

# Phylogeny and genetic relationship between hard ticks (Ixodidae) infesting cattle collected from selected areas of a wildlife-livestock interface ecosystem of Mikumi National Park, Tanzania.

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## Research

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# Abstract

## Background:

There is substantial increase in the number of tick species and tick-borne infectious agents in Tanzania. Due to their impact on human, livestock and wild animal health, increased knowledge of ticks is highly needed. So far, no published data on the phylogeny and the genetic distance between hard ticks collected from cattle is available in Tanzania.

## Methods

Ticks from cattle in 9 wards, which lie at the border of Mikumi National Park, were collected in dry season (November and December) 2019. Morphological identification of ticks was initially performed to genus level. To identify ticks to species level, molecular analysis based on mitochondrion 16S rRNA gene was performed. The evolutionary relationships and genetic distance between ticks were determined using Maximum Likelihood and Kimura 2-parameter methods respectively.

## Results

On the basis of morphology, two genera (*Rhipicephalus* and *Hyalomma*) were identified of the 630 adult ticks collected from a total of 252 cattle. Six species; *R. microplus*, *R. evertsi*, *H. marginatum*, *H. rufipes*, *H. truncatum* and *H. turanicum* were confirmed by BLASTn and phylogenetic analysis. The considerably mean and pairwise genetic distances for *Rhipicephalus* and *Hyalomma* genera were observed, whereas, the high overall mean and pairwise genetic distances were also recorded.

## Conclusion

The presence of different (clusters) phylogroups and considerably mean and pairwise genetic distances observed, reflecting possible biological diversity of hard ticks present in the study area. The outcomes of this study will be useful in the planning of integrated control strategies for ticks and tick-borne diseases in Tanzania.

## Background

Ticks are obligate blood-sucking ectoparasites of mammals, birds and reptiles (1). They are responsible for severe economic losses in livestock production (2). Through blood sucking, ticks can cause reduction in livestock weight, limit livestock production and induce anemia (2). Tick bites may cause irritation which leads to secondary bacterial infections, together resulting in reduced quality of hides (2). Certain tick species inject toxin to animals, which cause paralysis when feeding blood (1). In addition, ticks are vectors of several pathogens; viruses, bacteria, protozoa and filarial nematodes, which can cause

diseases in human, livestock and wild animals (3). Till recently, about 800 tick species are known worldwide, the most of which belong to the two main families, Ixodidae (hard ticks) and Argasidae (soft ticks) (1). Ixodidae (hard ticks) is the largest family of ticks, which are of great economic importance due to their negative social-economic impact on agriculture (1, 2, 3). Acaricides have been the first choice in tick control for cattle farmers, but hard ticks (Ixodidae) rapidly acquire resistance to these chemicals (4). Replication slippage and recombination drive genetic diversity in tick populations (4, 5, 6) generating point mutations and frame shifts within the genes targeted by acaricides, resulting in resistance (4, 7). In addition, resistance can quickly accumulate in a population due to the mating structure of ticks and their ability to produce multiple generations within one season (5, 8). It has, therefore, become increasingly important to clarify the level of genetic variation of hard tick population for the better development of tick control strategies.

Mikumi National Park is located in the Morogoro region of Tanzania and lies between latitudes 7° and 10° south of the equator and between longitudes 36° and 37° East of Greenwich (Fig. 1). This ecosystem is inhabited by a wide variety of wild animals and arthropods. Hence, the area is considered to be one of the epidemic foci of tick species and possibly tick-borne diseases (9, 10). In areas which lie at the border of Mikumi National Park, people practice nomadic pastoralism, keeping large number of indigenous cattle. Several cases of high tick infestation and tick-borne infections have been reported in livestock there in recent years (10, 11). Moreover, cattle trade in the country is largely unregulated, creating risks of tick and tick-borne disease dissemination that require detailed investigation. Studies on ticks prior to the present study have been conducted and three tick genera; *Hyalomma*, *Rhipicephalus* and *Amblyomma* were reported to be endemic (12, 13, 14).

However, there is no phylogenetic and genetic relationship (genetic distance) data for ticks of the family Ixodidae (hard ticks) infesting cattle in this area that have been published so far. Phylogeny utilizes the evidence of evolutionary relationship of species, whereas, genetic distance utilizes evidence from evolutionary divergence of species, both determine the genetic relationships of populations within species and was the main aspects of this study. The phylogenetic and genetic relationship data of Ixodidae ticks will provide valuable information to geneticists, farmers and acaricide suppliers about the differences and similarities of Ixodidae tick populations in the area. Therefore, accurate data from field studies is needed to inform rational control strategies and establish models to predict the changing epidemiology of ticks, tick-borne diseases and economic impact on livestock production.

In the present study, we analyzed the phylogeny and genetic relationship (genetic distance) of the hard tick of the genera *Hyalomma* and *Rhipicephalus* infesting cattle from the wildlife-livestock interface ecosystem of Mikumi National Park, Tanzania. Phylogeny and genetic distance of ticks were performed using the mitochondrion 16S rRNA gene. Because of the genetically high conservation and strictly maternal inheritance, the 16S rRNA gene appear to provide a reliable and convenient method for distinguish the lineage among diverse populations of hard ticks.

## Methods

# Ticks collection

Ticks from cattle were collected in dry season (November and December) 2019 in 26 survey points of the 9 wards which lie at the border of Mikumi National park, Morogoro region, Tanzania (Geographical coordinates S 7°00' to 8°00' and E 037°00' to 037°40') (Fig. 1). Cattle were restricted and kept standing and all body parts were examined. Only adult ticks were collected by plucking using blunt forceps. Collected ticks were preserved in 70% ethanol and stored at -20 °C in the laboratory, at the Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, Tanzania.

## Identification Of Ticks To Genus Level Based On Morphology

The morphological identification of tick genus was performed according to published taxonomic keys (1) using stereo microscopes with magnification up to 100X. This identification was conducted at the Entomology unit, Department of Parasitology and Entomology, Sokoine University of Agriculture, Tanzania.

### Identification of ticks to species level based on molecular techniques

To support morphological identification, the identified ticks were confirmed by molecular analysis of the partial sequences of the mitochondrion 16S rRNA gene. For each genus of tick identified morphologically, nine representative individuals were randomly selected for molecular analysis.

## DNA Extraction

A total of 18 adult ticks, representatives of the 2 genera identified morphologically, were washed twice in distilled water and air dried for 15 minutes. Each individual tick was transferred into a 2 ml micro-tube containing glass plating bead (ZymoResearch, California, USA). The tubes were frozen in liquid nitrogen for ten minutes and the ticks were ground into a powder using a Geno-grinder (SPEX Sample Prep; UK), followed by enzymatic digestion using Proteinase K (15). Genomic DNA extraction was then carried out using phenol-chloroform extraction (16). The DNA was then precipitated with absolute ethanol and re-suspended in 200 µL of 1 × TE buffer (Tris 10 mM, EDTA 1 mM, pH8) (15).

## DNA Amplification

A pair of specific primer set of 16S + 1(5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3') and 16S-1(5'-CCGGTCTGAACTCAGATCAAGT-3') was used to target the mitochondrial 16S rRNA gene of ticks. The PCR reactions were conducted in a final volume of 20 µl containing 10 µl of PCR NEB One Taq 2X Master Mix

with Standard Buffer, 6 µl of nuclease free water, 1 µl of 10 µmol/L of each primer and 2 µl of DNA template (15). The PCR was carried out using a Thermo Scientific Arktik Thermal Cycler (TC A0096). The protocol for the 16S rRNA gene amplification using 16S + 1/16S-1 was: initial denaturation 95 °C for 5 min; followed by 10 cycles of 92 °C for 1 min, 48 °C for 1 min and 72 °C for 1.5 min; 32 cycles of 92 °C for 1 min, 54 °C for 35 seconds, 72 °C for 1.5 min, followed by final extension of 72 °C for 7 min (460 bp) (15). For each PCR reaction, a negative control containing deionized distilled water was included.

## Agarose Gel Analysis And Purification Of Amplicons

PCR products were visualized in 1.4% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Blue light DNA Dye in Tris-Acetate-EDTA (TAE Buffer) under UV light after staining with Gel Red Nucleic Acid Stain(<https://biotium.com/product/gelred-nucleic-acid-gel-stain/>). A100bp DNA ladder was used as a standard marker. The amplicons were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The concentrations of purified PCR products were determined using a spectrophotometer.

## Sequencing Of The 16s rRNA Amplicons

Fragments were sequenced at Inqaba South Africa using the same forward and reverse primers as used to generate the PCR products. The labeled products were then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (<http://www.zymoresearch>). The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyzer with a 50 cm array using POP7 (<https://www.thermofisher.com>). Sequence chromatogram analysis was performed using Finch TV analysis software (<https://www.softpedia.com/get/Science-CAD/FinchTV.shtml>).

## Sequences Editing And Identity Confirmation

Sequences were edited using BioEdit in MEGA X software (17). To confirm the identity of each tick species, the sequences were compared with those available in the GenBank database using the BLASTn program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). For the BLASTn algorithm, a stringent E-value cut-off ( $10^{-6}$ ) was used as described previously (13). The identity of the query sequence was assigned to the best hit (highest bit score) returned from BLASTn. The query ID was regarded as confirmed when the best hit (highest bit score) had an E-value below  $10^{-6}$  (18).

## Sequences Alignment

Representative mitochondrion 16S rRNA gene sequences of *Rhipicephalus microplus*, *R. evertsi*, *Hyalomma rufipes*, *H. marginatum*, *H. truncatum* and *H. turanicum* were downloaded from GenBank. Downloaded sequences and sequences generated in the present study were aligned by multiple sequence

alignment using MUSCLE in MEGAX software (17, 19). The sequences acquired in this study have been deposited in the GenBank database with accession numbers MT0798663 to MT079880.

## Phylogenetic Analysis

To determine the genetic relationship between different tick species and to infer their evolutionary history, a phylogenetic tree was constructed. To build the tree, reference sequences of the mitochondrion 16S rRNA gene downloaded from GenBank database were aligned along with the sequences generated in the present study. The evolutionary history was inferred by using the Maximum Likelihood (ML) method with 100 replications in the bootstrap test and Tamura 3-parameter mode (17). Initial tree for the heuristic search was obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value (17). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 24 nucleotide sequences, 18 generated from the present study and the rest from GenBank. Evolutionary analyses were conducted in MEGA X software (19).

### Evolutionary divergence (genetic distance) of ticks

To further determine the evolutionary divergence of the ticks collected in this wildlife-livestock interface ecosystem, mean and pairwise genetic distance analysis were conducted using Kimura's 2-parameter (K2P) model (20). The analysis involved the sequences generated in the present study only. Standard error estimates were obtained by a bootstrap procedure (100 replicates). All ambiguous positions were removed for each sequence pair (pairwise sequence deletion option) (20). The mean and pairwise genetic distance analysis was conducted in MEGAX software (19).

## Results

### Ticks collection and identification

Table 1

Number of ticks collected from cattle in selected areas of the wildlife-livestock interface ecosystem of Mikumi National Park, Tanzania.

Ward	Number of cattle infested with ticks	Number of ticks collected	<i>Rhipicephalus</i>	<i>Hyalomma</i>
Mikumi	32	76	62	14
Doma	44	112	82	30
Kidodi	30	69	30	39
Ulaya	28	63	32	31
Melela	36	102	48	54
Kisaki	38	106	64	42
Kilangili	14	35	22	13
Ruhembe	20	37	18	19
Tindiga	10	30	13	17
<b>Total</b>	<b>252</b>	<b>630</b>	<b>371</b>	<b>259</b>

A total of 630 adult ticks were collected from 252 cattle in 9 wards, which lie at the border of Mikumi National Park, Tanzania (Table 1). On the basis of morphology, all ticks were classified into two genera; *Rhipicephalus* (371) and *Hyalomma* (259) (Table 1). Due to morphological similarity among ticks, all specimens were identified to genus level. Specimens of *Rhipicephalus* and *Hyalomma* genera were grouped by similarity and 9 representative individuals from each group were randomly selected. To confirm results of morphological identification, partial mitochondrion 16S rRNA gene was used as a molecular marker in species identification. A total of 18 specimens were sequenced, edited, aligned and the BLASTn analysis was performed. The lengths of the aligned sequences varied from 399 to 453 base pairs and the nucleotide components indicate that mitochondrion 16S rRNA gene of these ticks is highly A-T rich with average nucleotide frequencies of Thymine (36.77%), Cytosine (9.51%), Adenine (39.71%) and Guanine (13.99%). A bias toward Adenine and Thymine (76.48%) was consistent with the base composition of arthropod mitochondrial DNA. The results from molecular identification are summarized in Table 2. BLASTn matched morphological results with identity varying between 92 and 100% (Table 2). Molecular results revealed that, among the specimens of the genus *Rhipicephalus*, five were *R. microplus* and one was *R. evertsi*, whereas, for *Hyalomma* genus, five were identified as *H. marginatum*, three were *H. truncatum*, two as *H. rufipes* and two as *H. turanicam*. *R. microplus* and *H. marginatum* out numbered all other tick species and together they represent 56% of the total ticks sequenced in the present study (Table 2).

Table 2  
 Sampled tick species and percentage identity value with the references sequences

Sample accessions numbers	Sample collection ward	GenBank accession numbers	Percentage Identity	Tick species	Source country
MT079863	Mikumi	EU918187	99.50	<i>R. microplus</i>	Mozambique
MT079864	Mikumi	MK737650	98.76	<i>H. rufipes</i>	Egypt
MT079865	Doma	KC170742	100	<i>R. micropmus</i>	Thailand
MT079866	Doma	KU130478	97.15	<i>H. truncatum</i>	South Africa
MT079867	Kidodi	KP776645	98.99	<i>H. marginatum</i>	France
MT079868	Kidodi	KT391063	94.24	<i>H. turanicum</i>	Israel
MT079869	Ulaya	KP776654	94.70	<i>H. marginatum</i>	France
MT079870	Ulaya	EU918187	99.75	<i>R. microplus</i>	Mozambique
MT079871	Melela	L34307	97.72	<i>H. marginatum</i>	USA
MT079872	Melela	KU130478	96.89	<i>H. truncatum</i>	South Africa
MT079873	Kisaki	KC170742	99.26	<i>R. microplus</i>	Thailand
MT079874	Kisaki	KU130465	99.49	<i>H. rufipes</i>	South Africa
MT079875	Kilangili	KJ613642	98.42	<i>R. evertsi</i>	South Africa
MT079876	Kilangili	EU918187	99.50	<i>R. microplus</i>	Mozambique
MT079877	Ruhembe	KT391063	91.54	<i>H. turanicum</i>	Israel
MT079878	Ruhembe	KP776645	98.50	<i>H. marginatum</i>	France
MT079879	Tindiga	KU130478	96.37	<i>H. truncatum</i>	South Africa
MT079880	Tindiga	KP776645	97.47	<i>H. marginatum</i>	France

## Phylogenetic Analysis Of The Mitochondrion16s rRNA Gene

Eighteen sequences representing the two identified tick genera generated in the present study and six reference sequences downloaded from GenBank database were used to infer phylogenetic relationship of tick species. To determine the evolutionary relationship of species, sequences from each species generated in the present study were aligned along with the representative sequences downloaded from the GenBank. The Maximum Likelihood tree from mitochondrion 16S rRNA gene sequences derived from *Rhipicephallinae* and *Hyalomma* sub-families generated five (clusters) phylogroups (A, B, C, D and E) (Fig. 2). Group A, constitutes *Rhipicephalus* genus with two monophyletic groups; *R. microplus* and *R. evertsi*. Group B, was members of *H. truncatum*, group C comprises *H. rufipes*, group D comprises members of *H. turanicum* and group E comprises *H. marginatum* members (Fig. 2). There was strong bootstrap support among the species of *Rhipicephalus microplus*, *R. evertsi*, *Hyalomma truncatum* and *H. turanicum* (Fig. 2). The results provided weak support in bootstrap analysis for the clusters containing *Hyalomma rufipes* and *H. marginatum* (Fig. 2). The high bootstrap value (100%) supported the close relationship between *Rhipicephalus microplus* and *R. evertsi* (Fig. 2).

### **Evolutionary divergence (genetic distance) of ticks**

**Due to technical limitations, Table 3 is provided in the Supplementary Files section.**

Using K2P model, sample divergences at various taxonomic levels are shown in Tables 3 and 4. To determine the evolutionary divergence (distance) within the species, sequences from each species generated in the present study were aligned and the K2P distance model was then used to determine the mean and pairwise distances. *H. marginatum* recorded the highest mean intraspecies distance value, whereas, *R. microplus* recorded the lowest (Tables 3 and 4). The highest pairwise intraspecies value was recorded in *H. marginatum* and the lowest pairwise intraspecies values were observed in *R. microplus* and *H. truncatum* (Tables 3 and 4). Likewise, 16S rRNA gene sequences from each genus generated in the present study were aligned to determine the evolutionary distance of tick within the genus. The K2P distance model was thereafter applied to infer the mean and pairwise distances. The highest pairwise intragenus value was recorded in *Hyalomma* genus, whereas, the lowest pairwise intragenus value was recorded in *Rhipicephalus* genus (Table 4). Notably, the mean evolutionary divergences were recorded to be considerably high in both genera (Table 4). Finally, to determine the overall genetic distance of the overall tick community, all 16S rRNA sequences generated in the present study were aligned and the K2P model used to estimate genetic distance. The overall high mean distance of  $0.11 \pm 0.01$  (Table 4) and the high pairwise maximum distance value of 0.23 were recorded in the present study (Table 3).

Table 4  
The mean, maximum and minimum evolutionary divergence

Species	Mean distance	Pairwise distance	
	values	Minimum	Maximum
<i>Rhipicephalus microplus</i>	0.01 ± 0.00	0.003	0.02
<i>Hyalomma marginatum</i>	0.04 ± 0.01	0.015	0.06
<i>Hyalomma truncatum</i>	0.01 ± 0.00	0.003	0.015
<i>Hyalomma rufipes</i>	0.02 ± 0.00	0.02	0.02
<i>Hyalomma turanicum</i>	0.03 ± 0.00	0.03	0.03
Genus			
<i>Rhipicephalus</i>	0.04 ± 0.01	0.003	0.099
<i>Hyalomma</i>	0.06 ± 0.01	0.003	0.139
Overall tick community			
Tick community	0.11 ± 0.01	0.003	0.234

## Discussion

In the present study, we analyzed the phylogenetic relationship and genetic distance of the hard ticks (Ixodidae) infesting cattle using the mitochondrion 16S rRNA gene. The phylogenetic and genetic relationship data will provide valuable information to geneticists, farmers and acaricide suppliers about the differences and similarities of Ixodidae tick populations in the area and could also be useful in the planning of integrated control strategies for ticks and tick-borne diseases in Tanzania.

As shown in the results, the Maximum Likelihood tree generated in the present study recorded several clusters (groups) of the mitochondrion 16S rRNA gene sequences, indicating high divergence of the gene sequences of hard ticks (21, 22, 23) present in this wildlife-livestock interface ecosystem. In that way, clusters of similar sequences represent species clearly separated from other clusters (species) (24, 25, 26). Therefore, this is a reflection of high biological diversity of ticks within Ixodidae (hard ticks) (27, 28, 29) present in this wildlife-livestock interface ecosystem.

From the intraspecies genetic distance data generated in the present study; *Hyalomma marginatum*, *H. rufipes* and *H. turanicum* recorded considerably high mean and pairwise intraspecies genetic distances. On the other hand, *Rhipicephalus microplus* and *Hyalomma truncatum* recorded low mean and pairwise intraspecies genetic distances. The considerably high genetic distances recorded in *H. marginatum*, *H. rufipes* and *H. turanicum* suggest that different groups of these species might represent distinct species or subspecies, or that the species are species complexes with high genetic diversity (30). The low value of

divergence recorded in *R. microplus* and *H. truncatum* might indicate hybridization (30) or misidentification among the species (31, 32).

From the intragenus genetic distance data generated here, *Hyalomma* spp., recorded considerably high mean and pairwise genetic distance. The considerably high mean and pairwise genetic distances recorded in *Hyalomma* genus suggest high biological diversity in this group (genus) of hard ticks (29, 32). Therefore, either new species or subspecies closely related to those recorded in the present study might exist or that these ticks form species complexes with divergent lineages in this wildlife-livestock interface ecosystem (32). The interbreeding between *Hyalomma rufipes*, *H. marginatum* and *H. turanicum* populations has already been demonstrated (33, 34) and these hybrid forms can be transported to different regions by migratory birds (34, 35, 36, 37, 38). Therefore, it is possible to find hybrid forms as a result from the interbreeding of two or three species in regions where these species are found (37, 38). This could explain for the wide sequence variability (divergences) for *Hyalomma* spp., particularly for *Hyalomma marginatum*, *H. turanicum* and *H. rufipes* (34, 38) as observed in the current study.

The mean and pairwise genetic distances in the overall tick community recorded in the present study were highly similar to other studies (32, 33). Such considerably genetic distance values reflect the high biological diversity within the hard tick community present in this wildlife-livestock interface ecosystem (32).

As expected, the mean genetic distances recorded in the present study increased with higher taxonomic ranking;  $0.01 \pm 0.00$ ,  $0.06 \pm 0.01$  and  $0.11 \pm 0.01$  for intraspecies, intragenus and overall tick community respectively. This makes the mitochondrion 16S rRNA gene to be a good marker for supraspecific differentiation and mainly, for taxa grouping purposes (28, 29, 39). According to Ros et al. (2007) (40), DNA barcoding assumes that the genetic distances between species are greater than within species.

## Conclusion

The presence of different (clusters) phylogroups and considerable genetic distances observed, reflect the possible biological diversity of hard ticks present in the study area. Therefore, further work is required to delineate species boundaries and to develop a more complete understanding of hard tick diversity over larger scale.

## Abbreviations

BLAST: Basic Local Alignment Search Tool, H: *Hyalomma*, K2P: Kimura's 2-parameter, R: *Rhipicephalus*, ML: Maximum Likelihood, MCL: Maximum Composite Likelihood, NJ: Neighbor-Joining, MEGA: Molecular Evolution Genetic Analysis.

## Declarations

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## **Availability of data and materials**

The data supporting the conclusions of this article are included within the article. The sequences generated in the present study were submitted to GenBank under the accession numbers MT079863 to MT079880

## **Author's contributions**

DD designed the investigation, performed the experiments, conducted the investigation and drafted the manuscript. MD designed the investigation, conceived the study and revised the manuscript. JJW and MB conceived the study, designed the investigation and revised the manuscript. All authors read and approved the final manuscript.

## **Ethics approval and consent to participate**

No specific permits were required for this study. The study did not involve endangered or protected species. Therefore, the local ethics committee deemed that approval was unnecessary.

## **Consent to publication**

Not applicable.

## **Competing Interests**

The authors declare that they have no competing interests

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