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# Phenotypic and Genotypic Identification of Ticks Sampled from Wildlife Species in Selected Conservation Sites of Kenya

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## Abstract

Hard ticks are blood feeding ectoparasites that infest humans and animals and are vectors of pathogenic micro-organism that cause severe infectious diseases. Morphological identification has been the main approach of identifying ticks but the technique is considered inaccurate and difficult. Molecular techniques have recently been considered to be appropriate approaches for accurate and rapid identification and Internal Transcribed Spacer 2 (ITS 2) has been shown to differentiate genus of hard ticks. Currently, genetic identification of ticks using ITS 2 has not been carried out in Kenya. In this study, 80 tick samples were collected from Lake Nakuru and Tsavo National Parks and were identified morphologically using appropriate identification keys. DNA was extracted from the appendages using DNA extraction kit followed by partial amplification of ITS 2 gene. The PCR products were then analyzed by gel electrophoresis and positive PCR products were sequenced. Of the tick samples four genera were identified morphologically; *Amblyomma*, *Hyalomma*, *Rhipicephalus* and *Dermacentor*. Of the tick samples identified and compared with the sequences in the GenBank, six and seven samples showed 98-100% homology with *A. variegatum* and *R. pulchellus* respectively and they clustered in their respective monophyletic group in the phylogeny tree with a bootstrap of 99%. Two samples showed 92% homology with *H. dromedarii* and the study sequences clustered with the reference sequence with a bootstrap of 99% while six samples showed 95% homology with *H. marginatum* rufipes, however, only four of these samples clustered together with the reference sequence in the phylogeny with a bootstrap of 95%. One sample showed 91% homology with *A. humerali* and did not cluster together in the phylogeny tree. Congruency between both techniques was high with a correlation coefficient of 0.941. This is the first report of phenotypic and genotypic traits of tick species in Kenya and the findings will add value to the existing knowledge in identification of ticks.

**Keywords:** Ixodidae; ITS 2; Kenya

## Background

Ticks are ubiquitous blood feeding ectoparasites of humans and animals and are vectors of a plethora of pathogens that cause severe infectious diseases in humans and livestock [1]. Tick infestation and the diseases they transmit result in huge economic losses in livestock production and continue to cripple the industry, especially in sub-Saharan Africa [2]. The diversity and number of wildlife species is enormous all over the world and this diversity sustain a large and diverse tick- borne vectors of pathogens [3,4]. Most wild herbivores, including buffaloes and elephants are believed to be significant reservoirs of tick-borne pathogens that affect humans and animals [5] as they support a large population of tick species [6].

Reliable and quick identification of ticks is therefore, important in the control of spread of tick-borne diseases. Morphological characterization based on phenotypic traits has been the traditional method of identifying ticks. Morphological identification method is further weakened by the fact that traits used to differentiate species tend to overlap between species or vary within species or according to age and size [7]. Additionally, morphological identification of physically damaged ticks due to poor handling and preservation is often inaccurate. The advent of molecular techniques and development of markers that can identify ticks have revolutionized our understanding of other insect vectors and may be useful in studies of tick taxonomy and diversity.

To comprehend the epidemiology of tick-borne pathogens and develop effective strategies for controlling the diseases, accurate identification of the vector is vital. Currently, the tick diversity in Kenya is based on historical records that were determined by morphological methods [8] and yet the method has inherent challenges and weakness that weaken accuracy of species identification. Although, ticks can be identified directly using genetics, it is important to describe its distinctive phenotypic features and test for congruency with genetic classification. Currently, genetic identification techniques have not been effectively used in identification of tick in Kenya. Still, there is no evidence that the phenotypic and genotypic traits of tick species in Kenya are congruent. Therefore, incongruency in identification based on the two techniques will justify the need for taxonomic re-classification. Additionally, the information generated will quicken future identification of tick species. As such, the purpose of this study is to genetically identify tick samples and confirm whether the distinctive phenotypic traits for tick species differentiation are congruent with genetic traits.

# Methods

## Ethics statement

The ethics committee of the Kenya Wildlife Service (KWS) approved the study as it was based on opportunistic tick collection from immobilized elephants, buffaloes and rhinoceros during translocation, collaring and veterinary exercises in Lake Nakuru and Tsavo National Parks. KWS guidelines on Wildlife Veterinary Practice-2006 were followed and all KWS veterinarians follow the Veterinary Surgeons and Veterinary Para-Professionals Act 2011, Laws of Kenya, which regulates veterinary practices in Kenya.

## Tick collection

A total number of 80 ticks were collected randomly during scheduled veterinary management activities within the Lake Nakuru and Tsavo National parks. The animals were immobilized by the Kenya Wildlife Service veterinarians using a combination of etorphine and xylazine (Norvatis, PTY, Ltd, South Africa). Ticks were pulled off manually from the animals by hands and with forceps and placed in sterile loosely capped plastic vials and transported to the laboratory in dry ice. In the laboratory the ticks were stored at -80°C

## Morphological identification of ticks

Sampled ticks were kept at room temperature to thaw, and then washed twice with sterile water to remove excess particulate contamination from animal skin, rinsed once with 70% ethanol. They were mounted on slides and examined using a stereoscope microscope at a magnification of × 40, × 80, and × 100. Identification of the ticks was by sex and species using appropriate identification keys [8]. The identified ticks were transferred to sterile vials, and stored at -80°C until processing at the KWS Veterinary Laboratory, Nairobi.

## Molecular analyses of ticks

**DNA isolation and PCR:** DNA was extracted from each sample using DNA extraction kit (DNeasy blood & Tissue Kit, QIAGEN (Germany) following the manufacture's protocol. Genomic DNA extracted from the 28 ticks' tissues was amplified. Each species identified morphologically was represented by more than one sample. A pair of degenerate primers designed for ITS 2 amplification [9] were used; forward 5´-YTGCGARACTTGGTGTGAAT-3´ and reverse 5´-TATGCTTAARTTYAGSGGGT-3´.

Amplification of DNA was carried out in a final volume of 25 µl containing 15.8 µl of dH20, 2 µl of the genomic DNA, 2 µl of each primer (forward and reverse), 5 µl PCR buffer, 0.2 µl of Taq DNA polymerase. The tubes were then placed into a programmed Applied Biosystems Veriti 96 well thermocycler (Germany) where the reactions mixture was subjected to 2.5 min DNA denaturization at 94°C, 35 cycles of denaturization at 94°C for 30 sec, annealing at 50°C for 1 min and elongation at 72°C for 1 min. The reaction was completed by a further 30 min step at 72°C. The procedure was repeated twice for each sample in order to yield a final volume of 50 µl.

## Electrophoresis and purification of PCR products

The amplified products were analyzed by electrophoresis on 1% Agarose gel by aliqouting 4 µl of PCR products and 1 kb DNA ladder and run on the gel stained with ethidium bromide for 1 hour at 80 volts. The PCR products were purified using QIAGEN PCR Purification Kit (Germany) following the manufacturer's protocol.

## Sequencing and analysis

Purified DNA products were sent to Biosciences Eastern and Central Africa (BecA-ILRI) for sequencing. Sequencing was carried out through ABI310 DNA sequences. Sequences were taken through Bioedit, and aligned using multiple alignment program ClustalX. The default 6.66 and 15 values for gap extension and gap opening respectively were used. Misaligned nucleotides were detected and realigned manually. BLASTN searches were done in the GenBank so as to identify matches to the sample sequences.

## Congruency test

Correlation statistic were computed to test for congruency between morphological and phenotypic traits at P=0.01.

# Results

## Morphological identification

Tick species that were correctly identified morphologically by genus from Lake Nakuru and Tsavo National Parks from elephants, buffaloes and rhinoceros. Four genera were correctly identified namely *Amblyomma*, *Hyalomma*, *Rhipicephalus* and *Dermacentor* (Table 1). Some of tick species identified morphologically include; *Hyalomma arbiparmatum*, *Amblyomma tholloni*, *Rhipicephalus pulchellus*, *Amblyomma gemma*, *Amblyomma variegatum*, *Hyalomma marginatum rufipes* (Figures 1A, 1B, 1C, 1D, 1E and 1F).

| Name | Stage | Number | Host | Location |
|---|---|---|---|---|
| *Amblyomma gemma* | Adult | 13 | (*Loxondota Africana africana*) Elephant | Tsavo National Park |
| *Amblyomma variegatum* | Adult | 7 | (*Syncerus caffer*) Buffaloes | L. Nakuru National Park |
| *Amblyomma thollonia* | Adult | 1 | (*Loxondota Africana africana*) Elephant | Tsavo National Park |
| *Rhipicephallus pulchellus* | Adult | 24 | (*Loxondota Africana* | Tsavo National Park |

| | | | | |
|---|---|---|---|---|
| | | | *Africana)* Elephant | |
| *Hyalomma marginatum rufipes* | Adult | 7 | (*Loxondota Africana Africana*) Elephant | Tsavo National Park |
| *Hyalomma Truncatum* | Adult | 17 | (*Loxondota Africana Africana*) Elephant | Tsavo National Park |
| *Hyalomma Dromedarii* | Adult | 3 | *(Loxondota Africana Africana)* Elephant | Tsavo National Park |
| *Hyalomma Albiparmatum* | Adult | 5 | (*Loxondota Africana Africana*) Elephant | Tsavo National Park |
| *Dermacentor rhinocerinus* | Adult | 3 | (*Diceros bicornis*) Rhinoceros | L. Nakuru National Park |

**Table 1:** Number of tick species that were correctly identified morphologically from diverse animal species by genus from Lake Nakuru and Tsavo National Parks. Four genera were correctly identified namely *Amblyomma*, *Hyalomma*, *Rhipicephalus* and *Dermacentor*.

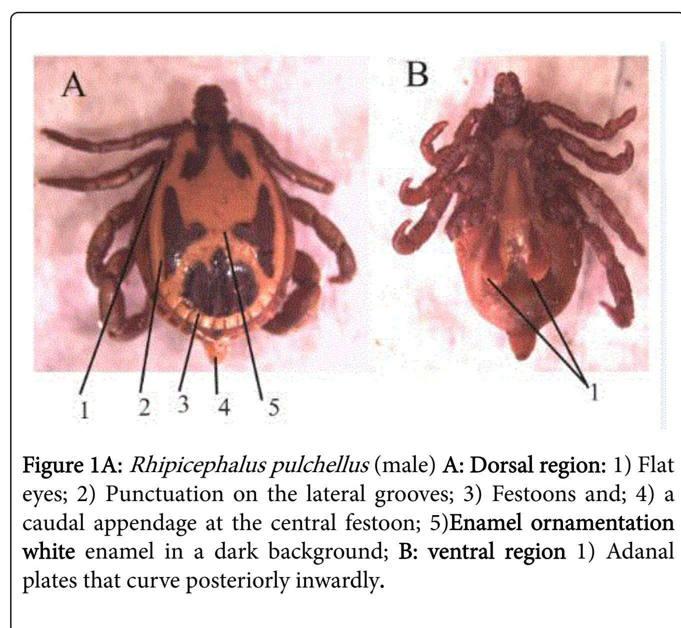



**Figure 1A:** *Rhipicephalus pulchellus* (male) **A: Dorsal region:** 1) Flat eyes; 2) Punctuation on the lateral grooves; 3) Festoons and; 4) a caudal appendage at the central festoon; 5)**Enamel ornamentation white** enamel in a dark background; **B: ventral region** 1) Adanal plates that curve posteriorly inwardly.

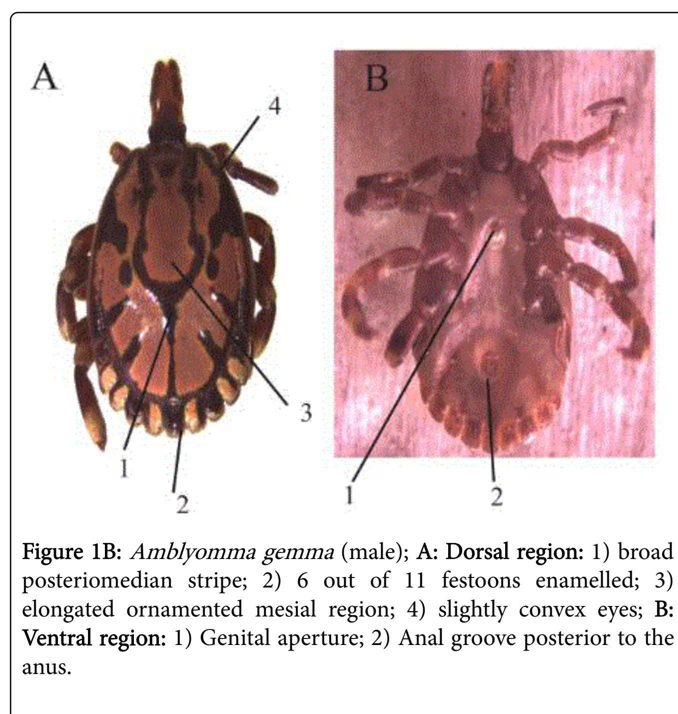
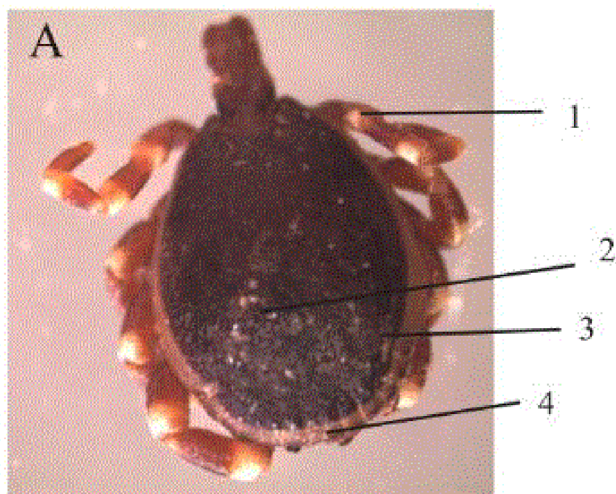



**Figure 1B:** *Amblyomma gemma* (male); **A: Dorsal region:** 1) broad posteriomedian stripe; 2) 6 out of 11 festoons enamelled; 3) elongated ornamented mesial region; 4) slightly convex eyes; **B: Ventral region:** 1) Genital aperture; 2) Anal groove posterior to the anus.



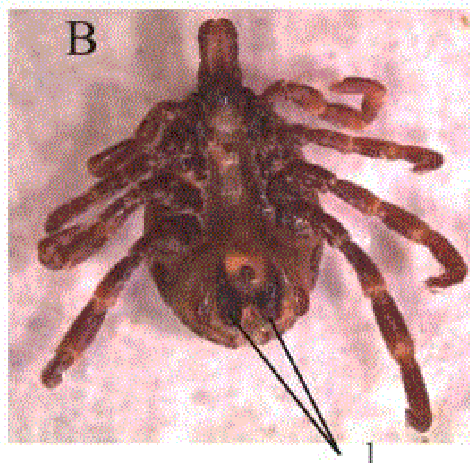




**Figure 1C:** *Hyalomma marginatum rufipes* **A: Dorsal region** 1) Legs with pale rings; 2) scutum rugged large punctuations; 3) short lateral grooves; 4) presence of paracentral festoons; **B: Ventral region** 1) adanal plates, length twice the width and the distance separating the pates is less compared to the width of their ends.

## Genetic Identification

DNA was isolated from 28 tick samples and after partial amplification of the PCR products, 22 samples were positive and yielded products of approximately 900-1200 bp. Different species could be discriminated from the ITS 2 fragment size. The fragment size markers of *R. pulchellus* was estimated at 1100 bp, *A. variegatum* 1200 bp, H. *marginatum* 1500 bp and *H. dromedarii* 1200 bp. A comparison of the ITS 2 sequence identified in this study with the sequence deposited in the GenBank registered different homology with different genera. From the data, six samples showed between 98-100% homology corresponding to *R. pulchellus* sequences obtained from Australia. Another seven samples showed 98% homology with *A. variegatum* sequences obtained from the U.S.A. Two Samples showed 92% homology with *H. dromedarii* while sample one showed 91% homology with ITS 2 sequence of *A. humerali* from Brazil in the GenBank The study sequences were deposited in the GenBank with the following accession numbers; KM819710 for *R. pulchellus*, KM819712 for *A. variegatum*, KM819713 and KM819711 for *H. marginatum rufipes* and *H. dromedarii* respectively (Table 2).

**Figure 1D:** *Hyalomma truncatum* (male); **Dorsal region** 1) Dark and shiny conscutum; 2) Pale rings on the legs; 3) Festoons; and caudal depression; **Ventral region** 1) Sub-anal plates; 2) Adanal plates with squared-ends; 3) Anus.

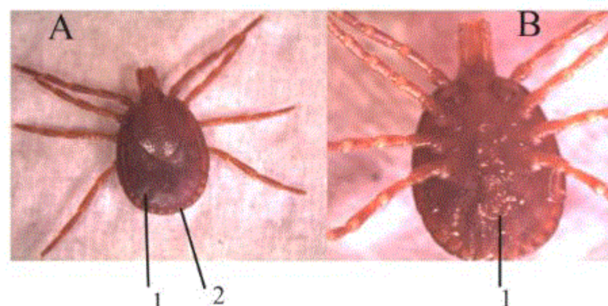



**Figure 1E:** *Amblyomma tholloni* (female) **A Dorsal region** 1) inornate scutum; 2) non-enamelled festoons; **B Ventral region** 1) Anus.

Sequence from this study and reference sequence obtained from the GenBank were aligned using multiple alignment program Clustal X. Maximum Composite Likelihood method was used in computing the evolutionary distance. Figure 1 shows the Phylogenetic tree generated from partial ITS sequence data. The branch length represents evolutionary changes that have taken place over time and the amount of genetic change is represented by a scale of 0.1. The number of substitution which is related to the clustering together of the taxa as a bootstrap test, is represented by a percentage value; that is, the number of substitution per 100 nucleotide sites and is shown above the branches. The tree was rooted to the genus Ixodes.

From the figure, *R. pulchellus* and *A. variegatum* haplotypes clustered together at their respective group with a high bootstrap value of 99%. *H. marginatum rufipes* also clustered together with the reference sequence while sample 39s and 40s which shared recent ancestor origin with *H. anatolicum* with a bootstrap support of 79%. *H. dromedarii* clustered together with the reference *H. dromedarii* ITS

2 sequence deposited in the GenBank with a high bootstrap (99%) while *A. humerali* is clustered together with *A. tuberculatum* than *A. humerale* with a low bootstrap support of 51%. *Oxidi dammini* was used as an out group making the tree is a rooted one (Figure 2).

| Genus | Accession Number | Tick species | ITS fragment Size | % Identity | Reference sequence | Origin Country |
|---|---|---|---|---|---|---|
| | | *R.pulchellus* | 1100 | 100 | AF271275 | Australia |
| | | *R.puchellus* | 1100 | 100 | AF271275 | Australia |
| | | *R.pulchellus* | 1200 | 98 | AF271275 | Australia |
| | | *R.pulchellus* | 1200 | 98 | AF271275 | Australia |
| | | *R.pulchellus* | 1100 | 99 | AF271275 | Australia |
| *Rhipicephalus* | KM819710 | *R.pulchellus* | 1100 | 99 | AF271275 | Australia |
| | | *A.variegatum* | 1200 | 98 | HQ856759 | U.S.A |
| | | *A.variegatum* | 1200 | 98 | HQ856759 | U.S.A |
| | | *A.variegatum* | 1200 | 98 | HQ856759 | U.S.A |
| | | *A.variegatum* | 1200 | 98 | HQ856759 | U.S.A |
| | | *A.variegatum* | 1200 | 97 | HQ856759 | U.S.A |
| | | *A.variegatum* | 1200 | 98 | HQ856759 | U.S.A |
| | | *A.variegatum* | 1200 | 98 | HQ856759 | U.S.A |
| *Amblyomma* | KM819712 | *A.humerali* | 800 | 91 | AY887111 | Brazil |
| | | *H. marginatum* | 1500 | 95 | JQ737104 | China |
| | | *rufipes* | | | | |
| | | *H. marginatum* | 1500 | 95 | JQ737104 | China |
| | | *rufipes* | | | | |
| | | *H. marginatum* | 1500 | 95 | JQ737104 | China |
| | | *rufipes* | | | | |
| | | *H. marginatum* | 1500 | 95 | JQ737104 | China |
| | | *rufipes* | | | | |
| | | *H. marginatum* | 1600 | 95 | JQ737104 | China |
| | | *rufipes* | | | | |
| | KM819713 | *H. marginatum rufipes* | 1600 | 95 | JQ737104 | China |
| | | *H.dromedarii* | 1200 | 95 | JQ733570 | India |
| *Hyalomma* | KM819711 | *H.dromedarii* | 1200 | 92 | JQ733570 | India |

**Table 2:** Identity of tick species and their bp sizes, and percentage similarity value with the references sequences.

## Morphological and molecular characterization of tick samples

Diverse ticks were identified using each technique were compared. The number of *R. pulchellus, A. variegatum* and H. *dromedarii* identified both morphologically and genetically were the same; that is, 6, 7 and 2 respectively. Under the genus *Hyalomma* 4 species were identified H. *marginatum rufipes* morphologically and 6 genetically. Two ticks were first identified as H. *marginatum rufipes* based on morphology but then by genotyping and comparison with reference sequences they were reclassified as H. *truncatum* and H. *albiparmatum.* Similarly, one tick was morphologically identified as D. *rhinocerinus* but by genotyping the tick was reclassified as A. *humerali* (Table 3). Congruency between morphological and genetic traits was high as depicted by correlation co-efficient of 0.941 (P=0.01).

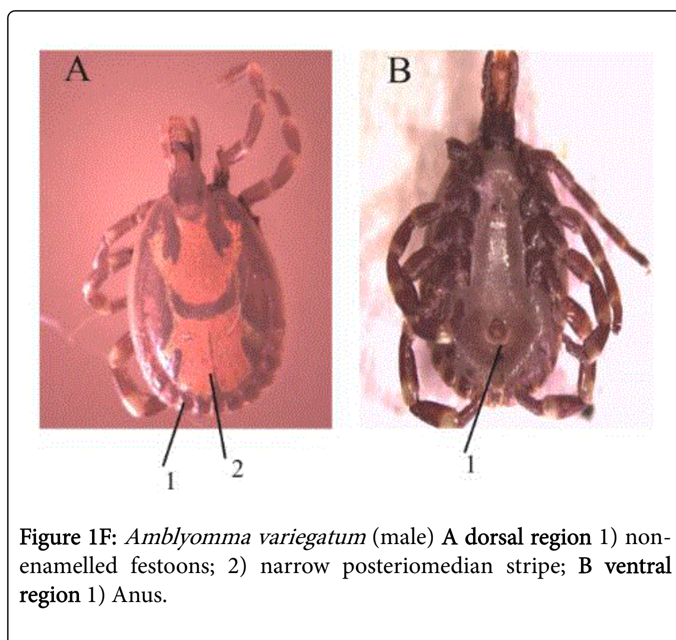



**Figure 1F:** *Amblyomma variegatum* (male) **A dorsal region** 1) non-enamelled festoons; 2) narrow posteriomedian stripe; **B ventral region** 1) Anus.

| Species | No. identified Morphologically | No. identified genetically |
|---|---|---|
| *R. pulchellus* | 6 | 6 |
| *H.marginatum rufipes* | 4 | 6 |
| *H.truncatum* | 1 | 0 |
| *H.dromedarii* | 2 | 2 |
| *H. albipармatum* | 1 | 0 |
| *A. variegatum* | 7 | 7 |
| *A. humerali* | 0 | 1 |
| *D. rhinocerinus* | 1 | 0 |

**Table 3:** Number of tick samples identified using phenotypic and phylogenetic techniques.

## Discussion

Various tick genera are associated with various diseases, for example *Amblyomma* species are associated with viruses noted to be mosquito-borne. More importantly, the impact of ticks on human health is on the rise and more novel microbial associations have continued to be described [10]. There is need therefore, for accurate identification of ticks in order to develop better control measures.

In this study, ticks collected from wild herbivores in L. Nakuru and Tsavo National Park was identified both morphologically and genetically. Morphologically, *Amblyomma* species and *R. pulchellus* were easily identified using Walker's identification keys. Male *R. pulchellus* were easily identified by their unique striped enamel on a brown-dark background over their conscutum as described by Beati & Keirans who observed that male *R. pulchellus* have characters that are useful for classification and easily distinguished [11]. Female *R. pulchellus* were easily identified by their association with the males and through their fine and sparse large punctuation on the scutum and U-shaped genital aperture. *Amblyomma* species were identified by ornamentation, long mouths and festoons in line with Piazark [12].

It was difficult to differentiate some Male *H. truncatum* from male *H. marginatum rufipes*. The distinctive features that differentiate the two species, the caudal depression present on *H. truncatum* and absent in H. marginatum rufipes; and dense even punctuation on *H. marginatum rufipes*. However, some species identified as *H. truncatum* had morphology of *H. marginatum*; that is, despite the presence of caudal depression, some species identified as *H. truncatum* had dense even covering of punctuations on their conscutum, a feature of *H. marginatum rufipes*. As such, it was difficult using Walker's identification to distinguish between *H. truncatum* and *H. marginatum rufipes*, though the offspring were regarded as *H. truncatum*. These observations are similar to other studies which have reported difficulties in identifying *Hyalomma* species using nomenclatures [7]. This is attributed to natural hybridization in the genus *Hyalomma* [13]. The authors reported that initial hybridization between *H. marginatum rufipes* and *H. truncatum* resulted in a hybrid with more morphological characteristic of *H. truncatum*. Similar observations were made by Hadani & Cwilich who noted that hybridization of *H. excavatum* and *H. marginatum* resulted to male offpsprings with morphological characterization of both *H. margiatum* and *H. excavatum* [14]. In this study, difficulties observed in differentiating *Hyalomma* species may also be attributed to such natural hybridization between the two species in the wild.

Other tick samples could not be identified due to lost body parts and physical damages. For example, in the genus *Amblyomma*, some tick samples had partially lost the conscutum tampering with the ornamentation, making it difficult to identify a problem observed by Abdigoudarzi et al. who noted that morphological changes that occur on ticks' surface body may result to misleading identification [7].

Recent molecular work on tick species has used the ITS 2 as a marker in identifying ticks especially in differentiating closely related genus [15-17]. Amplification of the entire ITS 2 have been done by various researchers and mixed results were observed including Zahler et al. and Murell et al. who attributed the unsatisfactory to specific nature of the ITS 2 region in ticks and some repeated fragments in the region [18,19].

In this study, partial sequence of the ITS 2 region was used to identify tick samples and different genera were differentiated based on the fragment sizes. The *Rhipicephalus* species was estimated at 1100 bp and *Amblyomma* species, 1200 and *Hyalomma* species, at 1500 bp. The results are similar to findings by Abdigourdarzi et al. who reported similar sizes for the three genera [7].

Sampled ticks were compared and identified with the sequences from the GenBank, and *R. pulchellus* and *A. variegatum* had a percentage similarity ranging between 98-100%. This suggested that both species could be regarded according to the genotypic identification given that in the phylogenetic tree, both species fell in their respective monophyletic group, clustering together, with a high bootstrap of 99%. The low percentage similarity value (92-95%) shown by *Hyalomma* species to the corresponding accession sequences in the GenBank could be due to diverse evolution as a result of geographical separations [20].The difference could be attributed to single nucleotide substitution between both sequences due to individual variation as a result of their wide range of distribution; an observation that is in agreement with other studies which showed that sequence divergence may be due to phylogeographical units in a given species [21].

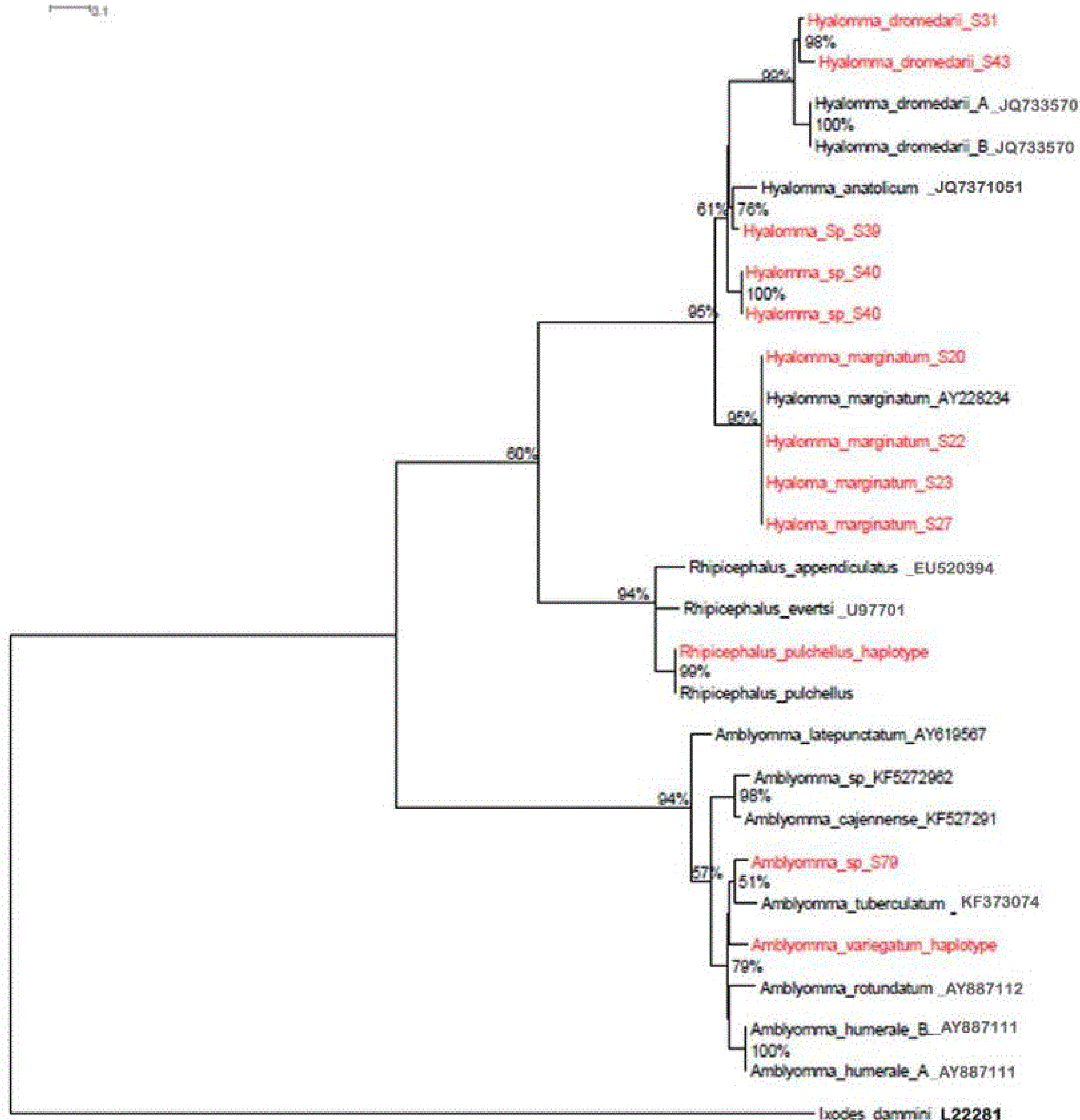



**Figure 2:** Sequence from the study and reference sequence obtained from the GenBank aligned using multiple alignment programs Clustal X. Evolutionary distance computed using the Maximum Composite Likelihood. The amount of genetic change is represented by a scale of 0.1. The number of substitution is represented by a percentage value; that is the number of substitution per 100 nucleotide sites and is shown above the branches. The reference sequences are in bold. The tree was rooted to the genus *Ixodes*.

This was also noted in *H. dromedarii*, with a low similarity value (92%) while the study sequence clustered together with the reference sequence with a high bootstrap of 99%. Such similarity value could be associated with the cryptic hybridization factor which according to Rees et al. results to nucleotide substitution [13]. Two samples considered *H. marginatum rufipes* failed to cluster together with the rest of the species in the phylogenetic tree. Phenotypically, the tick samples were identified as *H. truncatum* and *H. albiparmatum*; and therefore, the high dissimilarity value and failure to cluster together with the rest of *H. marginatum rufipes* could be attributed to lack of corresponding sequences in the GenBank. This could also be the case in *A. humerale* which had been identified as D. rhinocerinus phenotypically.

In this study, congruency between morphology and genetic traits was high as the correlation coefficient was high at 0.942. Congruency was observed in *Rhipicephalus* and *Amblyomma* species as they had samples matching between morphological and genetical traits. Similar findings have been reported by Lu et al. who observed consistency in their morphological and molecular characteristic of identifying tick species in Japan [22]. Inconsistency was observed in genus *Hyalomma* and this could be attributed to lack of or few corresponding sequences deposited in the GenBank. It could also have been contributed by several nucleotides mismatches and insertion which could be as a result of geographical separation and hybridization among the species.

## Conclusions

From the findings of the study, genus *Rhipicephalus* and *Amblyomma* were easily identified using morphologically characteristics while some *H. marginatum rufipes* and *H. truncatum* could not be easily identified morphologically. Partial amplification of ITS 2 was successful in differentiating tick species in Kenya. Further studies should be done on a wide collection of Ixodidae from Kenya to contribute to the existing ITS nucleotide database in order to have sufficient genetic database to cover tick species.

## Competing Interests

The authors declare that they have no competing interests.

## Author Contributions

Vincent Obanda, Carolyn Wanjira Muruthi, Moses Otiende, J.N.Makumi conceived and designed experiments. Carolyn Wanjira Muruthi, Vincent Obanda, Moses Otiende, Olivia Wesula Lwande, Steven Runo conducted the experimental work. Carolyn Wanjira Muruthi, Vincent Obanda, Moses Otiende, Olivia Wesula Lwande analyzed the data. Carolyn Wanjira Muruthi, Vincent Obanda, Moses Otiende, J.N. Makumi, Olivia Wesula Lwande, Steven Runo contributed to the manuscript. All authors approved the final version for submission.

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