

Characterization of Two Cuban Strains of *Rhipicephalus Microplus* Ticks

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Abstract

Background: *Rhipicephalus microplus* (Canestrini, 1888) is one of the species with medical and economic relevance that had been reported in the list of Cuban tick species. Some morphological characterizations about the *R. microplus* species in Cuba have been published, however, molecular studies are lacking. Molecular phylogenetic analyses have revealed a common ancestor for *R. annulatus*, *R. australis* and three clades of *R. microplus* within the *Boophilus* subgenus. These five clades were grouped in a complex named *R. microplus*. The present study aimed the accurate taxonomic classification of *R. microplus* tick strains established as colonies in the Cuban National Laboratory of Parasitology.

Methods: Morphological characterization of adult specimens from two Cuban strains of the *R. microplus* ticks were carried out by using Scanning Electron Microscopy. The sequences of three mitochondrial genes: 12S rRNA, 16S rRNA and the subunit I of cytochrome c oxidase gene (COXI) and one nuclear gene: internal transcribed spacer 2 (ITS2) were used for phylogenetic analyses. The life cycle under laboratory conditions for both strains was also characterized.

Results: Tick specimens of both strains showed morphologic characteristics which were strongly coincident with those distinctive for the *R. microplus* species. Phylogenies based on mitochondrial gene sequences identified congruently the Cuban tick strains within the *R. microplus* clade A together with a Mexican reference strain and tick isolations from Argentina, Bolivia, Brazil, Mozambique, Costa Rica, Panama, Paraguay, Peru, Tanzania, United States, Uruguay and South Africa. Phylogenetic inferences based on nuclear ITS2 sequences also classified both tick strains as belonging to the *R. microplus* species but did not support the clades A, B and C previously described. The life cycle for both strains under established laboratory conditions averaged 65 ± 5 days and 2422 ± 295 and 2604 ± 304 larvae were obtained for each fully engorged female tick collected from CC and ML strains, respectively.

Conclusions: These results placed of Cuban tick strains as *Rhipicephalus microplus* clade A inside the *R. microplus* complex. This study constitutes the first molecular characterization of ticks from the *R. microplus* species in Cuba.

1. Background

There are 34 tick species described in Cuba of which 9 belong to the Ixodidae family or hard ticks [1]. Within monoxenous ticks in the *Rhipicephalus* genus only *Rhipicephalus microplus* (Canestrini, 1888) is present in the island. It is the major responsible of *Babesia bovis*, *Babesia bigemina* and *Anaplasma marginale* transmission to bovines in the country causing severe damages to livestock production [2].

R. microplus previously classified in the genus *Boophilus* with another 5 current valid species: *Rhipicephalus annulatus* (Say, 1821), *Rhipicephalus decoloratus* Koch, 1844, *Rhipicephalus australis* Fuller, 1899, *Rhipicephalus kohlsi* [3] and *Rhipicephalus geigy* [4] were reassigned to the genus *Rhipicephalus* based on substantial morphological and molecular data retaining *Boophilus* as a subgenus [5]. However, the taxonomic classification of species within the *Boophilus* subgenus based on

morphology has been largely complicated due to the great intraspecific variation in their structures that overlap between clades [6]. The recent use of mitochondrial genomes for phylogenetic analyses revealed a common ancestor for *R. annulatus*, *R. australis* and three clades of *R. microplus* within the *Boophilus* subgenus [7–9]. These five clades with specific geographic localizations were included within a complex named *R. microplus* [7]. *Rhipicephalus annulatus* is found across Southern Europe, Western and Central Asia, Northern and tropical sub-Saharan Africa, Mexico and the border regions of Texas in the USA [10]. *R. australis* was recently reinstated for *R. microplus* from Australia, New Caledonia and parts of Southeast Asia [11, 12]. *R. microplus* s.s. ticks from Asia, South America and Africa were classified as clade A closely related to *R. australis*, meanwhile *R. microplus* ticks from Southern China and Northern India were classified as clade B closely related to *R. annulatus* [7]. Finally, a clade C was defined for *R. microplus* ticks from Malaysia, Bangladesh, Pakistan and Myanmar [8, 9].

The most recent morphological studies of the *R. microplus* species in Cuba were performed by using specimens of the Tick Collection from the Cuban Institute of Ecology and Systematics [13]. However, molecular studies of the *R. microplus* ticks of the island are lacking. The aim of this study was the morphologic and molecular characterization of two Cuban strains of *R. microplus* ticks established at the National Laboratory of Parasitology of isolations from different locations in the country. The life cycle under laboratory conditions for both strains was also characterized. Morphological identification and measurements of the most relevant taxonomic structures were conducted by using Scanning Electron Microscopy. The sequences of three mitochondrial genes: 12S rRNA, 16S rRNA and the subunit I of cytochrome c oxidase gene (COXI) and one nuclear gene: internal transcribed spacer 2 (ITS2) were used to obtain phylogenetic inferences.

2. Methods

2.1 Tick specimens

R. microplus tick specimens of colonies established in the National Laboratory of Parasitology (LNP) from isolations of a small key in the north Cuba named Cayo Coco (22°30'32.45"N 78°24'25.1"W), and the Manga Larga locality (22°04'30"N 78°21'1"W) in the Ciego de Ávila province in Cuba were used to perform the present studies. These tick strains were named Cayo Coco (CC) and Manga Larga (ML), respectively. Tick specimens of a colony kept at the LNP from the Media Joya (MJ) strain of Mexican *R. microplus* was used as reference for phylogenetic analyses. This Mexican reference tick strain was kindly provided by CENAPA, Mexico and it was originally established at the National Institute of Forestry, Agriculture and Livestock (INIFAP) in Jiutepec, Morelos in 2001 from cattle infested with *R. microplus* ticks in the municipality of Tapalpa in the state of Jalisco, Mexico (19° 57' 0" N, 103° 46' 0"W) [14].

2.2 Life cycle of *R. microplus* strains under laboratory conditions

All procedures involving animals and samplings were carried out in accordance with the Guide for the Care and Use of Laboratory Animals [15] and were approved by the Ethic Committee of the LNP.

Tick-free cattle of the Cuban Siboney breed (5/8 Holstein and 3/8 Cebu) from uninfested pastures neither exposed to chemical treatments, aged between 1 and 2 years were individually housed in stalls at the LNP to study the parasitic phase of the CC and ML strains. During the experiment, animals were fed with forage and water *ad libitum* and supplemented with a pellet diet (produced by CENPALAB, Havana, Cuba). Forty thousand larvae of each tick strain were delivered on the back of bovines to allow free infestations. Three bovines were used for each tick strain. Random tick samples were removed at daily intervals from each bovine. The number of unfed larvae, fed larvae, unfed nymphs, fed nymphs, unfed adults, partially fed adults and fully engorged females were recorded in the daily batches.

In order to study non parasitic stage, twenty fully engorged females collected from each tick strain were placed into individual glass vials and maintained during oviposition period at 28°C and relative humidity of 80%. Egg masses were individually weighted and placed in the same incubation conditions. After hatching, unhatched eggs and eggshells from each egg mass were counted under stereoscope.

2.3 Morphological analysis of CC and ML tick strains

Morphological characterization was carried out by using 40 unfed adult specimens (20 female and 20 male ticks) from CC and ML strains. Unfed adults were collected from bovines around day 15 after larvae infestation, cleaned and fixated with glutaraldehyde 3% during 20 minutes at 4°C. After, they were washed three times with phosphate-buffered saline (PBS -135mM NaCl, 8mM Na₂HPO₄, 3mM KCL, 1.5mM KH₂PO₄, and pH 7.2) and finally submerged in 1% of osmium tetroxide (OsO₄) during 20 minutes at 4°C. Posteriorly, ticks were again washed three times with PBS and dehydrated in a bath series of increasing concentrations of ethanol from 50% to 99% during 10 min each, at 4°C. At the end, ticks were dried and lyophilized (Freezer Dryer Model FD- 10V) to -62°C and 1.2Pa during 18 hours and coated with a 10 nm gold layer with a Desk Sputter Coater DSR1. Main structures with taxonomic value were characterized by using a TESCAN MIRA-3 FE- Scanning Electron Microscope (SEM) operated at 5kV at the Center for Advanced Studies in Cuba. These structures were analysed and measured by using the Digital Micrograph™ program (Version 2.32.888.0). Measure averages of these structures from female and male ticks were compared by using a Student's t test performed on Prism (version 6.0 for Windows; GraphPad Software).

2.4 DNA extraction and Polymerase Chain Reaction (PCR)

Egg masses from the CC and ML strains were crushed in a mortar with liquid Nitrogen. Genomic DNA was extracted by using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's recommendations. The DNA integrity was verified by agarose gel electrophoresis. Primers were designed for specific amplification of 12S rRNA, 16S rRNA, COXI and ITS2 sequences. PCR reactions were performed in a thermal cycler (Minicycler™, MJ Research, Inc., USA) by using the System GoTaq® Green Master Mix (Promega, USA). Primers and conditions used in the PCR reactions were described in the Table 1 [7, 9]. The amplified DNA fragments were extracted from agarose gels using the QIAquick Gel

Extraction kit (Qiagen, Germany) and sequenced through the services of the Microsynth SeqLab GmbH (Germany).

2.5 Phylogenetic analyses

Sequence alignments were performed by using Clustalw Omega [16]. Phylogenetic trees were constructed with the “Maximum Likelihood (ML)” method based on the Tamura 3-parameter model [17] with 1000 replicates by using the MEGA 7 software [18]. The GenBank accession numbers of sequences included in the analyses were: *R. microplus* clade A (12S- EU921764, EU921761; 16S- EU918184, EU918183, EU918181, EU918180, EU918187, L34310, EU918178, EU918177, EU918176.1, EU918182; COXI- KP143546, KF200106, KP226177, KP226172, KP226169, KP226160, KP226174; ITS2- KC503273, U97715, EU520392), *R. microplus* clade B (12S- JF906025, JF906024, JF906019, JF906028, JF906029, JF906021; 16S- JX051068, JF979381, JX051072; COXI- JF758636, JQ625683, JF758630; ITS2- JF758640, JF758642, JQ625705, JQ625709, KC503274, KC203364), *R. microplus* clade C (12S- MG459958, MG459960, MG459959; 16S- HM536972, HM536977, EU918188, HM536976; COXI- MG459964, KP698516, KP698515, MG459961, MG459962, KP792580, KP318133, KP792579, KP792578, KP792586, MG459963, KP792587, KP792588, KP792589, KM246869, KM246868; ITS2- KC503264, KC503265, MG459967, MG459966, MG459965, KC503276, KC503272, JX974346), *R. annulatus* (12S- EU921773, KF219716, AF133058, KT335263; 16S- Z97877, L34311; COXI- KM235716, KM235715, KM494917, KF219739, AF132825; ITS2- KJ410770, KC503267) and *R. australis* (12S- EU921769, EU921767; 16S- EU918185, EU918186, EU918192, EU918190, EU918191; COXI- KC503255, AF132827; ITS2- U97712, KC503268). These sequences were selected only if a species’ validation was available according to the recent definition of the *R. microplus* complex [7-9]. Sequences from the *Amblyomma mixtum* tick species were used as outgroups (12S- KF527329; 16S- KT820359; COXI- KT820364 and ITS2- JN866886). The estimates of genetic distances among sequences of each gene were conducted by using the Tamura 3-parameter model [17] performed on Mega7 [18].

3. Results

3.1 Life cycle of CC and ML *R. microplus* strains under laboratory conditions

The parasitic life cycles of the CC and ML strains from *R. microplus* ticks were very similar (Figure 1). Free-living larvae period on cattle ranged between 1-2 days. One hundred percentages of larvae had begun feeding on the second and third day after infestation of CC and ML strains, respectively. For the sixth day, appeared first newly moulted nymphs for both tick strains. Fed nymphs were present from day 8 until day 15 in the parasitic cycle of both strains. First unfed adults appeared on day 15 for CC strain and on day 13 for ML strain. The initial feeding period of adults lasted from day 17 to 19 for CC strain and from day 17 to 21 for ML strain. Fully engorged females appeared on day 20 for both strains. For ML strain, fully engorged females were 100% of collected ticks from day 22 meanwhile for CC strain it took place from day 20. On day 23 was the maximum number of fully engorged female ticks collected from both strains and on days 32 and 30, all female ticks of CC and ML strains, respectively, had completed

their parasitic life cycle and had detached fully engorged. In summary, the parasitic life period averaged 23.5 ± 2.38 days for ML strain and 24.5 ± 2.5 days for CC strain.

Non parasitic stage was characterized for a pre-oviposition period between 2 and 4 days and an oviposition period average of 15 ± 2 days for both tick strains. Egg masses showed an average weight of 155.8 ± 0.034 mg for ML strain y 157.4 ± 0.032 mg for CC strain. The number average of eggs per female was 2993 ± 372 and 3027 ± 333 eggs for ML and CC strains. Pre-hatching periods averaged 22 ± 1.99 days for CC strain and 24 ± 2.3 days for ML strain. Average hatching rate was $80 \pm 6\%$, and $87 \pm 9.6\%$ for CC and ML tick strain, respectively. It means that 2422 ± 295 and 2604 ± 304 larvae were obtained for each fully engorged female tick collected of CC and ML strains, respectively. The average life cycle was determined as 65 ± 5 days for both strains under conditions established at the LNP to maintain these *R. microplus* tick colonies.

3.2 Morphological characterization of the CC and ML strains

Tick specimens of the CC and ML strains showed similar morphologic characteristics which were strongly coincident with those distinctive for the *R. microplus* species [19]. Each analysed morphologic character is shown only with a representative photograph. All specimens presented an oval body outline without festoons. Scutum was non-ornamented for both sexes with eyes on both sides of scutum at the level of coxae II with difficult to be detected (Figure 2 A-B). Mouthparts were anterior and short, with hypostome longer than palps for both female and male ticks (Figure 2 C-D).

In male ticks, the basis capituli was found hexagonal with a straight posterior border, triangular short cornua and few setae on its lateral margins and transversely along the dorsal surface (Figure 2A-C). In the ventral view, the inner margin of palpal article I was short and essentially straight. Palpal articles I, II and III presented ventral protuberances and setae (Figure 2E). The hypostome presented a 4/4 dentition whit 6-8 denticles per row and a well-defined corona with minute denticles (Figure 2E). The scutum was occupying all dorsal body with subtriangular and strong scapulae. Cervical grooves were observed wide and shallow and posteromedian groove was deep with a next pair of broader but shorter paramedian grooves. Abundant long setae were observed on the scutum that were absent on grooves and depressions (Figure 2A). In the ventral view, coxa I was observed triangular with two posterior spurs; the inner spur blunt and the external slender and more pointed whit an elongate, anterior process, curved dorsally and extending well beyond the scapula that is visible dorsally (Figure 2G). Coxae II and III presented broadly rounded internal and external spurs. Only a small spur was observed on coxa 4 (Figure 2G). The genital aperture was situated at the level of coxae II and the anal aperture was observed posterior to the coxae IV distant from them around two third of the total space between the last pair of coxae and the body posterior margin. Anal groove was absent (Figure 2G). Long adanal plates on both sides of the anus with spurs single sharp point ending and with a second indistinct posterior external spur observed in the majority of specimens. The accessory adanal plates were also present with only an internal spur (Figure 2G). A narrow caudal appendage was also observed (Figure 2A-G). The spiracular plates were subcircular and located behind the last pair of coxae (Figure 2G-I).

In female ticks, the basis capituli was found hexagonal with a straight posterior border and indistinct cornua and few short setae were only found in the lateral margins. Lateral angles of basis capituli were slightly pointed and porous areas were kidney-shaped separated from each other by a distance of approximately 1–1.5 times the major axis of one porous area, (Figure 2B-D). Dorsal internal margin of palpal article II was observed with a median indentation, which is continued transversely as a mild groove. In the ventral view, the internal margins of palpal article I were slightly concave and short without protuberances. Meanwhile, clear protuberances were observed on the internal margins of palpal articles II and III (Figure 2F). In general, hypostome in female ticks presented a 4/4 dentition, however a 5/5 dentition was observed in a female tick of the ML strain and a 4.5/4.5 dentition was observed in two female ticks of the ML strain (Figure 3). A well-defined corona with minute denticles was also observed in all cases. The scutum with smooth outline, was occupying only 1/3 of the dorsal body. The anterolateral margins of the scutum were straight, the posterolateral margins mildly sinuous and posterior angle rounded and relatively wide. Subtriangular and strong scapulae were also observed (Figure 2B-D). Setae were long and sparse, usually found along the anterolateral margins to the eye level, but absent from cervical grooves in the middle scutum (Figure 2B-D). Medium to long abundant setae were observed in alloscutum. Median and posterolateral grooves were well defined and elongated. The last ones were confluent with shallow depression on each side almost extending to the cervical grooves on the scutum (Figure 2B). In the ventral view, coxa I was observed also triangular with two rounded spurs smaller than those found on male ticks. Single external spurs were evident on coxae II, III and IV (Figure 2H). Genital aperture was located at the level of coxae II with long setae on the anterior surface (Figure 2H). The anal aperture was observed posterior to coxae IV and the spiracular plates were subcircular and located behind the last pair of coxae like in male ticks (Figure 2H-J).

There were no differences in the morphometry of key structures from the CC and ML strains of *R. microplus* ticks. These structure measures were summarized in the Table 2. Statistically significant differences were found on the total body length, the idiosome length, the length and breadth of gnathosome, the basis capituli breadth, the length and breadth of hypostome and the diameter of spiracular plates between male and female ticks.

3.3 Phylogenetic inferences based on molecular markers

GenBank accession numbers were assigned to the sequences amplified by PCR in the present study from the CC, ML and MJ strains of *R. microplus* ticks. The sequences corresponding to each tick strain were: for CC 12S- CC- MT499890, 16S- MT462222, COXI- MT584888 and ITS2- MT462236; for ML 12S- MT499888, 16S- MT462223, COXI- MT584887 and ITS2- MT462237 and for MJ 12S- MT499889, 16S- MT462224, COXI- MT584889 and ITS2- MT462238. The evolutionary analysis involved 21 nucleotide sequences with 167 positions, 28 nucleotide sequences with 353 positions, 37 nucleotide sequences with 129 positions and 25 nucleotide sequences with 641 positions in the final dataset for 12S rRNA, 16S rRNA, COXI and ITS2 genes, respectively after all positions containing gaps and missing data were eliminated.

The CC and ML Cuban tick strains clustered in the *R. microplus* clade A together with the MJ reference strain and tick isolations from Argentina, Bolivia, Brazil, Mozambique, Costa Rica, Panama, Paraguay, Peru, Tanzania, United States, Uruguay and South Africa with a reliability of 69%, 93%, and 98% according to the evolutionary histories inferred from the 12S rRNA, 16S rRNA and COXI sequence analyses, respectively (Figures 4, 5 and 6). These phylogenies based on mitochondrial gene sequences identified congruently the five clades previously included in the *R. microplus* complex as phylogenetically well delimited, and supported moderately the *R. microplus* clade A as a sister to *R. australis* clade. Meanwhile, the maximum likelihood tree based on nuclear ITS2 sequences was unable to split the *R. microplus* species in clades A, B and C (Figure 7).

A summary of intraspecific and interspecific genetic distances estimated among sequences used for phylogeny inferences was presented in the Table 3. The intraspecific genetic distances revealed differences ranging from 0 to 2% when mitochondrial gene sequences were used for the analyses and from 0 to 1% when ITS2 sequence was used. On the other hand, interspecific differences among the five clades of the *R. microplus* complex ranged from 2 to 18% for 12S rRNA sequences, from 1 to 3% for 16S rRNA sequences, from 4 to 29% for COXI and from 0 to 2% for ITS2 sequences.

4. Discussion

Morphological characteristics found on specimens of the CC and ML tick strains were useful to classify them as belonging to the *R. microplus* complex. The hexagonal shaped basis capituli, the short anterior mouthparts with the hypostome longer than palps, the circular spiracular plates, the presence of accessory and adanal plates in males and the absence of anal groove and festoons in posterior body found on the Cuban strains are distinctive characteristics of the members inside this complex [9, 12, 19, 20]. Significant differences found between female and male ticks confirmed remarkable dimorphism that exists between sexes in ticks which have been widely documented [21–24] and remains a valuable tool for tick taxonomic identification. For example, the caudal process observed in male specimens of the CC and ML strains allows differentiation from the *R. annulatus* species because this feature is absent in male ticks of that species [19]. Anyway, the great intraspecific variability in the morphological characters that possess the species in the *R. microplus* complex makes practically impossible the accurate taxonomic classification of these specimens based only on morphology [25]. An example of this variability was the 4/4, 4.5/4.5 and 5/5 dentition found on female ticks of the ML strain [26, 27] and the kidney shaped porous areas found on female ticks from both strains that differs from oval shaped porous areas observed on female ticks from other members of the *R. microplus* complex [19, 28].

Phylogenetic analyses conducted with 12S rRNA, 16S rRNA and COXI sequences as molecular markers allowed inferring with a strongly support of 16S and COXI that these Cuban tick strains belong to the *R. microplus* clade A together other tick strains classified as *R. microplus* s.s. These results are agreed with previous reports that pointed 16S and COXI mitochondrial genes as the best markers to solve phylogenetic relationships among the five clades described within the *R. microplus* complex [7–9]. The ITS2 marker did not support phylogenetic relationships within the *R. microplus* complex, though it has

been reported useful to support monophyly of the subgenus *Boophilus* and the relationships among species of the genus *Rhipicephalus* [7].

5. Conclusions

The results presented here allowed the classification of Cuban Cayo Coco and Manga Larga tick strains as *Rhipicephalus microplus* clade A inside the *R. microplus* complex. This study constitutes the first molecular characterization of ticks from the *R. microplus* species in Cuba.

Declarations

Ethics approval and consent to participate:

Not proceed

Consent for publication:

Not Proceed

Availability of data and material:

With corresponding author

Not Competing interests

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Authors' contributions

Conceptualization: PEEG and ARM; Data curation: PEEG, CFC and ARM; Formal analysis: PEEG, MPE and ARM; Funding acquisition: LM, MPE and ARM; Investigation: PEEG, CFC, AFC, YFA and YBS Methodology: PEEG, AFC, RRF, YFA, YBS, YGA, LM and ARM; Project administration: RRF, YGA, MPE and ARM; Supervision: LM, ADG, MPE and ARM; Writing original draft: PEEG, ALCA, CFC and ARM; Writing - review & editing: PEEG, AFC, RRF, YFA, LM, MPE and ARM.

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Tables

Table 1
Specific Oligonucleotides and conditions used for specific PCR amplifications

Gen	Name	OligonucleotideSequence (5'–3')	PCR Program
16S rRNA	Rm16S- (F)	GCTCAATGATTTTTTAAATTGCTGT	95 ⁰ C-2 min, 30 x [95 ⁰ C-30 s, 52 ⁰ C-30 s, 72 ⁰ C-30s], 72 ⁰ C-5 min
	Rm16S- (R)	CCGGTCTGAACTCAGATCAAGT	
12S rRNA	Rm12S- (F)	AAACTAGGATTAGATACCCT	95 ⁰ C-2 min, 30 x [95 ⁰ C-30 s, 50 ⁰ C-30 s, 72 ⁰ C-45s], 72 ⁰ C-5 min
	Rm12S- (R)	ATTCTATTTTAATTTTACTTTCAAATCC	
ITS2	RmITS2- (F)	AGGACACACTGAGCACTGATTC	95 ⁰ C-5 min, 35 x [95 ⁰ C-30 s, 52 ⁰ C-30 s, 72 ⁰ C- 1min], 72 ⁰ C-5 min
	RmITS2- (R)	ACTGCGAAGCACTRGACCG	
COXI	RmCOXI- (F)	CTCTTCACTTAGCTGGGGCAT	95 ⁰ C 2 min, 35 x [95 ⁰ C-30 s, 40 ⁰ C-30 s, 72 ⁰ C-30s], 72 ⁰ C-5 min
	RmCOXI- (R)	CTGTTAACAATATAGTAATAGCCCCTG	

Table 2
Measures (mean $\pm$ standard deviation) of key structures from CC and ML strains of *Rhipicephalus microplus* ticks

Structure	Female ticks		Male ticks	
	Length (mm)	Breadth (mm)	Length (mm)	Breadth (mm)
Gnathosome	0.57 $\pm$ 0.11**	0.68 $\pm$ 0.04**	0.36 $\pm$ 0.09**	0.44 $\pm$ 0.03**
Basis capituli	0.23 $\pm$ 0.07	0.68 $\pm$ 0.04**	0.16 $\pm$ 0.06	0.44 $\pm$ 0.03**
Hypostome	0.34 $\pm$ 0.05*	0.17 $\pm$ 0.02*	0.21 $\pm$ 0.05*	0.13 $\pm$ 0.01*
Idiosome	2.05 $\pm$ 0.08*	1.24 $\pm$ 0.13	1.84 $\pm$ 0.08*	1.18 $\pm$ 0.09
Scutum	0.85 $\pm$ 0.04	0.82 $\pm$ 0.08		
Spiracular plates	0.28 $\pm$ 0.02*		0.21 $\pm$ 0.03*	
Caudal appendage	-		0.25 $\pm$ 0.143	0.19 $\pm$ 0.09
Porose area	0.067 $\pm$ 0.009	0.18 $\pm$ 0.01	-	
Distance between porose areas	0.165 $\pm$ 0.04		-	
Total Body	2.54 $\pm$ 0.09*	1.24 $\pm$ 0.13	2.32 $\pm$ 0.12*	1.18 $\pm$ 0.09
Asterisks mean statistical significant differences (Student's t-test, * P < 0.05; ** P < 0.01)				

Table 3

Summary of genetic distance ranges (in percentages) within and among clades of the *Rhipicephalus microplus* complex for 12S rRNA, 16S rRNA, COXI and ITS2 sequences based on the Tamura-3 parameter model. In the table A, values for 12S rRNA are above diagonal on the right and values for 16S rRNA are below diagonal on the left. In the table B, values for COXI are above the diagonal on the right, and values for ITS2 are above diagonal on the left. Along the diagonal, in bold, are the intraspecific distances for each gene.

A						
Tick species	RmA	Rau	RmB	Ran	RmC	Am
<i>R. microplus</i> clade A (RmA)	0 0-1	2-4	4	4-5	4	11.9-12.4
<i>R. australis</i> (Rau)	1	0 1	2	2-3	3-4	12.5-12.7
<i>R. microplus</i> clade B (RmB)	1-2	2	0-1 0	2	2	14.1
<i>R. annulatus</i> (Ran)	1-2	2	1	0 0	3	13.8
<i>R. microplus</i> clade C (RmC)	2-3	2-3	1	1-2	0-1 0	18.3
<i>Amblyomma mixtum</i> (Am)	21	22	22-23	21-22	22-23	- -
B						
Tick species	RmA	Rau	RmB	Ran	RmC	Am
<i>R. microplus</i> clade A (RmA)	0 0-1	6-7	7-8	7-11	10-12	27-28
<i>R. australis</i> (Rau)	1	1 2	9-10	8-11	7-10	26
<i>R. microplus</i> clade B (RmB)	0	0-1	0 0-1	4-7	5-7	28
<i>R. annulatus</i> (Ran)	1	2	1	1 0-2	8-10	28-29
<i>R. microplus</i> clade C (RmC)	0	1	0	1	0 0-2	27-28
<i>Amblyomma mixtum</i> (Am)	98-100	98-99	98	99	98-99	- -

Figures

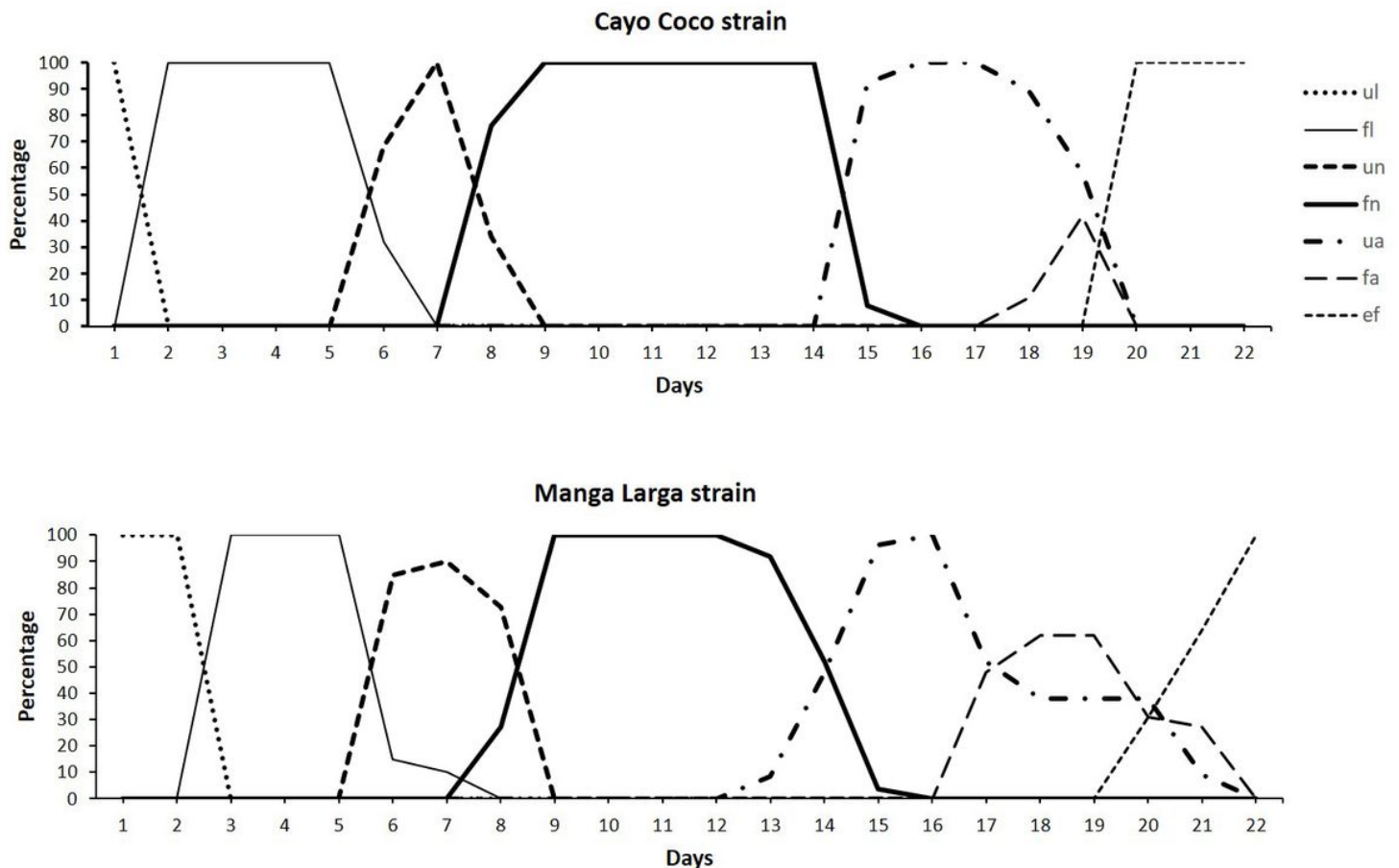


Figure 1

Parasitic life cycle of the Cayo Coco and Manga Larga strains of *Rhipicephalus microplus* ticks maintained at the Cuban National Laboratory of Parasitology. ul- unfed larvae, fl-fed larvae, un-unfed nymphs, fn- fed nymphs, ua- unfed adults, fa- partially fed adults, ef- fully engorged females.

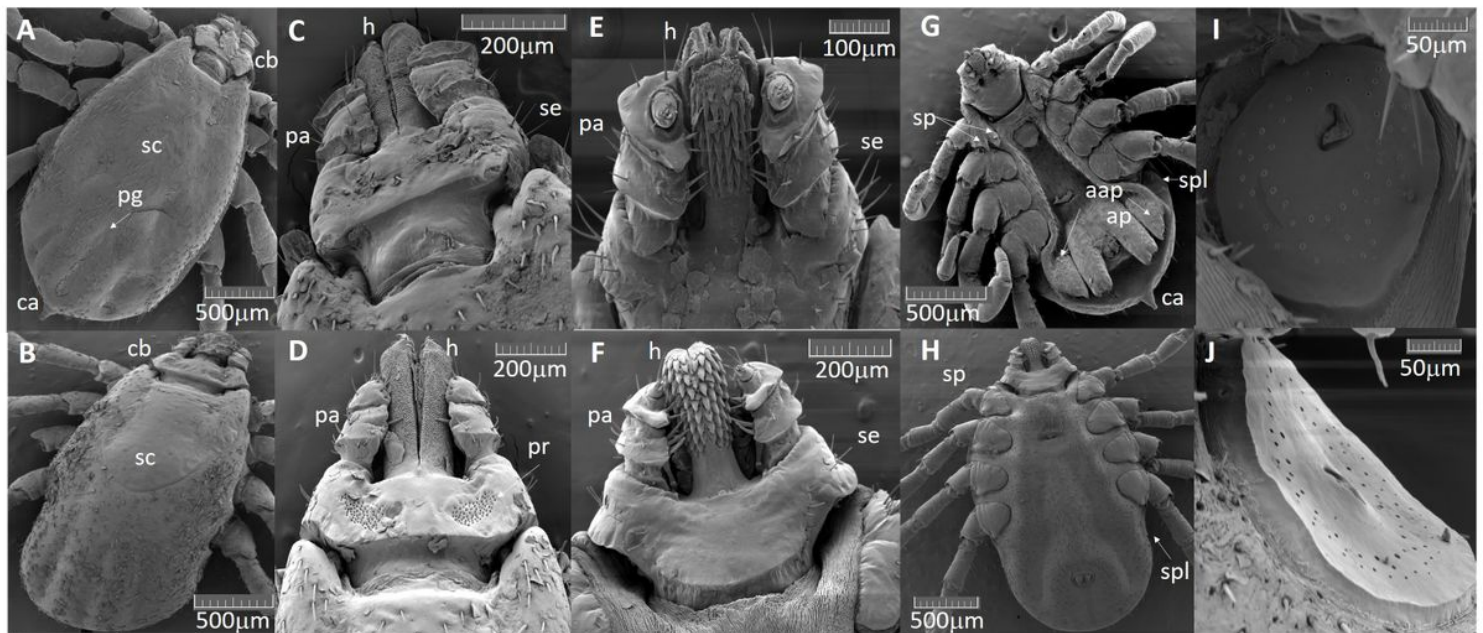


Figure 2

Scanning Electron Microscopy (SEM) of male and female ticks from Cuban *R. microplus* strains. (A) Dorsal view of a male tick; (B) Dorsal view of a female tick; (C) Capitulum dorsal view of a male tick; (D) Capitulum dorsal view of a female tick; (E) Capitulum ventral view of a male tick; (F) Capitulum ventral view of a female tick; (G) Ventral view of a male tick; (H) Ventral view of a female tick; (I) Spiracular plate of a male tick; (J) Spiracular plate of a female tick. cb- basis capituli; sc- scutum, pg- posteromedian groove; h- hypostome; pa- palps; se- setae; pr- porose area; sp- spur; ap- adanal plate; aap- accessory adanal plate; spl- spiracular plate.

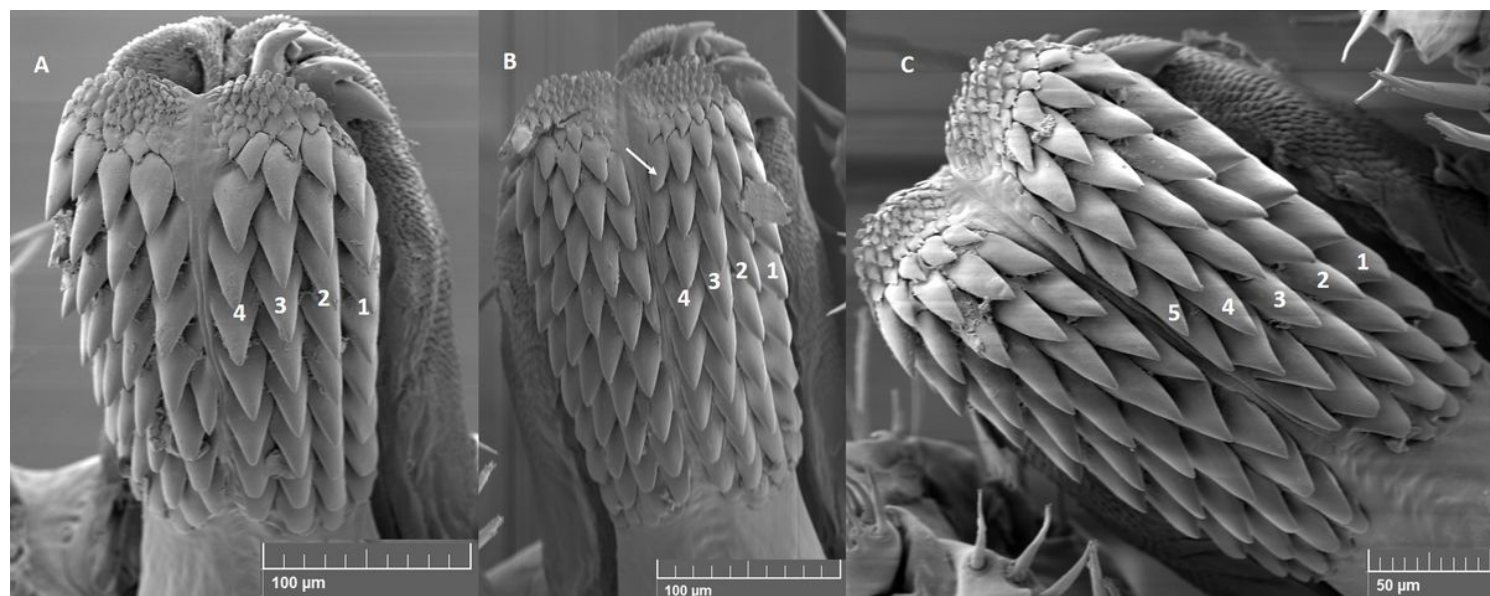


Figure 3

Hypostomes of female ticks from the ML strain. (A) Hypostome with 4/4 dentition present in most female ticks; (B) Hypostome with 4.5/4.5 dentition found in two female ticks; arrow points 0.5 dentition (C) Hypostome with 5/5 dentition observed only a female tick.



Figure 4

Phylogenetic analysis based on 12S rRNA sequences by using Maximum Likelihood method and the Tamura 3-parameter model [17]. The tree with the highest log likelihood (-465.40) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bar

represents 0.5 substitutions per site. Sequence data generated in the present study appear highlighted in red. Species names are preceded by GenBank accession numbers and followed by the location where they were collected. Each clade described for the *R. microplus* complex appears with a different colour. A sequence from the *Amblyomma mixtum* species was used as outgroup.

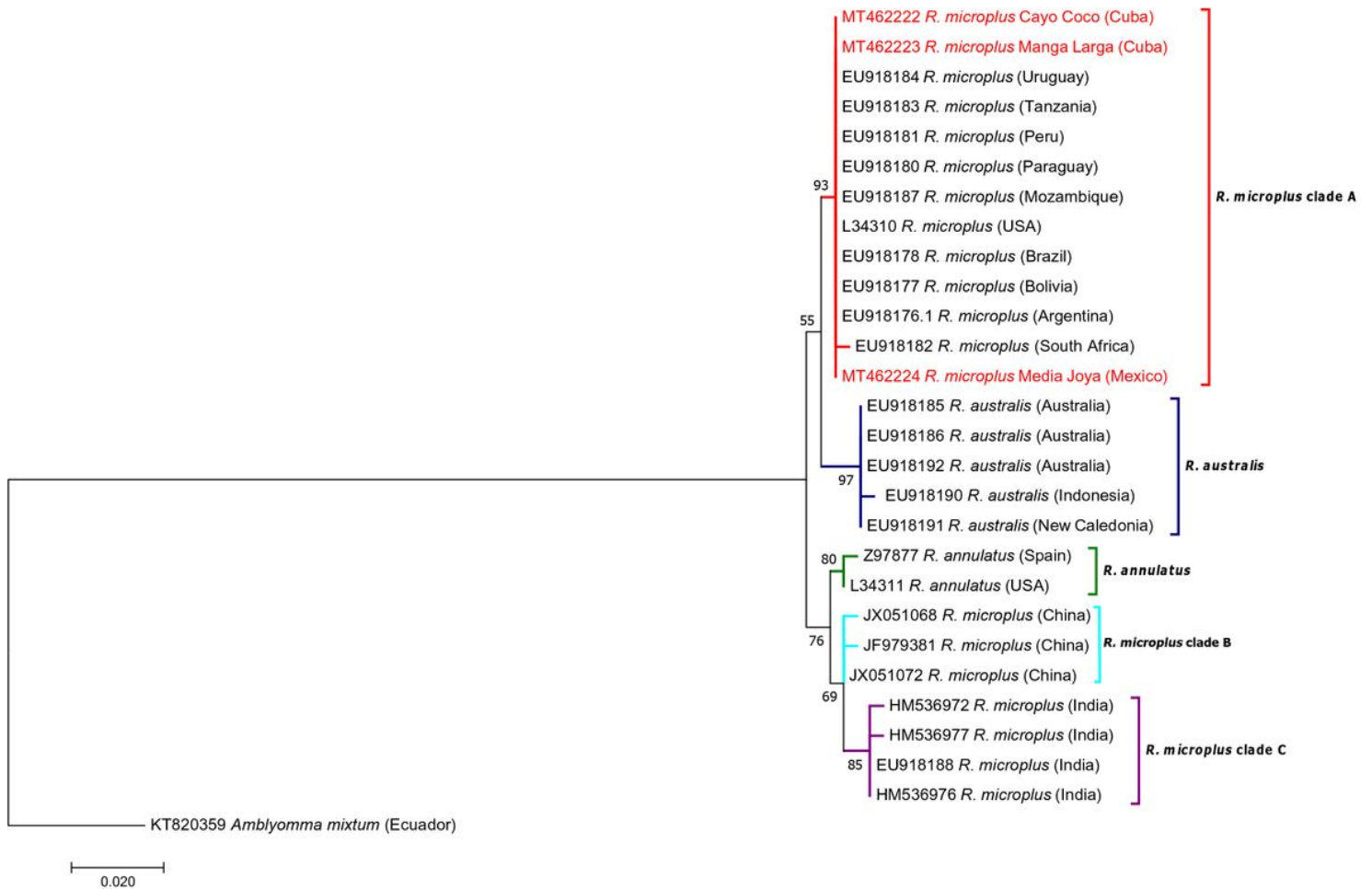


Figure 5

Phylogenetic analysis based on 16S rRNA sequences by using Maximum Likelihood method and the Tamura 3-parameter model [17]. The tree with the highest log likelihood (-786.15) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bar represents 0.02 substitutions per site. Sequence data generated in the present study appear highlighted in red. Species names are preceded by GenBank accession numbers and followed by the location where they were collected. Each clade described for the *R. microplus* complex appears with a different colour. A sequence from the *Amblyomma mixtum* species was used as outgroup.

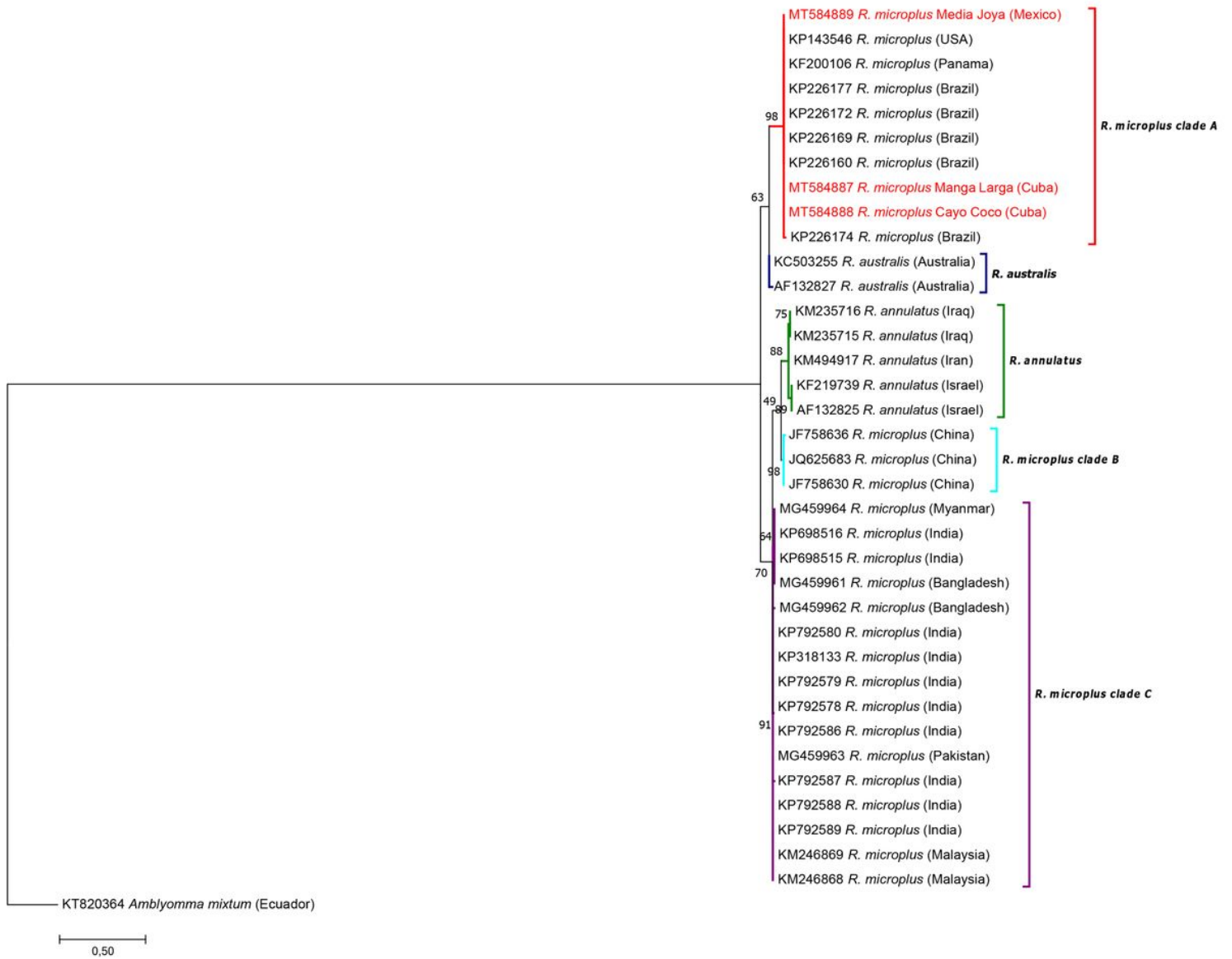


Figure 6

Phylogenetic analysis based on COXI sequences by using Maximum Likelihood method and the Tamura 3-parameter model [17]. The tree with the highest log likelihood (-411.24) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bar represents 0.5 substitutions per site. Sequence data generated in the present study appear highlighted in red. Species names are preceded by GenBank accession numbers and followed by the location where they were collected. Each clade described for the *R. microplus* complex appears with a different colour. A sequence from the *Amblyomma mixtum* species was used as outgroup.



Figure 7

Phylogenetic analysis based on ITS2 sequences by using Maximum Likelihood method and the Tamura 3-parameter model [17]. The tree with the highest log likelihood (-1752.71) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bar represents 0.1 substitutions per site. Sequence data generated in the present study appear highlighted in red. Species names are preceded by GenBank accession numbers and followed by the location where they were collected. Each identified clade appears with a different colour. A sequence from the *Amblyomma mixtum* species was used as outgroup.