, 147.5 mg, 7.8 days, 13.2 days, 59.5 mg, 485.5 × 348.7 μm, 41.2 % and 26.2 days, respectively. This study reports the first molecular identification of *H. leachi* in Nigeria.

Key words: *Cox1*; identification; Ixodidae; Nigeria; PCR; sequencing

INTRODUCTION

There are nearly 170 described species of ticks in the genus *Haemaphysalis* Koch, 1844 (Acari: Ixodidae) worldwide [12]. The subgenus *Rhipistoma* Koch, 1844 has 33 species occurring in a wide range of climatic regions ranging from rain forest to desert areas mostly in sub Saharan Africa, as well as in Europe, and Asia [12, 29]. Members of this subgenus primarily parasitize carnivores, rodents, hyraxes and hedgehogs [12, 15]. Renewed interest in the study of *H. (R.) spinulosa* subgroup of the *H. (R.) leachi* has led to the redescription of new taxa in addition to the four existing valid taxa in the group [2, 3, 28, 29]. In the tropical regions, ticks are ranked second to mosquitoes in terms of veterinary and public health importance. Specifically, *H. leachi* has been incriminated in the transmission of the infectious agent *Babesia rossi* to dogs and *Rickettsia conorii* to humans among others [18, 23].

Phenotypically, *H. leachi* possesses characteristic conspicuous lateral extensions to palp articles 2, forming mouthparts with a distinctive conical shape. Other distinctive features are the presence of medium length spurs on the coxae 4 of males compared to very conspicuous spurs on coxae 4 of *H. punctata* and *H. sulcata*. The presence of spurs on the ventral surface of palp articles 2 of female *H. spinulosa* differentiates it from *H. leachi*. However, *H. paraleachi*, *H. punctaleachi* and *H. moreli* are difficult to distinguish from *H. leachi* [29]. Therefore, ticks in the *H. (R.) leachi* group have been considered one of the most difficult from a taxonomic point of view [3]. This then calls for caution before making definite statements about the identity of specimens within this group. In this context, the conventional methods based on morphology and tick's ecology for species determination is inadequate, especially when dealing with morphologically similar taxa, damaged specimens, and where immature stages are not described or are engorged [22, 30]. This can be overcome by using molecular markers which are highly conserved and easy to amplify suitable regions of the genome using the polymerase chain reaction (PCR), sequence analyses and alignment of the data with reference sequences [4, 20].

The mtDNA encoded cytochrome oxidase subunit I (*Cox1*) gene has been identified as a species-level marker that produces a high standard barcode for phylogenetic and taxonomic studies of arthropods including ticks [9, 19, 20]. To date, there is no such study on identification of *Haemaphysalis* spp. using a well-defined molecular approach in Nigeria. Therefore, the aim of this study is to utilize the *Cox1* gene to verify the morphological status of *H. leachi* and to determine its biological characteristics in this African country.

MATERIALS AND METHODS

Ethical statement

Approval for this study was granted by the Animal Use and Care Committee (AUCC), National Veterinary Research Institute (NVRI) Vom, Nigeria, number AEC/03/21/15. Oral consent for permission to collect the ticks was obtained from dog owners.

Collection of ticks

Ticks were collected from naturally infested owned dogs in households or in veterinary clinics in 11 states and the Federal Capital Territory (FCT) Abuja, Nigeria between August 2018 to July 2019 (Fig. 1). Ticks were also removed from dog carcasses submitted for post-mortem at the veterinary pathology laboratory, College of Veterinary Medicine, FUNAAB. In all, ticks were removed from 472 dogs using forceps and kept in labelled ventilated tubes according to each host and transported to the Entomology Laboratory, National Veterinary Research Institute (NVRI), Vom, Nigeria.

Morphological identification of ticks

All tick samples were preliminary identified to ge-

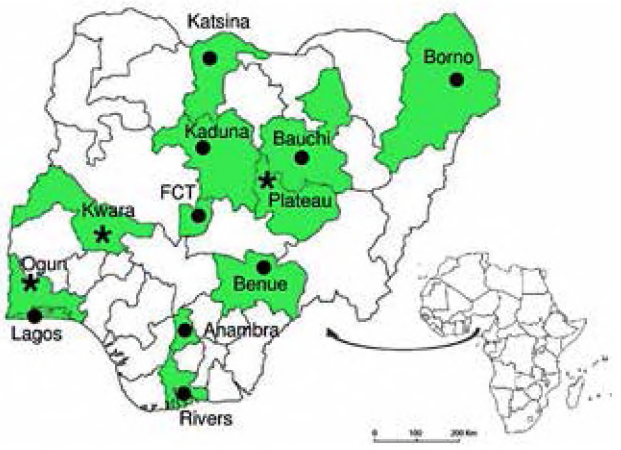

Fig. 1. Map of Nigeria, West Africa showing tick sampling locations (shaded) and states where *H. leachi* were identified (asterisk)

nus-level based on external morphological characteristics under a stereo microscope (Zeiss, Stemi DV4) using specific illustrated morphological taxonomic keys [28, 29]. *Haemaphysalis* ticks were identified based on characteristic conspicuous lateral extensions to palp articles 2, forming mouthparts with a distinctive conical shape, rectangular basis capitulum, absence of eyes, presence of festoons and absence of ventral plates in males [29]. Only ticks morphologically identified to belong to the genus *Haemaphysalis* were included in this study. Partially or fully engorged female ticks were placed individually in labelled ventilated tubes for fecundity studies.

Biological studies

Six engorged female ticks were weighed individually and placed in coded sterile ventilated tubes. The tubes were placed in an incubator at 27 °C, relative humidity of 85 % with 12-hour photoperiod. The ticks were monitored daily for the commencement of egg laying. Ticks were weighed and eggs laid counted daily. Ten eggs were randomly picked from each clutch daily and measured (length and width), under a calibrated microscope (Nikon, Eclipse E100). This was continued until egg laying ceased as evidenced by constant weight of the ticks over five consecutive days. Biological parameters; female engorgement weight (FW), egg mass weight (EMW), pre-oviposition period (POP); incubation period (IP); efficiency rates of female ticks in converting their food reservoir to eggs (ERCE), were calculated according to D i p e o l u et al. [7] and S z a b ó et al. [25].

Molecular identification of ticks

DNA extraction from ticks

Genomic DNA was extracted from 39 adult ticks morphologically identified as *H. leachi*. Ticks were removed from 70 % ethanol and washed in three changes of phosphate buffered saline (PBS), before DNA extraction using the QIAmp DNA Mini Tissue extraction kit (QIAGEN Hilden, Germany) according to manufacturer's instructions with slight modifications. Cell lysis was achieved at 56 °C overnight. Other procedures are as stated in the manufacturer's instructions. Eluted DNA were preserved at –20 °C until PCR analysis.

Amplification of cytochrome c oxidase 1 (*Cox1*) by conventional PCR

The identity of the ticks was molecularly confirmed by PCR targeting the 710 bp of the cytochrome c oxidase (*Cox1*) gene using the primers HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA ATC ATA AAG ATA TTG G-3') under a modified cycling conditions reported by F o l m e r et al. [11]. The reaction was carried out in a volume of 50 µl, which contained 1.25 U Taq DNA polymerase (Fermentas), 5 µl 10× Taq reaction buffer (including 15 mM $MgCl_2$), 5 µl PCR nucleotide Mix (0.2 mM each), 1.5 µl (1 µM final concentration) of each primer, 5 µl template DNA. PCR grade water (BioConcept, Switzerland) was used to make up the volume. The cycling conditions were as follows; an initial denaturation step at 95 °C for 2 min was followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. The PCR was carried out using the Applied Biosystem thermal cycler (GeneAmp 9700) in the Molecular Biology Unit, Parasitology Division, NVRI Vom.

The resulting PCR products were electrophoresed on a 1.5 % agarose gel stained with SafeView stain from Applied Biological materials Inc. (Canada) to check the size of the amplified fragments by comparison to 100 bp DNA Ladder from New England Biolabs (Ipswich, MA, USA) under a blue light transilluminator (Cleaver Scientific UK). The amplified products of the expected size were sequenced at a commercial facility (Macrogen Europe B.V Amsterdam, the Netherlands). The sequences obtained were manually corrected by visual analysis of the electrophoregram using Bioedit v7.0.5.3 [13] and were compared with sequences in the GenBank database by using the BLAST program (http:www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic and pairwise distance analyses

The sequences that allowed the species-level identification were aligned with the corresponding sequences of *Haemaphysalis* tick species available in GenBank using multiple sequence alignment with Clustal Omega (https://www.ebi.ac.uk/Toos/msa/clustalo/). Phylogenetic relationships based on the alignment of *Cox1* sequences were performed to analyze the phylogenetic status of the *H. leachi* in this study. Data were analysed using the Maximum likelihood (ML) approach while *Ixodes ricinus* (GenBank:

AY945440) was used as outgroup. The phylogenetic tree was performed using Kimura 2-parameter as substitution model in MEGA 5 [27]. Branch supports were estimated by the bootstrap analysis of 1000 replicates. Pairwise analysis was conducted on the nucleotide sequences obtained in this study and six nucleotide sequences in GenBank. The distance divergence was computed based on Kimura 2 parameter model.

Statistical analysis

Data generated in this study were entered into an Excel spreadsheet (Microsoft Corporation, Redmond, WA) and analyzed accordingly. Data were summarized using descriptive statistics to obtain the mean, range standard deviation and 95 % confidence interval for each parameter assessed in the study.

RESULTS

A total of 5,670 ticks (nymph, adult and larva) were collected from 472 owned dogs in 11 states of Nigeria and the Federal Capital Territory (FCT), Abuja between August 2018 to July 2019 (Fig. 1). The ticks were identified to genus level under a stereomicroscope based on the diagnostic morphological characters. Among all the 5,670 ticks collected, 166 (2.9 %) of them were morphologically identified to the genus *Haemaphysalis*, Kwara state (n = 4), Ogun state (n = 7) and Plateau state (n = 155) (Fig. 1). Based on the number of *Haemaphysalis* ticks from each study location a subset of 39 were selected for molecular identification. Amplification of the 710 bp of the *Cox1* was successful in 34 specimens; 2/2 (100 %) from Kwara state, 4/5 (80 %) from Ogun state and 28/34 (82 %) ticks from Plateau state. Sequencing results confirmed the ticks as *H. leachi* with 99 % identity to *H. leachi* in Genbank (GenBank: MN663156.1). Sequences generated in the study were deposited in the GenBank under the accession numbers MW558147-MW558149.

Phylogenetic analyses showed significant grouping of *H. leachi* sequences in this study with sequence in GenBank in independent monophyletic subclade with a bootstrap value of 100 %. The phylodendograms showed that the *H. leachi* sequences identified in this study were clustered together and with *H. leachi* from USA (GenBank: MN663156.1) with 100 % bootstrap value. Other *Haemaphysalis* species such as; *H. erinacei*, *H. longicornis*, *H. qinghaiensis* and *H. flava* as well as *Dermacentor* and *Rhipicephalus* species formed distinct clusters (Fig. 2).

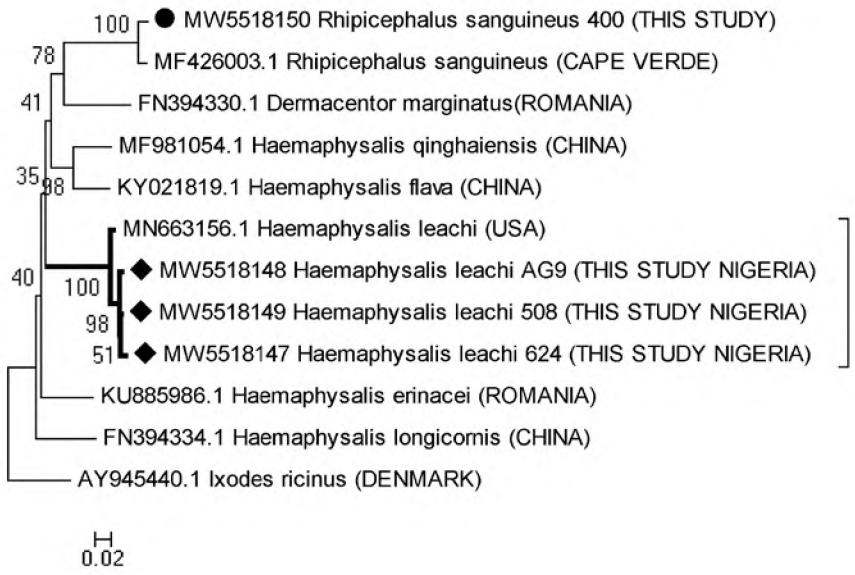

Fig. 2. Maximum Likelihood tree of 12 sequences (including one outgroup) from GenBank and sequences of *H. leachi* (black rhombus) and one sequence of *R. sanguineus* (black circle) identified in the present study. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

Table 1. Pairwise distance of *Cox1* of 3 *H. leachi* from this study with 5 *Haemaphysalis* and 1 *Ixodes* ticks

S/No.	Species (Accession number)	1	2	3	4	5	6	7	8	9
1	***H. leachi* (MW5518149)**	–	0.003	0.004	0.005	0.017	0.016	0.018	0.017	0.020
2	***H. leachi* (MW5518147)**	0.007	–	0.004	0.006	0.019	0.019	0.020	0.020	0.022
3	***H. leachi* (MW5518148)**	0.008	0.009	–	0.005	0.017	0.017	0.019	0.017	0.020
4	***H. leachi* (MN663156)**	0.019	0.022	0.016	–	0.017	0.015	0.018	0.016	0.019
5	***H. erinacei* (KU885986)**	0.150	0.166	0.154	0.145	–	0.016	0.017	0.016	0.019
6	***H. flava* (KY021819)**	0.174	0.190	0.178	0.159	0.138	–	0.015	0.011	0.017
7	***H. longicornis* (MF666902)**	0.182	0.184	0.182	0.171	0.144	0.151	–	0.014	0.018
8	***H. qinghaiensis* (MF981054)**	0.168	0.178	0.164	0.158	0.155	0.098	0.144	–	0.017
9	***I. ricinus* (AY945440)**	0.232	0.232	0.230	0.222	0.176	0.200	0.182	0.215	-

Table 2. Biological parameters of *H. leachi* maintained under standard conditions

Biological parameters		Tick sample code						Range	Mean ± SD
		159	163	597	610	616	588		
FW [mg]		128	124	144	127	150	212	124—212	147.5 ± 33.3
POP [days]		6	9	8	7	9	8	6—9	7.8 ± 1.2
OP [days]		12	11	12	13	15	16	11—16	13.2 ± 1.9
NEL		1690	1407	885	1095	1419	2190	885—2190	1447.7 ± 458.9
EMW [mg]		76	58	36	49	62	76	36—76	59.5 ± 15.6
ES (μm)	**Length**	465	455	474	427	465	465	427—474	485.5 ± 16.6
	Breath	344	316	353	362	352	365	316—365	348.7 ± 17.7
TFW		29	26	27	24	26	48	24—48	30 ± 3.66
WL (%)		99 (77.3)	98 (79.0)	117 (81.3)	103 (81.1)	124 (82.7)	164 (77.4)	98—164 (77.3—82.7)	117.5 ± 25 (79.8 ± 2.2)
ERCE [%]		59.4	46.8	25.0	38.6	41.3	35.8	25—59.4	41.2 ± 11.5
IP [days]		23	28	24	25	29	28	23—29	26.2 ± 2.5

Engorged female weight (FW); egg mass weight (EMW); pre-oviposition period (POP); oviposition period (OP); egg size (ES); incubation period (IP); efficiency rates of female ticks in converting their food reservoir to eggs (ERCE), weight loss (WL); number of eggs laid (NEL); tick final weight (TFW)

The pairwise distance analysis of *H. leachi* showed that the local species is genetically different from *H. leachi* from USA (GenBank: MN663156.1) with low genetic distance value of 0.02 % (Table 1). Genetic distance analysis of *H. leachi* collected from all the three localities also indicated a very low level of intraspecific diversity/variability of 0.016 %. However, interspecific distance analyzed by the pairwise comparison revealed that *H. leachi* sequences from this study genetically differ from other *Haemaphysalis* spp.; *H. erinacei*, *H. longicornis*, *H. qinghaiensis* and *H. flava* with slightly higher genetic variation values from 0.15—0.190 % (Table 1).

The average engorgement weight of female *H. leachi* in this study was 147.5 mg, ranging from 124—212 mg. On the average, a female tick lays 1448 eggs within 11—16 days oviposition period which was preceded by 6—9 days pre-oviposition period. The egg mass was correlated to the initial weight of the ticks (Table 2). The number of eggs laid increased daily reaching a peak at 3—5 days into oviposition before it gradually declined. Similarly, there was a gradual increase in the size of the eggs before it then reduced toward the end of the oviposition period. The maximum size, length × breadth (L × B) of the eggs recorded in this study was 474 μm × 365 μm [(mean ± SD) 485.5 ± 6.6 × 348.7 ± 17.7)]. The efficiency rates of female ticks in converting their food reservoir to eggs was 25—59.4 % resulting in the loss of 77.3—82.7 % of the initial weight (Table 2). The eggs were spherical to oval in shape and dark brown to mahogany in colour with glistening surfaces.

DISCUSSION

To a large extent, tick identification in Nigeria and other less scientifically developed countries relies on morphological criteria, which is fraught with challenges especially when dealing with ticks of similar morphological features, those with damaged mouth parts or immature stages [19, 30]. However, accurate identification of ticks is of great significance for disease investigation and formulation of effective tick control measures. *Haemaphysalis leachi*, also known as the yellow dog tick, is a three-host tick adapted to feeding on domestic dogs in tropical and sub-tropical areas, especially in sub-Saharan Africa [29]. The difficulty of the determination of ticks belonging to the *H. (R.) leachi* group from a taxonomic point of view has been reported [2]. Therefore, the main objective of this study was to employ PCR and sequencing approaches in order to overcome the limitations associated with the phenotypic method of tick identification and to obtain data on some biological characteristics of *H. (R.) leachi* group in Nigeria.

Ticks collected from different ecological zones of Nigeria and morphological identified as belonging to the genus *Haemaphysalis* were subjected to PCR targeting the *Cox1* gene. The results from this study showed that the *Cox1* gene was successfully amplified from ticks sampled from three states in Nigeria with high sequence identity to *H. leachi* in GenBank confirming for the first time by molecular approach the presence of this tick species in Nigeria. Indeed, the phylogenetic tree inferred by the ML method placed the sequences of *H. leachi* from this study in the same cluster with *H. leachi* sequences in the GenBank. So far, this tick species has been morphologically identified on dogs in three states located in the Guinea and the Savanna regions of Nigeria [17]. The results in this study supports the earlier morphological identification of *H. leachi* from some parts of Nigeria. It can be surmised from both the morphological and molecular studies that *H. leachi* is present in the Guinea and Savanna, but not the Sahel region of Nigeria. The low rainfall and high ambient temperature characteristic of the Sahel ecological zone of Nigeria may not favour the survival of this tick.

Pairwise and phylogenetic analyses revealed that genetic diversity at both inter- and intra-species levels between the tick samples in Nigeria and sequences deposited in GenBank was low; 0.007—0.82. This was depicted in the high bootstrap value in the phylogenetic tree, attesting to the utility of the mitochondrial *Cox1* gene is for determination of genetic variation either by interspecies or intraspecies of ticks. Similar findings have been reported among *H. punctata*, *H. hystricis* and *H. bispinosa* in Romania, Malaysia and India, respectively [4, 5, 9, 10]. Of interest is the fact that *H. leachi* has been reported as the putative vector of *B. rossi* [29], which is considered the most pathogenic of the large babesias infecting dogs [21]. Indeed, pallor of the mucosa was observed in carcasses of dogs examined at the Department of Veterinary Pathology, FUNAAB, where some of the ticks were collected. This could be due to anaemia as a result of blood sucking by the ticks or the effects of haemoparasite transmitted by the ticks. The confirmation of its presence in some states of Nigeria may explain

the frequent detection of *B. rossi* in Nigerian dogs [1, 16, 24]. Some doubts remain, however, as to whether *H. leachi* or *H. elliptica* or both are involved in the transmission of *B. rossi* to dogs, following the reclassification of *H. leachi* to *H. elliptica* in South Africa by Apanaskevich et al. [3]. It is intriguing that some yellowish coloured ticks sampled from dogs in this study did not yield readable sequences in the *Cox1* reaction. Indeed, the ticks phenotypically identified and confirmed as *H. leachi* by PCR and sequencing in this study were dark brown in colour, possibly due to blood engorgement. Therefore, more studies are needed in order to update, reconcile and resolve earlier phenotypic and genotypic identities of these species. None of the samples tested in this study had similar identity with *H. elliptica* sequences in GenBank on BLASTn. In fact, *H. elliptica* did not appear on the BLASTn search list. This may be due to lack of the sequences of *H. elliptica* in the GenBank, underscoring to the need for more studies on the members of *Haemaphysalis* genus.

Biological characteristic determined in this study showed that the mean female engorgement weight of *H. leachi* was 1147.5 mg. Several factors such as climate, hosts and tick species have been reported to influence the engorgement weight of female ticks [6, 26]. The daily egg laying in *H. leachi* follows a Type 1 pattern where there was an initial low egg production followed by a gradual increase reaching peak production before it starts to decline as previously observed in *A. ariegatum* [7]. The total number of eggs laid by individual *H. leachi* varied proportionately with initial engorgement weight in accord with earlier observation for other tick species [8, 14, 26]. This could be explained by the fact that the blood meal that constitute the bulk of the tick mass is required for the egg production process as well as other biological activities of the tick during the period of oviposition [6, 26]. This could be surmised from the fact that approximately 80 % of the initial weight of the ticks was lost during the period of oviposition. However, it has also been reported that there is a maximum effective engorgement weight beyond which the number of eggs laid is not influenced by the engorgement weight [6]. This phenomenon was not observed in the *H. leachi* ticks collected in this study. Other biological parameters such as, the POP, EMW, IP and the ERCE values obtained for *H. leachi* in this study differed from those reported for *R. sanguineus* collected from dogs [14, 26]. Furthermore, the egg morphology of *H. leachi* in this study differs from those of *R. sanguineus, A. maculatum* and *D. variabilis* [6, 7, 8]. This could be due to tick species and /or host differences.

CONCLUSIONS

In conclusion, the identity of *H. leachi* has been confirmed in Nigeria by the amplification and sequencing of the *Cox1* gene, also, preliminary data on its biological characteristic has been provided. As the putative vector of *B. rossi*, veterinarians should be cautious and accord priority to the proper diagnosis and treatment of this protozoan parasite as well as the control of this tick species. Additional studies are required to ascertain the various species of the *Haemaphysalis* genus in the various ecological zones of Nigeria and to determine their veterinary and public health significance.

ACKNOWLEDGEMENTS

The authors are grateful to all our field staff who facilitated the ticks sample collection.

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Received May 7, 2021
Accepted August 17, 2021