Molecular evidence confirms occurrence of Rhipicephalus (Boophilus) microplus in Kenya and suggests that an undifferentiated genotype is prevalent in the African continent

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Abstract

Background The tick vector Rhipicephalus (Boophilus) microplus which transmits Babesia and rickettsial pathogens has not been reported in Kenya since 1998. More recently, the pathogenic Babesia bovis has been detected in cattle blood DNA. The status of R. microplus in Kenya remains unknown. This study employed morphological and molecular tools to characterize R. microplus originating from Kenya and assess the genetic relationships between Kenyan and other African R. microplus genotypes. Methods Morphological identification and differentiation of 35 Kenyan and 11 reference tick specimens was implemented by following standard tick reference keys and identification guides. Genetic and phylogenetic relationships between the Kenyan and other annotated R. microplus reference sequences was investigated by analysis of the cytochrome c oxidase subunit I (COI) gene. The complete mitochondrial (mt) genome of four tick specimens which included two R. microplus was assembled from whole genome data to further characterize the Kenyan ticks. A B. bovis specific Taqman probe qPCR assay was used to detect B. bovis in gDNA from R. microplus ticks. Results Of the 35 Kwale tick specimens analysed, 23 were confirmed to be R. microplus by both morphology and molecular characterizations. The Kenyan R. microplus COI sequences showed very high pairwise identities (>99%) and clustered very closely with reference African sequences. We also found a low differentiation and lack of geographical sub-structuring among the African COI sequences. The mitochondrial genome sequences of the two Kenyan R. microplus ticks also clustered closely with reference genome sequences from Brazil, USA, Cambodia and India. No B. bovis was detected in the Kwale R. microplus DNA. Conclusions These findings confirm the presence of R. microplus in Kenya and suggest that a common undifferentiated genotype is prevalent in cattle in Africa. These and other recent findings of widespread occurrence of R. microplus in Africa provide a strong justification for urgent surveillance to determine and monitor the spread of R. microplus and vector competence of Rhipicephalid ticks for B. bovis in Africa, with the ultimate goal of strategic control.

Background

The Asian blue tick, *Rhipicephalus (Boophilus) microplus* has a widespread distribution in many tropical and subtropical areas of Asia, north-eastern Australia, South and Central America, southern and eastern Africa [1]. It was originally introduced into the African continent from southern Asia through Madagascar during the rinderpest epidemic in 1896 when *R. microplus*-infested cattle were imported into South Africa [2]. In the last decade it has been reported in many West African countries [3–8] and more recently in Angola [9], Cameroon [10] and Uganda [11].

Rhipicephalus microplus is considered more economically important than other African ticks including *R. decoloratus* and *R. annulatus* because of its invasive capabilities and higher competence for *Babesia bovis*, the causative agent for a fatal form of bovine babesiosis [1]. In addition to pathogen transmission, the tick also causes direct economic losses to productivity and through hide damage [12]. The spread of *R. microplus* to new areas in Africa is a big threat to livestock industries and livelihoods of rural populations, who often depend on livestock for their survival. Annual economic losses associated with

babesiosis and anaplasmosis in Kenya was estimated at about $6.9 million per year [13] and 70% of cattle are assumed to be at risk. The economic importance of *R. microplus* is also compounded by its propensity to displace other tick species, its higher vectoral capacity and ability to accumulate resistance to acaricides [14]. Already, widespread acaricide resistance has been reported in West Africa, where the tick is spreading [3, 5].

Rhipicephalus microplus was first recorded in Kenya in 1974 [15] and then later in 1998 [16] within a very limited area around Kwale County along the Kenyan Coast. However, more recently, *B. bovis* has been detected in cattle blood in Central and western Kenya [17, 18], strongly suggesting the presence of its vector, *R. microplus*. In order to prevent the spread of *B. bovis* validated methods to identify *R. microplus*, are required, discriminating between this species and the endemic *R. decoloratus* and other Rhipicephalid ticks. Based on cytochrome c oxidase subunit I (COI) and mitochondrial genome phylogenetics, four distinct geographical clusters of *R. microplus* species complex have been reported [19, 20]. They include clade A (Africa, Asia and S. America) and B (southern China and northern India) of Burger et al., (2014) [19]; clade C (Malaysia and India) of Low et al., (2015) [20] and *R. australis*. The clade B lineage which was found to be more closely related to *R. annulatus* than to ticks in clade A or C is thought to constitute a cryptic species restricted to China and parts of India [19].

Therefore, to determine if *R. microplus* does exist in Kenya, this study employed morphological and molecular tools to characterize and positively identify 35 tick specimens collected from Kwale County and 11 reference specimens. Genetic characterization of Kenyan ticks positively identified as *R. microplus* was undertaken to enable validation of the morphological and molecular assays and comparisons with other annotated African tick genotypes. Our confirmation of the occurrence of *R. microplus* in Kenya indicates the need for an urgent tick and babesiosis epidemiological strategy enabling monitoring and introduction of control measures in Kenya. With the current invasion and rapid spread of *R.* (*B). microplus* in many African countries, it is necessary to determine whether its occurrence is accompanied by the presence of the pathogenic *B. bovis* in cattle. Continent-wide population genetics analysis of *R. microplus* from cattle and other animal hosts using a combination of markers may reveal the gene flow patterns and population structure of *R. microplus* as it adapts into new areas in the continent.

Materials And Methods

Study site

Tick samples were collected in May 2019 from cattle herds in Kwale County, Kenya (Figure 1). Most of the *R. microplus* were from three localities (Matuga Tangini (MATAN), Shimoni (SHIM) and Shimoni Kidimu (SHIKI) (Figure 1). The County has an area of 8, 270.3 km^2 and borders Indian Ocean to the East and South East and Tanzania to the South West. It lies between latitudes 30.05º to 40.75º South and longitudes 38.52º to 39.51º East. The County has a coastal plain that lies 30 meters above sea level after which there is a foot plateau at an altitude of between 60 and 135 meters above sea level. A coastal

range characterized by hills rises steeply from the foot plateau to an altitude between 150 metres and 462 metres above sea level. The final zone is a semi-arid plateau that stands at an altitude of about 180 to 300 meters above sea level on the western boundary of the County. The County has a tropical type of climate influenced by monsoon seasons. The average temperature is about 23ºC with maximum temperature of 25ºC being experienced in March and minimum temperature of 21ºC experienced in July. On average, annual precipitation in the County is less than 800mm. Rainfall is bi-modal with a short rain season from October to December and a long rain season from April to July. There is a strong east to west gradient of decreasing precipitation with eastern (coastal) parts of the County receiving greater than 1000 mm of precipitation per year, while a majority of the central to west areas receive around 500-750 mm. Some areas along the western side of the County receive less than 500 mm of precipitation per year.

Tick samples

Live adult ticks were plucked directly from cattle by use of steel forceps and placed in tubes. However, most of the ticks were either partially fed, semi- or fully engorged and some died and decomposed during the long week sampling. Thirty-five tick specimens that survived were subjected to morphological and molecular analyses. These specimens were compared to 11 reference specimens that included three *R. australis* from the Tick Fever Centre, Queensland, Australia, two *R. microplus* and two *R. decoloratus* from Cameroon, two specimens of *R. microplus* from Laos and two of *R. decoloratus* from a laboratory colony maintained at the International Livestock Research Institute, Nairobi. Detailed descriptions of all the 46 tick specimens analysed are summarised in Table 1.

Morphological identifications

Key morphological features of the tick specimens were observed under a compound microscope following standard keys and identification guides in Walker et al., (2003) and Barker and Walker (2014) [21,22]. Images of key characteristic features were captured using a digital microscope (VHX-6000, KEYENCE Inc., Japan). Scanning electron microscopy (SEM) analysis of some reference specimens was done. Whole tick specimen was adhered to a mounting SEM stub (Ted Pella Inc., USA) with a double-sided carbon tape. The stub with the tick was then placed on a rotary planetary specimen stage within a K550X Sputter Coater unit (Quorum Technologies, Kent, UK) and coated with gold (Au) using the following parameters: current 25 mA, time 2:00 min and coating 15 nm. Once coated, the specimen was placed in a JEOL Neoscope, JCM-6000 (JEOL Inc., Nikon Inc., Japan) for imaging.

DNA isolation, PCR amplification and sequencing of the cytochrome c oxidase subunit I (COI)

Genomic DNA (gDNA) was isolated from a total of 43 specimens. These included 23 *Boophilus*, 8 *R. appendiculatus*, one *A. variegatum* specimens from Kwale and all the 11 reference specimens. Using sterile single-use scalpel blades, 3-4 cuts were made on the body of the tick sparing key features important in morphological identifications. All semi- and fully engorged ticks were cut into two halves along the abdomen. DNA was extracted from the cut specimens using the ISOLATE II Genomic DNA Kit (Bioline, Australia) by following the manufacturer's protocol. Total DNA was eluted into 70 µl of elution

buffer (Tris buffer, pH = 8.5, preheated to 70°C). A 604 nucleotide 5' fragment of the cytochrome c oxidase subunit 1 (COI) gene was amplified in a conventional PCR using the forward primer S0725 (F1) (5'-TAC TCT ACT AAT CAT AAA GAC ATT GG3') and reverse primer S0726 (R1) (5'–CCT CCT CCT GAA GGG TCA AAA AAT GA-3') [23]. MyTaq™ Red Mix (Bioline, Australia) was used for COI amplification in 25 μL reactions using 1 μL of each of the two primers (10 pmol) and 2 μL template DNA. The PCR run conditions were: Initial denaturation at 95°C for 5 min, followed by 34 cycles of 94°C for 10 s, 55 °C for 10 s and 72 °C for 15 s. A final extension at 72°C for 7 min was included. A positive control and a negative no-template water control were included in all the reactions which were performed in a T100™ Thermal Cycler (BioRad, Australia). PCR products were purified and sequenced at Macrogen Ltd (Seoul, South Korea).

Sequencing and assembly of mitochondrial DNA from whole genome sequencing data

Isolated genomic DNA was used for NEBNext® DNA Library preparation following manufacturer's recommendations. Indices were added to each of the four samples sequenced followed by the next-generation sequencing using 150 bp paired end Illumina HiSeq 2500 sequencing systems utilizing a depth of 1Gb of raw sequence data (Novogene, Singapore). The complete mitochondrial genome (mtDNA) of the four specimens was assembled from FastQ data using the MITObim pipeline available at https://github.com/chrishah/MITObim with the sequence of *R. (B.) micropus* complete mtDNA (KC503260) as bait. The assembly was repeated three times with varying percentage of the raw FastQ sequence data used (10–50%), keeping mtDNA coverage at 60–100×. The obtained mtDNA was annotated with the aid of MITOS Web Server available at http://mitos.bioinf.uni-leipzig.de/ and aligned with available *Rhipicephalus* species genomes. Complete mtDNA sequences were analysed in CLC Genomics Workbench 6.9.1. (CLC bio, Qiagen) for manual validation.

TaqMan qPCR assay for detection of mammalian and *Babesia bovis* DNA

A TaqMan qPCR assay targeting a mammalian housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was conducted to confirm the presence of mammalian DNA in the Kwale *R. (B). microplus* DNA samples. To detect *B. bovis* DNA, an assay targeting two different *B. bovis* specific genes was done. One set targeted the nuclear rDNA18S [24] and the other targeted the mitochondrial cytochrome b gene [25]. The primer and probe sequences used for detection of both mammalian and *B. bovis* DNA are listed in Table 2. The qPCR was conducted in a CFX96 Touch™ Real-Time PCR detection system (BioRad, Australia). The 20 μL reactions included 10 μL of SensiFAST 2× Probe Mix (Bioline Australia Pty Ltd., Alexandria, NSW, Australia), 0.8 μL of each oligonucleotide primer, 0.2 μL of the FAM or HEX labelled probe, and 2 μL of genomic DNA template. Temperature cycling conditions were: 95.0°C for 3 min followed by 39 cycles of 95 ◦C for 10 s, 54 ◦C for 15 s and 72 ◦C for 30 s. The sensitivity and efficiency of the *B. bovis* assay was determined by using serial 10-fold dilutions of *B. bovis* control DNA (48ng/μl) ranging from 1:10-1:10^7. Positive and negative controls (no-template PCR grade water) were included in each PCR run. The threshold was set to 100 relative fluorescence units (RFUs) for the three

assays and the cycle quantification (Cq) scores corresponding to the PCR cycle number at which the amplification curve of each sample intersected the threshold line were recorded for each sample.

Data analysis

COI sequence chromatograms were visually inspected and resulting sequences edited manually using CLC Main Workbench 20 software (CLC bio, Qiagen GmbH, Germany). Sequences were trimmed to remove low quality reads at the 5´ and 3´ ends and consensus sequences generated from the sequenced fragments. Molecular identity of the study ticks was confirmed via BLASTN [26] searches of the COI against the GenBank's non-redundant nucleotide sequence database. Multiple sequence alignments of the COI and mtDNA genomes were performed using ClustalW2 in CLC Main Workbench. COI sequences of the 23 *Boophilus* ticks were collapsed into haplotypes, using DnaSP v5.10.01 [27]. Percent identity analyses was performed using Clustal Omega multiple sequence analyses tool [28]; https://www.ebi.ac.uk/Tools/msa/clustalo/). A COI phylogenetic tree was constructed by employing the Maximum Likelihood (ML) algorithm implemented in MEGA X [29] using a total of 42 nucleotide sequences which included three Kwale *R. microplus* COI haplotype and one *R. appendiculatus* sequences, 11 sequences from reference ticks and 28 GenBank reference sequences. The best nucleotide substitution model which gave the lowest Bayesian Information Criterion (BIC) score (3833.705) was Tamura 3-parameter (T92+I) as determined using MEGA X. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 69.54% sites). All positions with less than 95% site coverage were eliminated (partial deletion option). Clade support was assessed via 1,000 bootstrap replications. Bootstrap values below 70% were collapsed. There were a total of 403 positions in the final COI dataset. A mtDNA phylogenetic tree was constructed using complete mtDNA genome sequences of two *R. microplus*, one *R. decoloratus* and one *R. appendiculatus* from this study and 13 reference tick mtDNA sequences available in GenBank. The best substitution model for the mtDNA tree which gave the lowest BIC score (148759.37) was General Time Reversible (GTR) [30]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5523)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 30.59% sites). The analysis involved 17 nucleotide sequences. There were a total of 18435 positions in the final mtDNA dataset. Evolutionary analyses were conducted in MEGA X.

Results

Morphological identification of tick species

Of the 35 ticks collected from Kwale, 16 specimens were semi-engorged, 11 were fully engorged, 3 were partially fed and 5 were slightly fed (Table 1). On microscopic examination, 14 specimens were found to have damaged or missing mouth parts while 21 specimens were intact. All the study tick specimens were examined for key morphological characteristics defining *Boophilus* species. In Kenya, *R. decoloratus* is endemic and widely distributed and is likely to be confused with *R. microplus*. Thus, reference *R. microplus* and *R. decoloratus* ticks were also analysed and compared to the Kwale ticks. Since most the

ticks were semi- or fully engorged and some had missing mouth parts, only those specimens that were intact could be tentatively identified based on morphological features described in the identifications guides and keys used.

Microscopically, the principal features used for identification and discrimination of ticks in the sub-genus *Boophilus* from other *Rhipicephalus* species included an inornate scutum, short mouth-parts with palps slightly extending beyond the mouth, small eyes, pale yellow legs, a round-shaped spiracular plate, absence of festoons, and presence of ventral anal plates with spurs in males. Among the features used to discriminate species within the *Boophilus* sub-genus are the structure of denticles in the hypostome in both males and females, presence of seta on palp article one in females, the length of spurs on coxa I and presence or absence of caudal appendages in males [21]. The basis capituli was the only feature that was clear in intact semi- or fully engorged adults as most of the other features become unclear when the tick body stretches after feeding.

As expected, reference *R. microplus* female specimens analysed in this study had four rows of denticles on each side of the hypostome and a concavity with no setae on the medial aspect of article one of the palpal segment (Figure 2). Eleven of the twenty-one ticks that had an intact mouth part were found to have morphological features characteristic of female *R. microplus* while 12 of the 14 ticks with damaged mouth parts had a spiracular plate (Figure 2) consistent with that of *Boophilus*, they were thus tentatively grouped as *Boophilus* with no species designation. There were no male *R. microplus* in the Kwale collections. Reference *R. australis* males from Queensland had four rows of denticles on each side of the hypostome and indistinct adanal plates with short spurs which did not reach the posterior body margin and were not visible dorsally (Figure 3). On the other hand, reference *R. decoloratus* females and males had three rows of denticles on each side of the hypostome. Females had a convex protuberance with setae on the first palpal segments while males had long distinct adanal plates with long spurs which extended beyond the posterior body margin and were clearly visible outside the scutum (Figure 3 and 4). No *R. decoloratus* specimen was found among the 35 ticks from Kwale. Ten tick specimens had features consistent with *R. appendiculatus* while two specimens had features consistent with the *Amblyomma* genus. Figure 5 shows mouth part images of representative specimens from 11 ticks from Kwale with features consistent with *R. microplus*. The tentative species identity of all the 35 tick specimens from Kwale based on observed morphological features is listed in Table 1.

Molecular identification and genetic characterization based on COI

Molecular analysis involving partial amplification and sequencing of cytochrome oxidase subunit I (COI) gene was performed on each of the 23 tick specimens tentatively identified as *Boophilus,* eight identified as *R. appendiculatus*, one *A. variegatum* and the 11 reference specimens. A total of 43 ticks were characterized based on the COI sequence. BlastN analysis of the COI sequences confirmed the 23 specimens from Kwale to be *R. microplus* as well as the *R. appendiculatus, A. variegatum* and the species identity of reference specimens.

The 23 *R. microplus* COI sequences exhibited nucleotide similarity >99.5%. Only two informative polymorphic sites were found, resulting in three haplotype sequences differing in two variable polymorphic sites at position 51 and 483 with a haplotype diversity (Hd) of 0.5692. Haplotype 1 consisted of six sequences which had the bases TG while fourteen sequences formed haplotype 2 with bases CG at the two variable positions. Haplotype 3 consisted of 3 sequences with bases CA at the two polymorphic sites. COI sequences of the 23 Kwale *R. microplus* and 11 COI sequences of reference ticks appear in GenBank under the Accession numbers MT181192-MT181227 while the three haplotype sequences appear under Accession numbers MT181228-30 (Table 1).

Pairwise identity comparisons between the three *R. microplus* haplotype sequences and reference sequences obtained in this study or from GenBank are indicated in Supplementary Table S1. The three Kwale haplotypes had a high similarity of >99.6%. They matched with >99% to each of the 10 *R. microplus* reference sequences from Africa. These included a Kenyan *R. microplus* sequence (Rm_KE_KX228549), sequences from Cameroon (Rm_CF4, Rm_CF5, Rm_CM__MK648412, Rm_CM_MG983832, Rm_CM_MG983831), Congo (Rm_DRC_MF45873), South Africa (Rm_SA_KY678117), Benin (Rm_BN_KY678120) and Madagascar (Rm_MA_KY678118). They also had a high similarity (99.5%) with GenBank *R. microplus* reference sequences from USA (Rm_USA_KP143546), Colombia (Rm_CL_KT906181) and Philippines (Rm_PH_KX228548) (Supplementary Table S1).

The Kwale *R. microplus* haplotypes had an identity of 94.5% to *R. australis* sequences (Rm_QLDF1, Rm_QLDF4, Rm_QLDF6) obtained in this study and two other Australian GenBank reference sequences (Rm_AU_KC503255 and Rm_QLD_AF132827). Sequences from reference *R. microplus* specimens from Laos and GenBank reference sequences from China matched with a lower identity of 92%. The Kwale haplotypes matched to GenBank *R. annulatus* reference sequences from India (Ran_IN_KX228542), Cameroon (Ran_CM_MK648411) and Burkina Faso (Ran_BF_KY678123) with between 92-93% identity. They matched to *R. decoloratus* sequences from reference specimens from Kenya (Rd_KBF6 and Rd_KBF7) and Cameroon (Rd_CdF1 and Rd_CdF6) with about 88%, the same with other *R. decoloratus* reference sequences from GenBank (Rd_SA_KY678130, Rd_SA_AF132826) from South Africa, (Rd_CM_MK648413) from Cameroon and (Rd_Bfaso_KY678127) from Burkina Faso (Supplementary Table S1).

COI phylogeny and genetic relationships

Phylogenetic analysis was undertaken to determine the genetic relationships between the Kwale *R. microplus* haplotype sequences and reference *R. microplus*, *R. australis* and *R. decoloratus* isolates analysed in this study and other annotated reference sequences available in GenBank. A tree (Figure 6) was constructed using the three *R. microplus* COI haplotype sequences, the 11 reference sequences analysed in this study and 28 GenBank reference sequences. Two *R. appendiculatus* sequences, one from GenBank (Rap_AF132833) and another obtained in this study (Rap_KF18) were used as outgroups. Only significant bootstrap values above 70% are shown.

In the COI tree (Figure 6), four major clusters (Clade A-D) of *R. microplus* complex were observed. The Kenyan sequences (Rm_KW_H1-H3) clustered in a major clade (Clade A) with sequences from Cameroon (Rm_CF4 and Rm_CF5) analysed in this study and three GenBank reference sequences (Rm_CM__MK648412, Rm_CM_MG983832, Rm_CM_MG983831), one from Congo (Rm_DRC_MF45873), South Africa (Rm_SA_KY678117), Benin (Rm_BN_KY678120) and Madagascar (Rm_MA_KY678118), USA (Rm_USA_KP143546), Colombia (Rm_CL_KT906181) and Philippines (Rm_PH_KX228548). The cluster was strongly separated (100%) from a GenBank *R. microplus* sequence from Kenya (Rm_KE_KX228549). Clade A was strongly separated (92%) from a *R. australis* sister clade (clade B). Clade A and B formed a larger *R. microplus* complex cluster which was moderately separated (65%) from a *R. annulatus* clade. Two sequences of *R. microplus* specimens from Laos (clade C) analysed in this study clustered with GenBank reference sequences from China (clade D). The Lao sequences were moderately separated from the Chinese sequences (68%). The Laos/China cluster was strongly separated (96%) from the *R. microplus/R. annulatus* cluster (Figure 6). The Kenyan *R. decoloratus* sequences analysed in this study (Rd_KBF6, Rd_KBF7) clustered closely in one clade with those from Cameroon (Rd_CdF1 and Rd_CdF6) and other reference sequences from South Africa (Rd_SA_KY678130, Rd_SA_AF132826), Cameroon (Rd_CM_MK648413) and Burkina Faso (Rd_Bfaso_KY678127).

Mitochondrial genomes characterization and phylogeny

MITOS, a web server for automatic annotation of metazoan mitochondrial genomes was used to annotate proteins, tRNAs and non-coding RNAs in the four complete mtDNA genomes analysed in this study. Multiple sequence (MS) analysis was performed on the four mtDNA sequences and 13 reference genomes from GenBank. These included six *R. microplus*, one *R. annulatus*, one unverified *R. decoloratus*, one *R. geigyi,* a partial *R. appendiculatus*, one *R. sanguineus*, one *R. turanicus* and one *H. longicornis* reference genome sequences. Figure 7 shows the 13 proteins, 22 tRNAs and two rRNAs annotated by MITOS for the four mtDNA sequences analysed in this study.

From the MITOS prediction, the arrangement and length of the annotated mitochondrial features in the two *R. microplus* (Rm_KF13 and Rm_KSF2) genomes was very similar (Figure 7). The program predicted the presence of two trnE genes (65bp) in the two genomes. The first occurred in the plus strand in position 4614-4678 while the second is in position 4744-4808 for both Rm_KF13 and Rm_KSF2. Both predictions had a similar e-value of 3.313e x 10^{-05}. In the MS alignment, this region was part of control region I which occurs within the tandem repeat region annotated between trnE and nad1 in other reference *R. microplus* genomes.

The program also predicted pseudo copies of tRNA genes for trnA (gca), trnR (cga), trnN (aac), trnS1(aga) and trnE (gaa) (Figure 7) in position 4854-5184 of the *R. decoloratus* (Rd_KBF6) mt genome. The MS alignment showed the first prediction in position 4368-4610 to be conserved and common to the reference mtDNAs. The pseudo prediction from position 4854-5184 was a 331bp long AT rich sequence lacking in the unverified *R. decoloratus* reference sequence and in other reference genomes. It occurs as

part of the tandem repeat region annotated between trnE and nad1 in other reference *R. microplus* genomes.

MITOS could not locate the gene for trnS1 in the *R. apendiculatus* (Rap_KF10) genome. In the reference mtDNA annotations, the trnS1 gene is reasonably conserved located upstream of the trnN gene in position 4557-4612 for Rm_KF13 and Rm_KSF2 and 4555-4610 for Rd_KBF6. In the *R. appendiculatus* Rap_KF10 sequence, the region (4543-4599) is predicted by MITOS to be a pseudo non-standard tRNA feature (trnX) (Figure 7). In the MS alignment, two nucleotides are missing from the 5' start of the gene in Rap_KF10 and the partial *R. appendiculatus* Zimbabwe reference genome when compared to the predicted trnS1 gene of the *R. microplus* reference genomes. MITOS also predicted a short nad4 gene and two nad5 genes in the minus strand in Rap_KF10 (Figure 7). The first nad5 (1500bp long) at position 8251-9750 with a quality value of 2.05×10^8 and a second tiny pseudo fragment (45 bp) at position 11081-11125 with a value of 375.9. With such a lower value, the 2nd prediction is highly unlikely. In the MS alignment, this fragment is part of nad4 which appears to be conserved across the other reference genomes.

Pairwise identity matches and phylogenetic analysis were performed on the four mtDNA sequenced in this study and the 13 reference sequences from GenBank. The mtDNA genome sequences appear in GenBank under the Accession numbers MT430985-88. The percent identities observed are shown in Supplementary Table S1. The two *R. microplus* sequences Rm_KF13 and Rm_KSF2 sequenced in this study had very high nucleotide similarity (99.97%). They matched with an identity of greater than >98% to the *R. microplus* reference genome sequence from India, Cambodia, Brazil and USA. They matched to the *R. australis* reference genome (Rm_AU_KC503255) with a similarity of 96.07% and to the China sequence (Rm_CHI_KC503259) with an identity of 94.34%. Their similarity to the *R. decoloratus* sequence (Rd_KBF6) was 87.15%, 94.26% to the reference *R. annulatus* (Ran_ROM_KC503256), 87.32% to the *R. geigyi* (Rgei_BF_KC503263) and 82.5% to the *R. appendiculatus* sequence (Rap_KF10). The *H. longicornis* sequence (HL_MK450606) was used as an outgroup. It matched to the other sequences analysed with an identity ranging between 72-75%.

In the mtDNA phylogenetic tree (Figure 8), four major clusters were observed with all the nodes strongly supported by 100% bootstrap value. The two *R. microplus* genome sequences (Rm_KF13 and Rm_KSF2) clustered closely with *R. microplus* reference sequences from Brazil (Rm_BZ_KC503261), USA (Rm_USA_KP143546), Cambodia (Rm_CA_KC503260), India (Rm_MK234703 INDIA) and Australia (Rm_AU_KC503255). This *R. microplus* cluster was separated from a sister clade consisting of a reference sequence from China (Rm_CHI_KC503259) and a *R. annulatus* (Ran_ROM_KC503256) sequence. The *R. decoloratus* sequence (Rd_KBF6) clustered with the unverified sequence (Rd_SA_KY457525) from South Africa and were separated from the *R. geigyi* sequence. This *R. decoloratus* sequence matched with an identity of 99.13%, 90.54% and 82.81% to the unverified, *R. geigyi* and *R. appendiculatus* (Rap_KF10) sequences respectively (Supplementary Table S1). The *R. appendiculatus* (Rap_KF10) sequenced in this study clustered with the reference partial sequence (Rap_ZM_KC503257) from Zimbabwe (Figure 8). The percent identity matches between these two

sequences was 99.18%. The *R. appendiculatus* Rap_KF10 sequence matched to the reference *R. geigyi* sequence with an identity of 83.49% and showed identities of 82.81% and 83.69% to the *R. decoloratus* (Rd_KBF6) and the reference *R. annulatus* sequences respectively (Supplementary Table S1). In the tree, *R. sanguineus* and *R. turanicus* sequences clustered in a final clade with similarities of 89.2%.

Molecular detection of *B. bovis* in *R. microplus* ticks

Genomic DNA samples from 21 specimens confirmed by molecular analysis to be *R. microplus* were subjected to sensitive molecular quantitative qPCR assays to detect presence of bovine blood and consequently *B. bovis* parasites. Presence of cattle blood DNA was tested in undiluted (neat) gDNA by amplifying glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene found in all mammalian cells.

The cycle quantification (Cq) values ranged from a high of 39.45 in KSF3 to a low of 23.40 in KF16. At a threshold of 100 relative fluorescent units (RFU), five samples had Cq values below 30 while 14 had values that ranged from 30-39. Bovine DNA was not detected in 2 samples (KF-K6 and KSF5). Analysis of a 1:10 diluted DNA of these two samples resulted in the detection of bovine DNA (Cq value of 37.26) in KSF5. No DNA was detected in the diluted KF-K6 sample.

Having confirmed the presence of bovine DNA in the extracted tick DNA, *B. bovis* specific primers were used to amplify specific regions of cytochrome b (Cytb) and 18S rDNA genes to detect presence of *B. bovis* parasites. At a threshold of 100 RFU, all material was considered negative for *B. bovis* DNA using either assay (Cq values = >40.00).

Discussion

The invasive *Rhipicephalus Boophilus microplus*, a highly efficient vector of tick fevers caused by *Babesia bovis, Babesia bigemina*, and *Anaplasma marginale* has rapidly spread into many African countries over the last decade [3–11]. In many areas, it has been reported to progressively displace, the indigenous *R. decoloratus* and *R. annulatus* [3, 5, 11, 32–35] sometimes establishing itself in areas predicted to be climatically unsuitable for its survival [7, 32, 35]. The latest findings of widespread occurrence of *R. microplus* in eastern Uganda [11] and recent introduction and spread of the tick in Cameroon [10] have been attributed to uncontrolled live animal movements from neighbouring countries and cross-border animal trade.

R. microplus was first recorded in Kenya in 1974 in a limited area along the Kenyan coast [15]. This study confirms that *R. microplus* still occurs in the region, although *B. bovis* was not detected in the limited number of ticks analysed. Of the 35 Kwale tick specimens analysed, 23 were confirmed by both morphology (Fig. 5) and genotyping (Figs. 6 and 8) to be *R. microplus*. Those with intact mouth parts had the characteristic 4 + 4 rows of denticles on each side of the hypostome (Fig. 5). This feature was clearly distinct from the 3 + 3 rows of denticles observed in *R. decoloratus* (Fig. 4). Since most of the ticks were either semi-or fully engorged, other detailed morphological features were not apparent. The coastal plain

in Kwale is usually hot and humid with an average temperature above 25 °C and precipitation greater than 1000 mm annually [36]. In southern Africa, *R. microplus* has been shown to prefer warm and humid conditions [37]. Warm temperatures of at least 15–20 °C are required for egg laying and larval hatching to occur. Thus, the eco-climatic conditions in Kwale are suitable for the survival of *R. microplus*. The tick is widespread over most of the neighbouring Tanzanian mainland [35]. The cross-border animal market at Tanga and transhumance at Shimoni port could be contributing to the re-introduction and spread of *R. microplus* from mainland Tanzania and the Indian ocean Islands into Kenya.

The COI gene has previously been shown to have greater intraspecies resolution within the *R. microplus* complex compared to 12S, 16S or ITS2 genes [19, 20, 38]. Based on COI phylogenetics, five distinct taxa have been shown to occur within the *R. microplus* species complex [19, 20]. These include clade A (ticks from Africa, Asia and S. America) and B (Southern China and Northern India) of Burger et al., (2014) [19]; clade C (ticks from Malaysia and India) of Low et al., (2015) [20], *R. australis* and *R. annulatus* which has been found to be closely related to ticks in clade B sensu Burger et al., (2014) [19]. The Kenyan *R. microplus* sequences analysed in this study clustered with other African *R. microplus* reference sequences into Clade A sensu Burger et al., (2014) [19] (Fig. 6). The cluster included sequences from Kenya, Congo, Cameroon, Benin, Madagascar and S. Africa as well as sequences from USA, Brazil, Colombia, and Philippines. The single Kenyan reference sequence was however, significantly separated (100%) from the rest of the sequences in this cluster. *R. australis* formed a strongly supported (92%) distinct sister clade as previously observed [19, 20]. In our analysis, *R. annulatus* was more closely related to the clade A/*R. australis* complex. These three taxa were strongly separated (96%) from a cluster composed of reference ticks from Laos (clade C) and sequences from China (clade D) (Fig. 6) which are thought to constitute a cryptic species [19].

Previous studies on genetic diversity and population structure of *R. microplus* have reported a low genetic variation and differentiation. Low et al., (2015) [20] reported a relatively low but significant genetic differentiation of *R. microplus* in Malaysia while in S. African a low variation within the species was observed with COI and 16S rDNA genes [38]. A similar low divergence was also recently observed in Cameroonian *R. microplus* ticks by Silatsa et al., (2019) [10] using the two mitochondrial genes. Low levels of genetic differentiation and lack of population structuring amongst *R. microplus* populations based on microsatellite markers has also been observed [38–40]. In this study, we also found a low divergence and lack of geographical sub structuring among the African COI sequences analysed (Supplementary Tables S1 and Fig. 6). The same low divergence has also been observed with the 16S rDNA gene [10, 20]. *R. microplus*, a one-host tick that completes its entire life cycle attached to a single vertebrate host can be classified as a host specialist [41]. Cattle are thought to be the only effective maintenance vertebrate hosts of *R. microplus* and although it can infest sheep, goats and other wild bovidae, infestations of other hosts only occur when a population of ticks is maintained on cattle [21]. De Meeûs et al., (2010) [40] reported the divergence of *R. microplus* into two differentiated host-specific genetic groups with little or no genetic exchange within a short period of time after the tick was introduced in New Caledonia. One group was specific to cattle and another specific to the rusa deer which often co-grazes with cattle. This strict host specialization and the observed sympatric isolation can limit

genetic exchange and flow resulting in very low genetic differentiation. All the sequences analysed in this study were derived from ticks collected from cattle except one Kenyan GenBank reference sequence (Rm_KE_KX228549) that had been collected from a migratory Zebra [42] (Fig. 6). Interestingly, the Kenyan reference sequence was strongly separated (100%) from the rest of the African ticks. A genetic drift caused by tick population bottlenecks such as a small effective population size or a founder event where a new population arises from few individuals such as was observed in New Caledonia can result in a decrease in allele frequencies leading to the low genetic variation and differentiation observed with *R. microplus* [40]. In West and Central Africa, founder events such as few ticks arriving with imported animals could cause the undifferentiation and low genetic variation observed. Analysis of a larger set of ticks from geographically separated areas in the continent using a combination of markers may reveal the gene flow patterns and population structure of *R. microplus* as it adapts into new areas.

Although *R. microplus* was first recorded in Kenya in the mid-70 s, it had been reported in Tanzania in the late 60 s [43]. These occurrences were attributed to the spread of the tick from South Africa where it had arrived from South East Asia [2] spreading to the rest of southern and eastern Africa regions [21]. The recent W. African COI genotypes would be expected to be differentiated from the East African genotype which is assumed to have originated from S. Africa and Asia in the late 18th Century. Although the numbers analysed in this study are few and from one general area, when combined with available data on other African genotypes, the sequence identities and COI clustering observed suggest a lack of phylogeographic structuring of *R. microplus* in Africa. The close clustering of African genotypes and low differentiation has also been observed with the 16S rDNA gene [10, 20]. Given the historical origins of the tick in the two geographically separated regions of West and East Africa, it was anticipated that isolation-by-distance would result in the eastern Africa ticks being genetically divergent from those from Central and West Africa. However, our findings reveal that there is very low divergence of *R. microplus* populations from East, South, West and Central Africa suggesting the existence of an undifferentiated gene pool with a monophyletic genotype circulating in cattle in the African continent. Since *R. microplus* was only recorded in W. Africa in the last decade, presumably from Brazilian cattle imports, these findings could suggest that the undifferentiated, highly conserved Brazilian and East African genotypes originated from the same ancestor, have remained in a closed genetic pool with no introgression, genetic flow or divergent population expansion over time. However, this is highly unlikely for a randomly breeding tick species such as *R. microplus* which has been shown to be easily dispersed across distant geographical areas through anthropogenic movement of cattle hosts. A continent-wide sampling and phylogenetic analysis of *R. microplus* from cattle and other animal hosts including wildlife may identify the factors contributing to the low genetic variation and limited divergence observed with the African *R. microplus*.

Previous studies have reported the genetic distinctiveness of *R. australis* and the close genetic similarities of *R. microplus* and *R. annulatus* [19, 44, 45]. We observed a divergence of 6% between the Kenyan and *R. australis* COI sequences with a strong support (98%) for a distinct *R. australis* sister clade (Fig. 6). The same clustering was also observed with the mtDNA tree (Fig. 8). The two Kenyan *R. microplus* mtDNA sequences diverged from *R. australis* and China mtDNA sequence with 4% and 6% respectively. Burger et al., (2014) [19] had also observed distinct separation of *R. australis* from a clade containing the Brazil

and Cambodia sequences. In our analysis, the *R. microplus* complex cluster was separated from the *R. annulatus* clade in the COI tree though not with a significant bootstrap support (65%-not shown) while in the mtDNA tree (Fig. 8), the *R. annulatus* mtDNA sequence clustered with a China sequence. Both species share many morphological similarities [21] and differential diagnosis can be difficult. The divergence of the Kenya COI sequences from reference *R. annulatus* sequences from India, Cameroon and Burkina Faso was between 6–8% while it was about 6% for the reference *R. annulatus* mtDNA from Romania. In the mtDNA tree, *R. annulatus* is genetically more closely related to the Chinese *R. microplus*. A higher divergence of 8% was observed with reference *R. microplus* COI sequences from Laos which were significantly separated and genetically distinct from the *R. microplus/R. annulatus* cluster (Fig. 6). Although they formed a sister clade, these sequences were more closely related to the China cryptic species reported by Burger et al., (2014) [19].

Analysis of whole mitochondrial (mt) genomes of two Kenyan *R. microplus* confirmed the high conservation of mtDNA genome within the *R. microplus* complex as observed by Burger et al., (2014). The arrangement and length of the 13 mitochondrial proteins, 22 tRNAs (trn) and two rRNAs (rrn) in the two Kwale *R. microplus* genomes is similar to the reference genomes reported by Burger et al., (2014). The pseudo trnE gene annotated by MITOS in the two Kenyan *R. microplus* genomes appears to be part of the tandem repeat region found to be present in all members of the *R. microplus* [19, 46]. This 150 bp tandem repeat consists of the 3' end of trnS, trnE, the Tick-Box motif and the 3' end of nad1. The Tick-Box is a 17 bp motif which intersperses the 3' ends of nad1 and 16S rRNA during transcription in tick mt genomes [47]. Pseudo copies of tRNA genes for trnA (gca), trnR (cga), trnN (aac), trnS1(aga) and trnE (gaa) were predicted in *R. decoloratus* (Rd_KBF6) mt genome (Fig. 7). This 331 bp insert occurred within the variable tandem repeat region annotated in other *R. microplus* reference genomes. It is lacking in the unverified *R. decoloratus* reference sequence and in other reference genomes. Two nucleotides were found to be missing from the 5' start of the trnS1 gene in the annotated *R. appendiculatus* mtDNA and the partial *R. appendiculatus* Zimbabwe reference genome when compared to the predicted trnS1 gene of *R. microplus* reference genomes.

R. decoloratus, the African blue tick, which also transmits *B. bigemina* and *A. marginale* is the most common and widespread of the *Boophilus* ticks in Kenya [21]. Although it has a characteristic 3 + 3 row of denticles (Fig. 4), morphological identification and differentiation of *R. microplus* and *R. decoloratus* is usually difficult because of their small size and they share many morphological similarities. Few *R. decoloratus* were found on cattle in Kwale during the survey by Zulu et al., (1998) [16]. None was found in the current study, though very few localities and numbers were sampled and analysed. The *R. decoloratus* COI sequences analysed in this study diverged from *R. microplus* haplotypes with about 12% while the mtDNA sequences diverged with about 13% from the Kenyan *R. microplus* mtDNA (Supplementary Table S1). *R. decoloratus* formed a distinct species clade both in the COI tree (Fig. 6) and the mtDNA tree (Fig. 8). The Kenyan *R. decoloratus* COI sequences clustered closely with African reference sequences from Cameroon, S. Africa, and Burkina Faso. However, one S. African COI reference sequence (Rd_SA_KY678130) appeared genetically distinct and was significantly separated (99% bootstrap) from the majority cluster (Fig. 6). A divergence of 13% was observed between the Kenyan mtDNA sequences

and *R. geigyi* while a divergence of 17.5% was observed with the *R. appendiculatus* mtDNA sequence. Based on the complete mtDNA phylogenetics (Fig. 8), *R. decoloratus* was found to be more genetically related to *R. geigyi* than to *R. microplus* and *R. annulatus* as was observed by Burger et al., (2014) [19].

Although *R. microplus* has only been recorded in a small focus along the Kenyan coastline, *B. bovis* DNA has recently been reported in cattle blood from Central and western Kenya. Based on *B. bovis* spherical body protein-4 (SBP-4) nested PCR amplification and subsequent sequencing of the amplicons, Moumouni et al., (2015) [18] detected *B. bovis* in 19 of 110 blood samples from a dairy cattle farm in Ngong area, Kajiado County, southwest of Nairobi. Another 9/25 blood samples from a farm in Machakos County Southeast of Nairobi were *B. bovis*-positive. In the Ngong farm, *B. bigemina* was found in 65 blood samples and 5 from the Machakos farm. Using a reverse line blot (RLB) assay targeting specific rRNA gene V4 hypervariable regions of all *Theileria* and *Babesia* species, Njiiri et al., (2015) [17] detected *B. bovis* (10/453) and *B. bigemina* (1/453) in blood samples collected from indigenous calves in Busia County, western Kenya. These observations attest to the possibility that *B. bovis* may already occur in Kenya but is probably disregarded in most studies screening for tick hemoparasites in the mistaken belief that the vector, *R. microplus* has not been recorded recently in Kenya. Failure to discriminate the "rarer" *R. microplus* from the more widespread *R. decoloratus* could be one of the reasons why *R. microplus* has not been reported in Kenya in the recent past. In Kenya and most of Africa, *B. bigemina* is endemic and more widespread than *B. bovis* because of the wide distribution and vector competence of *R. (B). decoloratus* and *Rhipicephalus evertsi evertsi*. The increased dispersion and geographical spread of *R. microplus* into new areas in eastern and southern Africa has caused a displacement of the indigenous *R. decoloratus* [11, 32–35]. With the current invasion and spread of *R. microplus* into new regions in West, Central and East Africa, it is important to determine the current status of the two Babesia parasites in areas where the tick co-occur especially with the widespread displacement of the endemic *R. decoloratus*.

In Kenya and in many other African countries, diagnosis of tick-borne diseases including babesiosis is mostly achieved through observation of clinical signs and microscopic examination of blood smears, methods which are rapid and inexpensive but not specific or sensitive especially in immune carrier animals. For epidemiological studies, serological antibody detection of tick-borne parasites is employed. The use of sensitive and specific molecular assays may increase and improve detection of tick-borne hemoparasites as indicated by the detection of *B. bovis* in Kenya [17, 18]. Conventional PCR-based techniques have been used to detect Babesia parasites with high sensitivity and specificity [48–51]. Highly sensitive quantitative PCR (qPCR) assays based on SYBR green [48] and Taqman probes [24, 25] able to detect, quantify and differentiate *B. bovis* from *B. bigemina* are also available. In this study, we used the two Taqman probes qPCR assay to screen the Kenyan *R. microplus* DNA for presence of *B. bovis* but none was detected. The use of species specific sensitive molecular surveillance tests will allow for detection and differentiation of *B. bovis* from *B. bigemina* which could co-infect animals in regions where they co-occur. Validated sensitive diagnostic and epidemiological assays should be employed to screen and confirm the status and distribution of *B. bovis* in Kenya and other Africa countries where *R. microplus* has recently spread.

Conclusion

Our study has confirmed the occurrence of *R. microplus* in Kenya but no *B. bovis* was detected in the limited sample of Kenyan ticks. The Kenyan *R. microplus* COI sequences clustered very closely with reference African genotypes. We found very low levels of genetic differentiation and lack of geographical sub structuring among the African COI sequences analysed suggesting the existence of monophyletic genotype circulating in cattle in the African continent. With current invasion and rapid spread of *R. microplus* in many African countries, it is important to determine whether its occurrence is accompanied by the presence of the pathogenic *B. bovis*. Our findings and the recent reports of widespread occurrence of *R. microplus* in Africa provide the fundamental basis and rationale to implement diagnosis, urgent strategic control and surveillance to determine and monitor the spread of *R. microplus* and *B. bovis* in Kenya and in the region.

Abbreviations

BLASTN: Basic local alignment search tool; BIC: Bayesian Information Criterion; COI: cytochrome *c* oxidase subunit I; Cq: cycle quantification; DNA: deoxyribonucleic acid; dNTPs: deoxyribonucleotide triphosphates; ILRI: International Livestock Research Institute; GTR: General Time Reversible; mt: Mitochondrial; mtDNA: Mitochondrial DNA; MS: Multiple Sequence; rDNA: ribosomal Deoxyribonucleic Acid; qPCR: quantitative PCR; SEM: Scanning electron microscopy; trn: transfer RNA; rrn: ribosomal RNA

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The nucleotide dataset(s) supporting the conclusions of this article are available in the GenBank repository (http: //www.ncbi.nlm.nih.gov/genbank/). The 23 Kenyan *R. microplus*, haplotype and reference ticks COI sequences are under the Accession numbers MT181192-MT181230. The whole mitochondrial genome DNA datasets appear in GenBank under the Accession numbers MT430985-MT430988. The whole genome sequence data is available at SRA NCBI BioProject: PRJNA611067 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA611067).”

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EGK, NG, EKN and RPB conceived the research idea. EGK, NG, EKN and DE developed the research concept. DE: Provided funding for the laboratory consumables. EGK and NG carried out field work. DE and JS designed, coordinated and supervised the morphological and molecular studies, data analysis and results presentation. EGK, DE and JS drafted the manuscript. All authors reviewed the manuscript to its current form and approved the final manuscript.

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Tables

Due to technical limitations, Table 1 is only available as a download in the supplemental files section

Table 2: Details of primers used for mammalian DNA and *B. bovis* qPCR detection assays

Target gene	Primer/probe name	Sequence	Size	Fluorescence label
18S reverse	S0933_BoR_18S_Kim	AGTCGTGCGTCATCGACAAA	20	
18S forward	S0934_BoF_18S_Kim	AGCAGGTTTCGCCTGTATAATG	22	
18S probe	S0935_BoP_Kim	CCTTGTATGACCCTGTCGTACCGTTGG	27	5'-FAM-3'
Cytochrome b forward	S0936_bovisF160_Cytb_Zhang	ATATGTTTGCATTTGCTG	18	
Cytochrome b reverse	S0937_bovisR249_cytb_Zhang	CTCCAAACCAATATGAAAG	19	
Cytochrome b Probe	S0938_bovisPb_cytb_ Zhang	CAAACCATAAAGTCATCGGTATATCCTAC	29	5-'HEX-3'
GAPDH forward	S0631_DOG_F	TCAACGGATTTGGCCGTATTGG	22	
GAPDH reverse	S0634_DOG_R	TGAAGGGGTCATTGATGGCG	20	
GAPDH probe	S0632_DOG_Probe	CAGGGCTGCTTTTAACTCTGGCAAAGTGGA	30	5'-HEX-3'

Figures

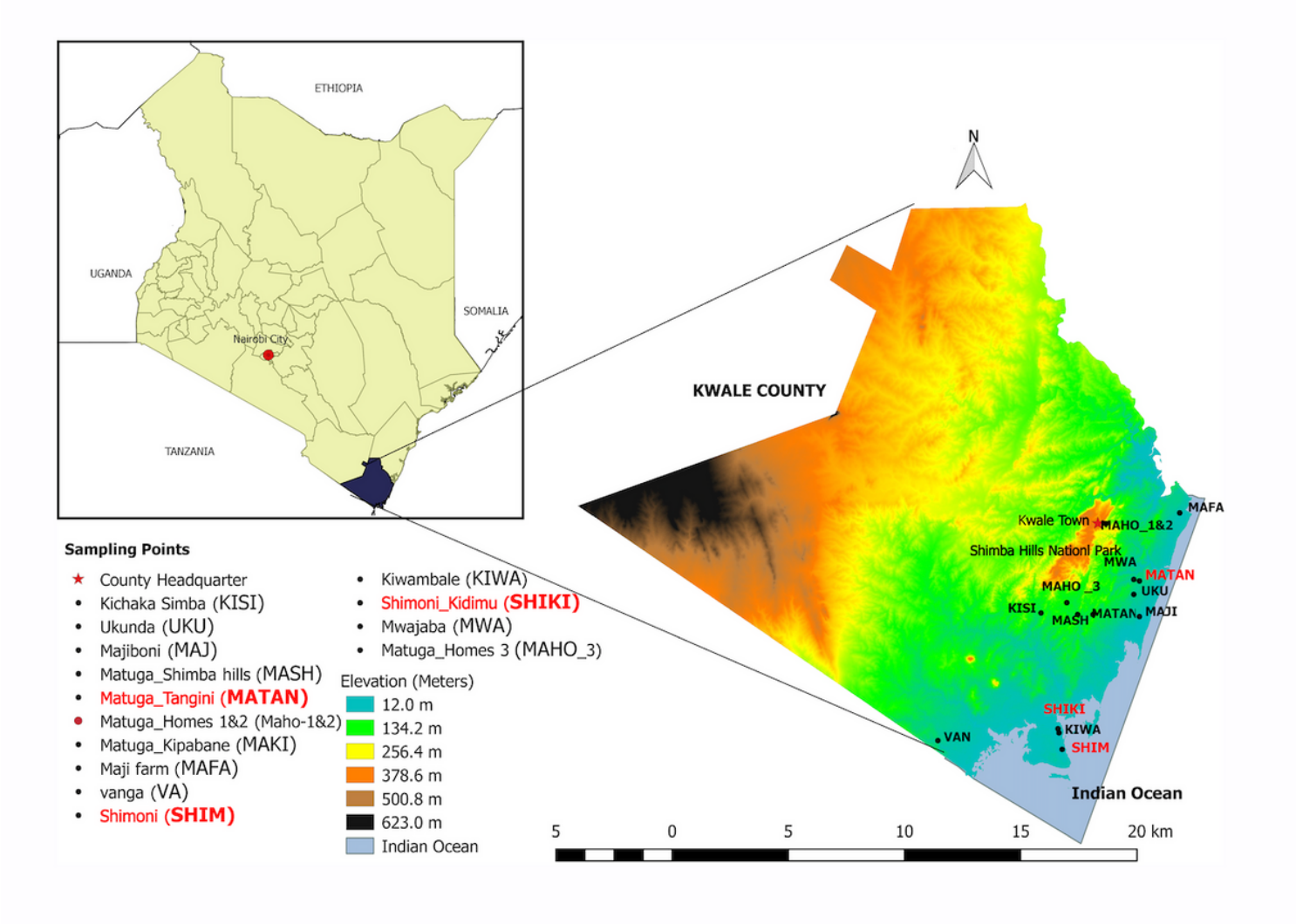

Figure 1

Map of Kenya showing Kwale County. Tick samples were collected from Matuga Tangini (MATAN), Shimoni (SHIM) and Shimoni Kidimu (SHIKI).

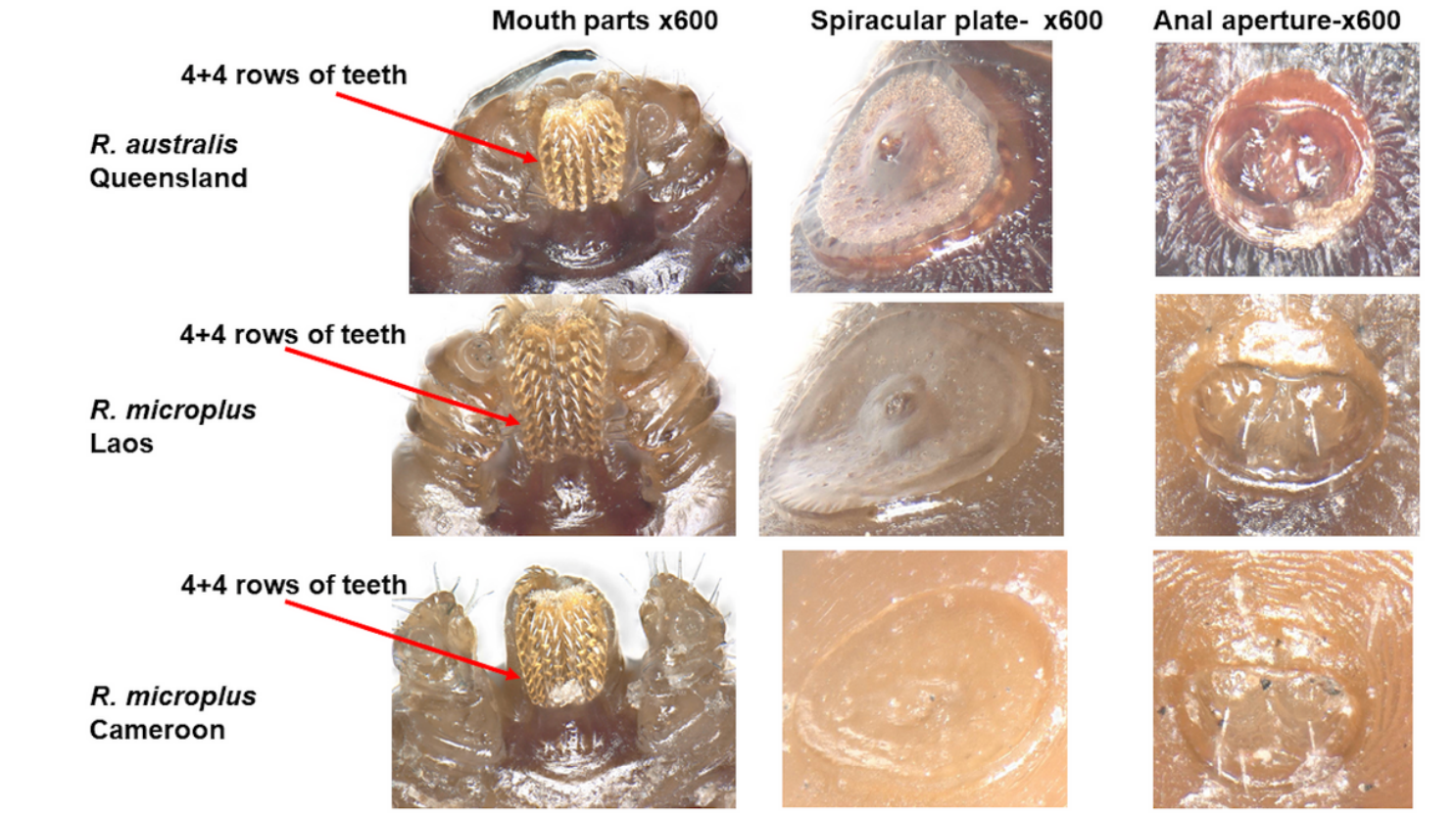

Figure 2

Mouth parts, spiracular plates and anal aperture images of R. microplus reference specimens from Queensland, Laos and Cameroon.

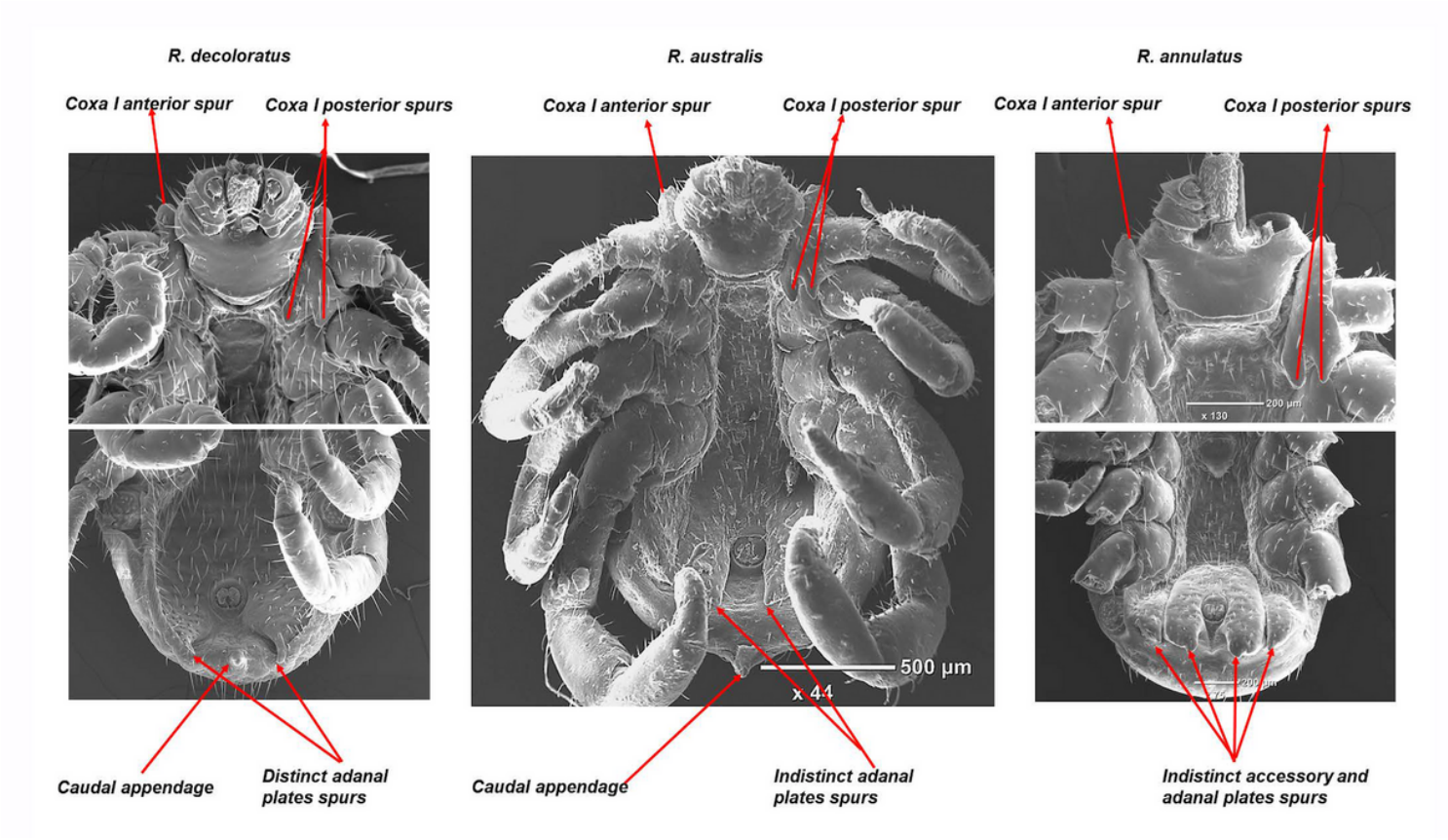

Figure 3

SEM images (ventral view) of male reference R. decoloratus, R. australis and R. annulatus showing some characteristic morphological features.

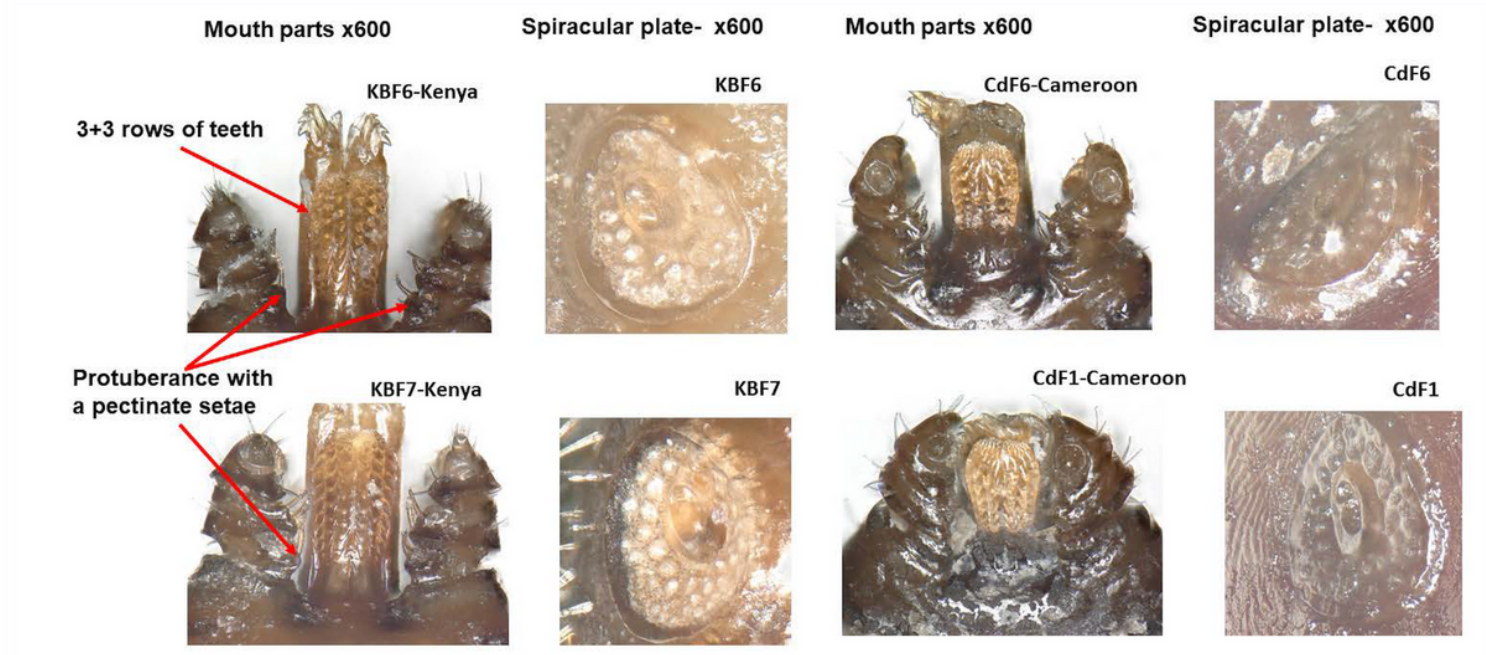

Figure 4

Mouth parts and spiracular plates images of R. decoloratus reference specimens from ILRI, Kenya and Cameroon.

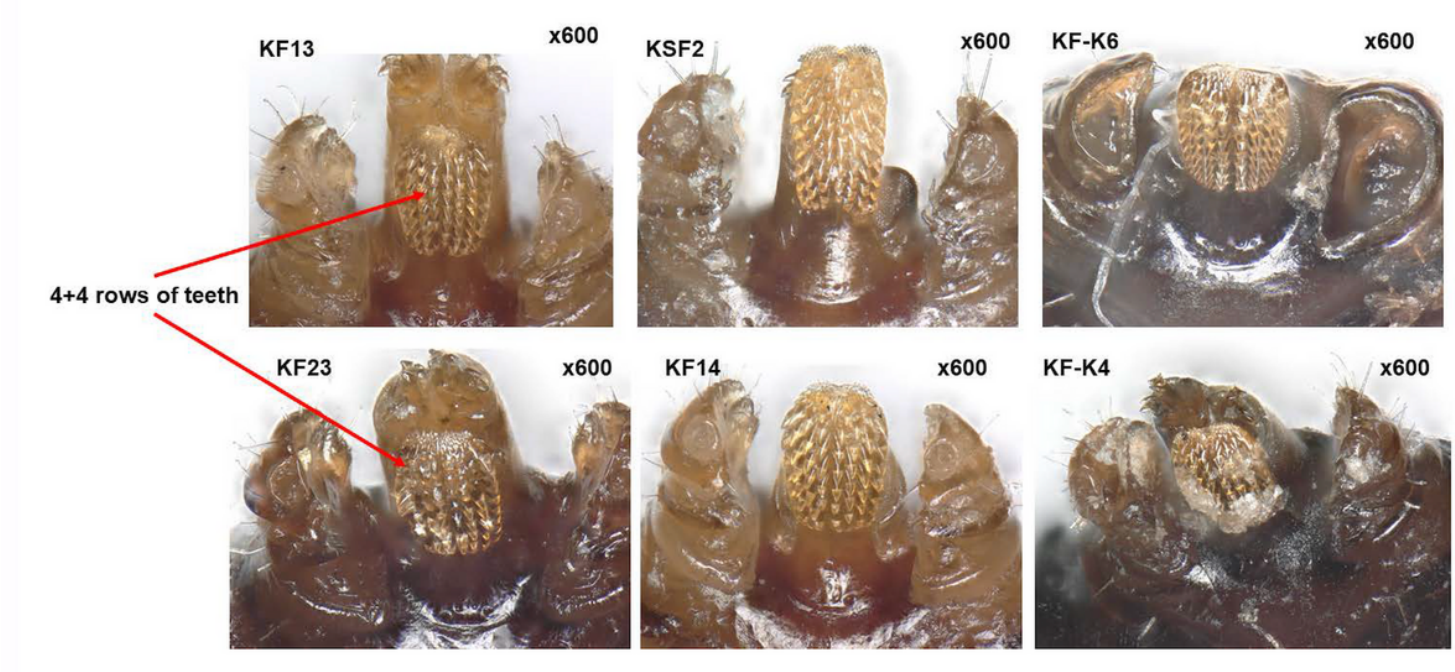

Figure 5

Mouth parts images of 6 female R. microplus specimens from Kwale County.

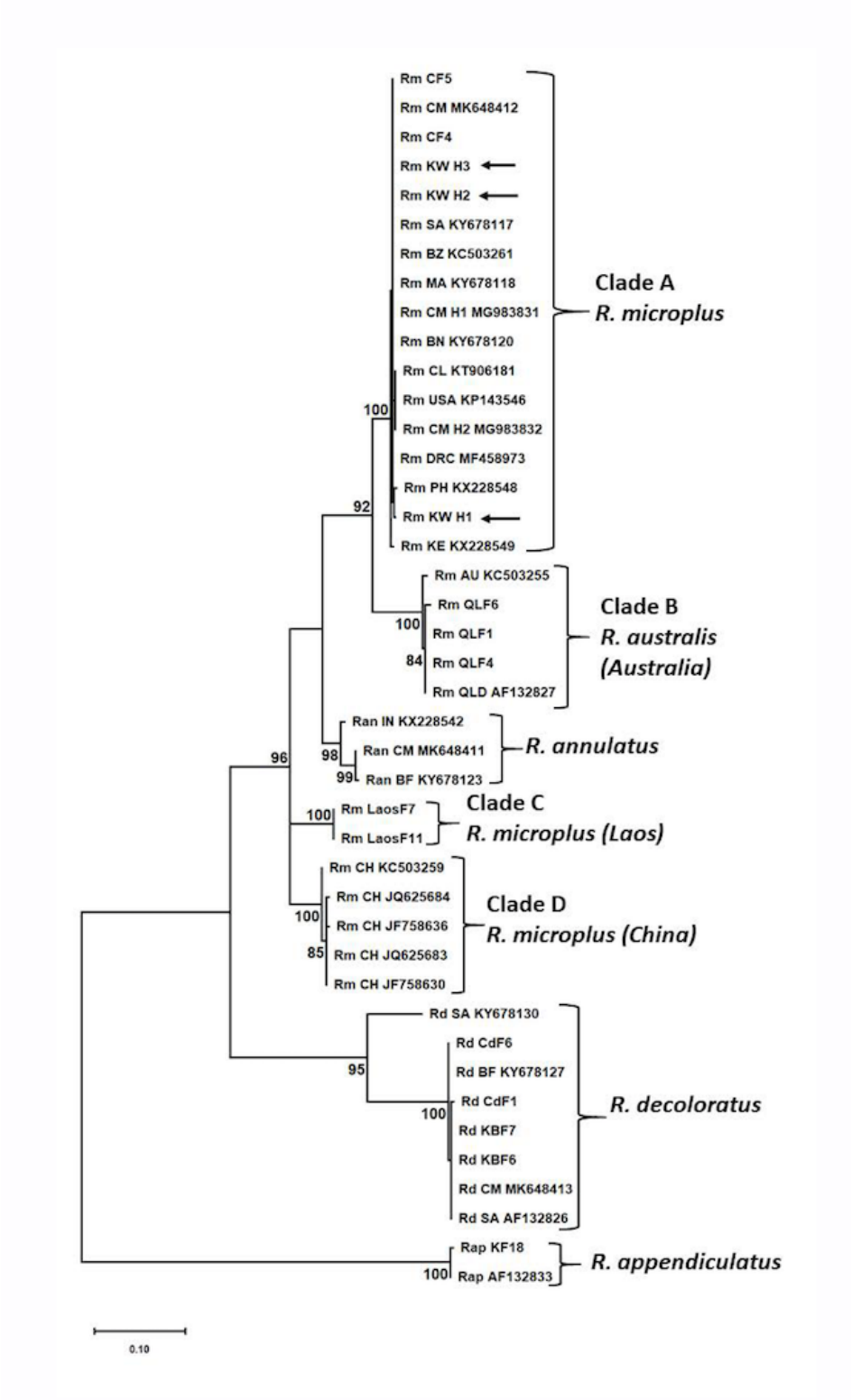

Figure 6

Maximum Likelihood (ML) tree showing the phylogenetic relationships between Kenyan R. microplus COI haplotype sequences (shown in arrows), 11 sequences of reference ticks analysed in this study and 28 GenBank reference sequences. The tree was constructed based on Tamura 3-parameter (T92) model [31]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 69.54% sites). This analysis involved 42 nucleotide sequences. The scale represents 0.10 nucleotide substitutions per site.

There were a total of 403 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [29]. Bootstrap values (1000 replications) above 70% are shown.

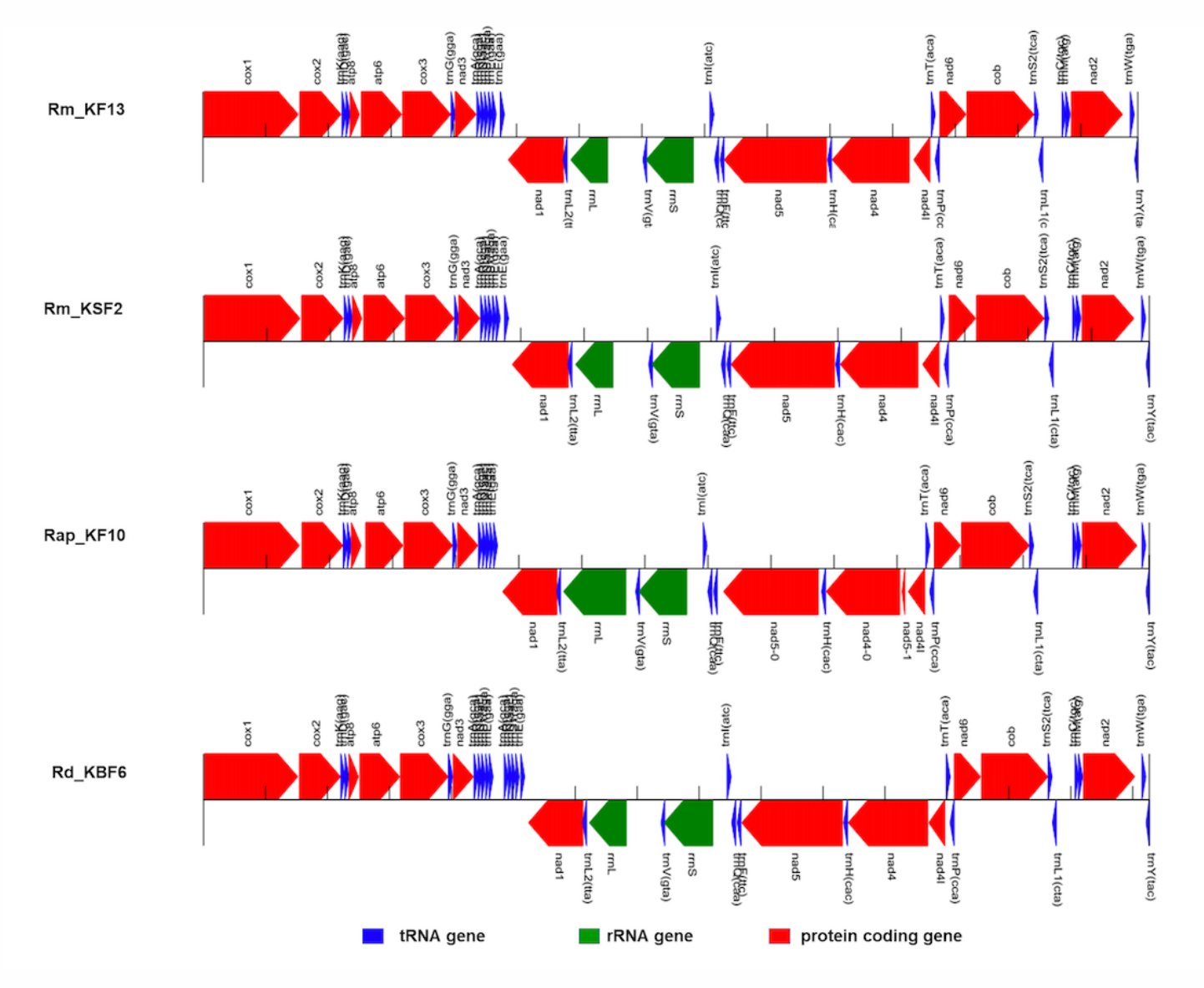

Figure 7

Graphical overview of the 13 mitochondrial proteins, 22 tRNAs and 2 rRNAs annotated by MITOS in the four mitochondrial genomes sequenced in this study. Genes located on the plus strand are drawn in the upper part. Genes annotated on the minus strand are shown in the lower region. A small vertical line is drawn every 1,000 nt. Rm_KF13 and Rm_KSF2 refer to genomes of R. microplus, while Rap_KF10 and Rd_KBF6 are genomes of R. appendiculatus and R. decoloratus respectively.

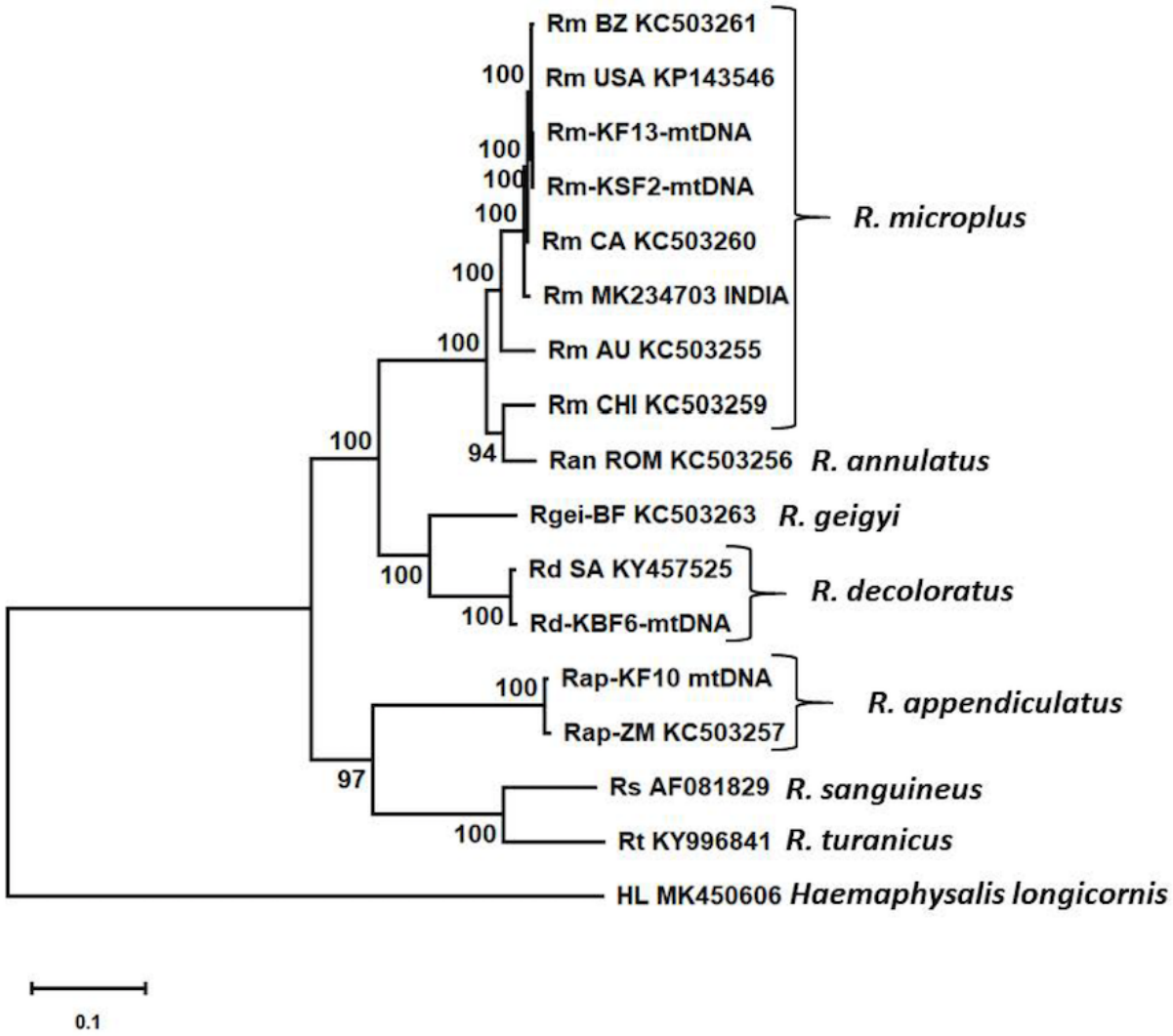

Figure 8

Maximum Likelihood (ML) tree inferred from 17 nucleotide sequences that included four mitochondrial genomes sequenced in this study and annotated reference genomes available in GenBank. R. microplus Rm_KF13 and Rm-KSF2, R. appendiculatus Rap_KF10 and R. decoloratus Rd_KBF6 were obtained in this study. Six R. microplus genomes, one genome each for R. annulatus, R. geigyi, R. sanguineus, R. turanicus and H. longicornis from GenBank were included. A partial R. appendiculatus (Rap_ZM_KC503257) and an unverified R. decoloratus (Rd_SA_KY457525) genome available in GenBank were used to compare the genome sequences of R. appendiculatus (Rap_KF10) and R. decoloratus (Rd_KBF6) respectively as no annotated complete genomes were available for the two species. The tree was reconstructed using the General Time Reversible (GTR) (Nei and Kumar (2000) in MEGA X. There was a total of 18, 435 positions in the final dataset.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx
- FinalGraphicalAbstract352020.tif
- Table1.xlsx