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New insights from molecular characterization of the tick *Rhipicephalus (Boophilus) microplus* in Brazil

Novos pontos de vista sobre a caracterização molecular em carrapatos *Rhipicephalus (Boophilus) microplus*, no Brasil

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Abstract

The *Rhipicephalus (Boophilus) microplus* complex currently consists of five taxa, namely *R. australis*, *R. annulatus*, *R. (B.) microplus* clade A *sensu*, *R. microplus* clade B *sensu*, and *R. (B.) microplus* clade C *sensu*. Mitochondrial DNA-based methods help taxonomists when they are facing the morpho-taxonomic problem of distinguishing members of the *R. (B.) microplus* complex. The purpose of this study was to perform molecular characterization of ticks in all five regions of Brazil and infer their phylogenetic relationships. Molecular analysis characterized 10 haplotypes of the COX-1 gene. Molecular network analysis revealed that haplotype H-2 was the most dispersed of the studied populations (n = 11). Haplotype H-3 (n = 2) had the greatest genetic differentiation when compared to other Brazilian populations. A Bayesian phylogenetic tree of the COX-1 gene obtained strong support. In addition, it was observed that the population of *R. (B.) microplus* haplotype H-3 exhibited diverging branches among the other Brazilian populations in the study. The study concludes that the different regions of Brazil have *R. (B.) microplus* tick populations with distinct haplotypes.

Keywords: *Rhipicephalus (Boophilus) microplus*, cattle tick, COX-I gene, ITS-2 gene, Brazil.

Resumo

Carrapatos do complexo *R. (B.) microplus* se distribuem em cinco taxa: *R. australis, R. annulatus, R. (B.) microplus* clado A *sensu R. microplus* clado B *sensu*e clado C *sensu*. Métodos baseados no DNA mitocondrial podem auxiliar taxonomistas quando há dificuldades em estabelecer diferenças morfológicas para distinguir membros do complexo *R. (B.) microplus*. O objetivo deste estudo foi a caracterização molecular e a inferência de relações filogenéticas em carrapatos de todas as cinco regiões geográficas do Brasil. Para o gene COX-1, a análise molecular caracterizou 10 haplótipos. Na análise molecular em rede foi observado que o haplótipo H-2 é o mais disperso entre as populações (n=11). O haplótipo H-3 (n=2) foi o que obteve maior diferenciação genética ao ser comparado com outras populações brasileiras. A árvore filogenética Bayesiana de gene COX-1 gerou suporte robusto e foi observado que a população de *R. (B.) microplus* haplótipo H-3 apresentou ramificação com divergência entre as outras populações brasileiras apresentadas neste estudo. Conclui-se que as populações brasileiras possuem diversidade haplotípica com divergência entre as diversas populações de *R. (B.) microplus* no Brasil.

Palavras-chave: *Rhipicephalus (Boophilus) microplus*, carrapato-do-boi, gene COX-I, gene ITS-2, Brasil.

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Introduction

The bovine tick *Rhipicephalus (B.) microplus* can be found in multiple tropical and subtropical regions worldwide (ESTRADA-PEÑA et al., 2006). This parasite may transmit pathogens that cause babesiosis (*Babesia bovis* and *B. bigemina*) and anaplasmosis (*Anaplasma marginale*) (PETER et al., 2005; de la FUENTE et al., 2008). Infestation of herds with *R. (B.) microplus* cause losses estimated at US$3.24 billion per year in Brazil (GRISI et al., 2014).

In most cattle-producing countries, control methods against this tick are costly and require the use of acaricides. However, acaricide-resistant *R. (B.) microplus* populations have become a worldwide problem, and molecular ecology studies and new tick control technologies are now required to preserve cattle production (RODRÍGUEZ-VIVAS et al., 2007; ANDREOTTI et al., 2011; GUERRERO et al., 2014).

Six years ago, Labruna et al. (2009) used 12S and 16S molecular markers to show a lack of divergence among populations in the Americas, including Brazil. Later, Burger et al. (2014) used the COX-1 molecular marker to show that the *R. (B.) microplus* populations of Brazil (GenBank: KC503261) are organized in clade A, along with populations of *R. (B.) microplus* from China (GenBank: HM193863) and Cambodia (GenBank: KC503260). Thus, molecular markers may resolve very close taxonomic relationships and provide new knowledge about population structure, allowing a deeper understanding of *R. (B.) microplus* populations (BURGER et al., 2014).

Although the history of the dissemination of *R. (B.) microplus* is not well documented, the species is known to have originated in India (HOOGSTRAL, 1986). According to Barré & Uilenberg (2010), *R. (B.) microplus* originated in the southern and southeastern regions of Asia and was spread throughout the tropical and subtropical belts via cattle, arriving in Brazil between the 16th and 17th centuries.

Mitochondrial and nuclear genome markers are increasingly being used to better understand phylogenetic relationships in order to elucidate whether evolution has occurred among populations of a species (KANDUMA et al., 2012).

The DNA barcode has been proposed as a universal tool for identifying biological diversity, and can be used as a molecular marker (HEBERT et al., 2003). DNA barcoding is based on information gathered from a fragment of approximately 688 base pairs of mitochondrial DNA base sequences (mtDNA) from the cytochrome oxidase I gene (COX-I) of different species. A number of studies have shown that the DNA barcode is a universal code highly effective for the identification of species (HEBERT et al., 2004; BARRETT & HEBERT, 2005).

In addition to the DNA barcode, ribosomal DNA (rDNA) may be used as a nuclear molecular marker. The internal transcribed spacer 2 (ITS-2) is located between the 5.8S and 28S ribosomal subunits of rDNA (CRUICKSHANK, 2002). These genes are arranged in repeated units known as ribosomal cistrons, which have repeated copies and have been used to study phylogenetic relationships (CAMPBELL et al., 1993; SONG et al., 2011; BURGER et al., 2014).

The objective of this study was to infer the phylogenetic and phylogeographic relationships among *R. (B.) microplus* tick populations in Brazil based on COX-I mitochondrial DNA gene sequences and ITS-2 nuclear DNA, and compare them with *R. (B.) microplus* tick populations from other countries.

Materials and Methods

Tick collection

Ticks were obtained from 22 locations in different geographical regions of Brazil (Figure 1, Table 1). A pool of larvae and 22 engorged females were collected from natural environments. The morphology and identification key was based on Barros-Battesti et al. (2006). Samples were stored in an ultra-freezer at –80 °C for subsequent analysis.

DNA extraction and PCR amplification

DNA extraction was performed on a pool of larvae and individual samples of engorged females using DNAzol® Reagent (Invitrogen) according to the manufacturer's recommendations. The DNA was quantified using a Nano Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and an A260nm/A280nm ratio above 1.6 was established as acceptable. The sample concentration was corrected to 50 ng μL^{-1}. Next, the DNA was used in a polymerase chain reaction (PCR) for COX-I and ITS-2 region amplification with new primers designed by our group. PCR generated products of 643bp and 580bp for COX-1 and ITS-2, respectively.

COX-I gene fragments were amplified using the COX-I.2F (5'-CTTCAGCCATTTTACCGCGA-3') and COX-I.2R (5'-CTCCGCCTGAAGGGTCAAA-3') starter oligonucleotides. ITS-2 fragments were amplified with the ITS-2F (5'-CGGATCACATATCAAGAGAG-3') and ITS-2R (5'-CCCAACTGGAGTGGCCCAGTTT-3') primers. The PCR was standardized for a final volume of 25 µL with 1X buffer, 1.5 mM MgCl, 25 mM dNTPs, 10 µM of each primer, 0.5 unit of Platinum® Taq DNA Polymerase High Fidelity (Invitrogen by Life Technologies™, Massachusetts, USA), and 1µL of DNA at 50 $ng\mu L^{-1}$. PCR conditions were optimized for each reaction, and the annealing temperature was adjusted to suit the primers used. General PCR conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 56 °C/60 °C (COX-I/ITS-2, respectively) for 30s, 68 °C for 1 min, and a final extension of 68 °C for 3 min. PCR products were visualized in a 2% agarose gel stained with ethidium bromide. The PCR-amplified products were purified using a Purelink®kit (Invitrogen by Life Technologies™, Massachusetts, USA). Fragments were then cloned with pGEM®-T Easy Vector Systems cloning vector according to the manufacturer's manual (Promega, Madison, WI-USA) and sequenced as described below.

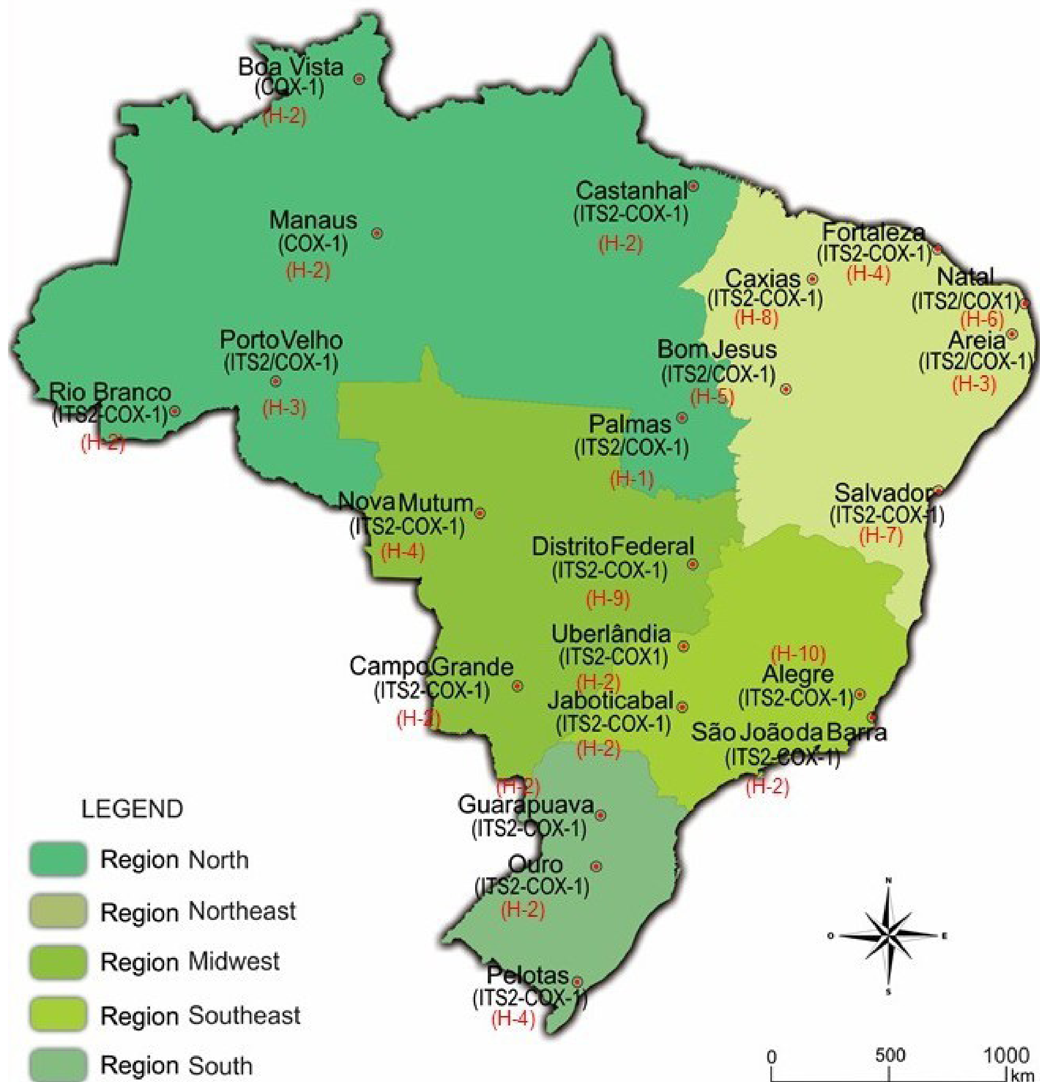

Figure 1. Geographic locations where *Rhipicephalus (Boophilus) microplus* ticks were collected in Brazil.

Genetic analysis

All analyses generated in this study were performed *in silico*. Sanger sequencing was performed in a 48 well plate with robotic instrumentation using T7 Transcription Start (5'-CTAATACGACTCACTATAGGG-3') universal sequencing primer (Promega, Madison, WI-USA). Samples were sequenced using an Applied Biosystems^{TM} ABI 3730 DNA Analyzer and the conditions described by Song et al. (2011). The sequencing reactions were done using the Big Dye® Terminator v3.1 cycle sequencing kit. The runs were performed in 36-cm capillaries using the POP7 polymer, and the sequences were generated by the Sequencing Analysis Software v5.3.1 through the Caller KB Phred program (EWING & GREEN, 1998; EWING et al., 1998).

Plasmid sequences were identified and removed using an NCBI (VecScreen) tool (ALTSCHUL et al., 1997), and a hash search algorithm was used to remove the contaminant sequences. Con*sensu*s sequences were aligned with the GenBank data using the BLASTN program (ALTSCHUL et al., 1997).

The partition homogeneity test was conducted using the ARLEQUIN program, version 3.5.1.2 (EXCOFFIER & LISCHER, 2010). The aligned COX-I and ITS-2 sequences comprised 681bpsand 649bps, respectively. Database sequence *R. (B.) microplus* MS- Brazil (GenBank: KC503261) clade A *sensu* Burger et al. (2014), was used for alignment with samples from this study. This sequence alignment revealed 10 haplotypes (H1-H10) based on COX-I genes alone (Figure 2). Distinct sequences of COX-I (KP226159-KP226180) and ITS-2 (KP226139-KP226158) were deposited in NCBI GenBank.

A median-joining analysis implemented in the program Network Version 5.0 (BANDELT et al., 1999) was used for the intraspecific analysis of the evolutionary relationships among haplotypes. Uncorrected (p) pairwise genetic distances were calculated using ARLEQUIN program, version 3.5.1.2

Table 1. Brazilian states, geographic coordinates related to the collection and GenBank accession number of this study.

Region	State	Geographic coordinates	GenBank (COX-1)	GenBank (ITS2)
North	AC	9°59'30"S/67°48'36"W	KP226159	KP226139
	AM	3°6'0"S/60°1'0"W	KP226162	-
	PA	1°17'49"S/47°55'19"W	KP226163	KP226153
	RO	8°45'43"S/63°54'14"W	KP226178	KP226142
	RR	2°49'10"S/60°40'17"W	KP226161	-
	TO	10°11'4"S/48°20'01"W	KP226180	KP226146
Northeast	MA	4°51'32"S/43°21'21"W	KP226160	KP226157
	CE	3°43'6"S/38°32'34"W	KP226177	KP226140
	RN	5°47'42"S/3512'32"W	KP226172	KP226145
	PB	6°57'46"S/35°41'31"W	KP226176	KP226141
	PI	9°4'26"S/44°21'32"W	KP226174	KP226148
	BA	12°58'16"S/38°30'39"W	KP226168	KP226156
Midwest	MT	13°49'44"S/56°04'56"W	KP226167	KP226143
	GO	15°47'56"S/47°52'00"W	KP226166	KP226151
	MS	20°26'34"S/54°38'45"W	KP226164	KP226155
Southeast	ES	20°45'50"S/41°31'58"W	KP226175	KP226154
	MG	18°55'8"S/48°16'37"W	KP226171	KP226150
	RJ	21°38'24"S/41°3'3"W	KP226165	KP226149
	SP	21°15'18"S/48°19'19"W	KP226170	KP226158
South	PR	25°23'42"S/51°27'28"W	KP226179	KP226147
	SC	27°20'27"S/51°37'4"W	KP226173	KP226144
	RS	31°46'19"S/52°20'34"W	KP226169	KP226152

(EXCOFFIER & LISCHER, 2010) to assess the genetic divergence of *R. (B.) microplus* in both COX-I and ITS-2 genes. To assess the level of genetic differentiation, we used the program DnaSP 5.0 (LIBRADO & ROZAS, 2009). Haplotype diversity (Hd), nucleotide diversity (pi), number of haplotypes (h), and genetic differentiation (FST) were determined.

The levels of genetic differentiation were defined as FST > 0.25 (great differentiation), 0.15 to 0.25 (moderate differentiation) and FST< 0.05 (negligible differentiation) (WRIGHT, 1931). The levels of gene flow were defined as *Nm*> 1 (high gene flow), 0.25 to 0.99 (intermediate gene flow) and *Nm*< 0.25 (low gene flow). The distinct COX-I and 16S rRNA haplotypes identified in the Brazilian *R. (B.) microplus* ticks were aligned with all representative sequences of Rhipicephalus taxa available in the NCBI GenBank. *R. annulatus* was used as an outgroup for the construction of Bayesian phylogenetic trees based on Brazilian *R. (B.) microplus* ticks COX-I only; *Dermacentor nitens* was used as an outgroup for the construction of Bayesian phylogenetic trees based on COX-I and ITS-2 sequences. The Bayesian phylogenetic analysis performed using MrBayes 3.1 (HUELSENBECK & RONQUIST, 2001; RONQUIST & HUELSENBECK, 2003) were plotted using FigTree software version 1.4.2 (TREE BIO, 2016).

The ARLEQUIN software revealed population structure by means of AMOVA, which uncovers the existence of population differentiation at both intra- and inter-population levels (EXCOFFIER & LISCHER, 2010).

3. Results and Discussion

Nucleotide analyses and Haplotype

The phylogenetic and phylogeographic relations of the Brazilian *R. (B.) microplus* were inferred from the COX-I sequences (22 samples), *R. (B.) microplus* Brazil (GenBank: KC503261) and the ITS2 sequences (20 samples) obtained from all five Brazilian geographic regions (Figure 1).

This is the only study that has compared a representative sample of *R. (B.) microplus*, which together represent the whole country and the authors were careful to represent each region by just having differentiated biogeography. Assays performed by Dantas-Torres (2015), can demonstrate that certain factors such as climate change and biodiversity boost the expansion of tick populations.

The COX-I dataset showed haplotype diversity (0.767) greater than that revealed by the ITS-2 (0.567) gene. However, the ITS-2 showed greater nucleotide diversity (0.00277) than the COX-I (0.00432) dataset. Haplotype network analysis (COX-I) revealed ten distinct haplotype clusters among Brazilian populations, with no clear separation by tick populations/geographical areas, indicating that an overlap of the ten genetically divergent haplotypes of *R. (B.) microplus* (Figure 2A) exists.

Haplotype H-2 was the most widespread haplotype (n = 11) in Northern, Midwest, Southeast and Southern populations (Figure 2B). Haplotypes H-5, H-6 and H-8 (Northern region) originates from H-4 and H-2 haplotypes, as shown in Figure 2B.

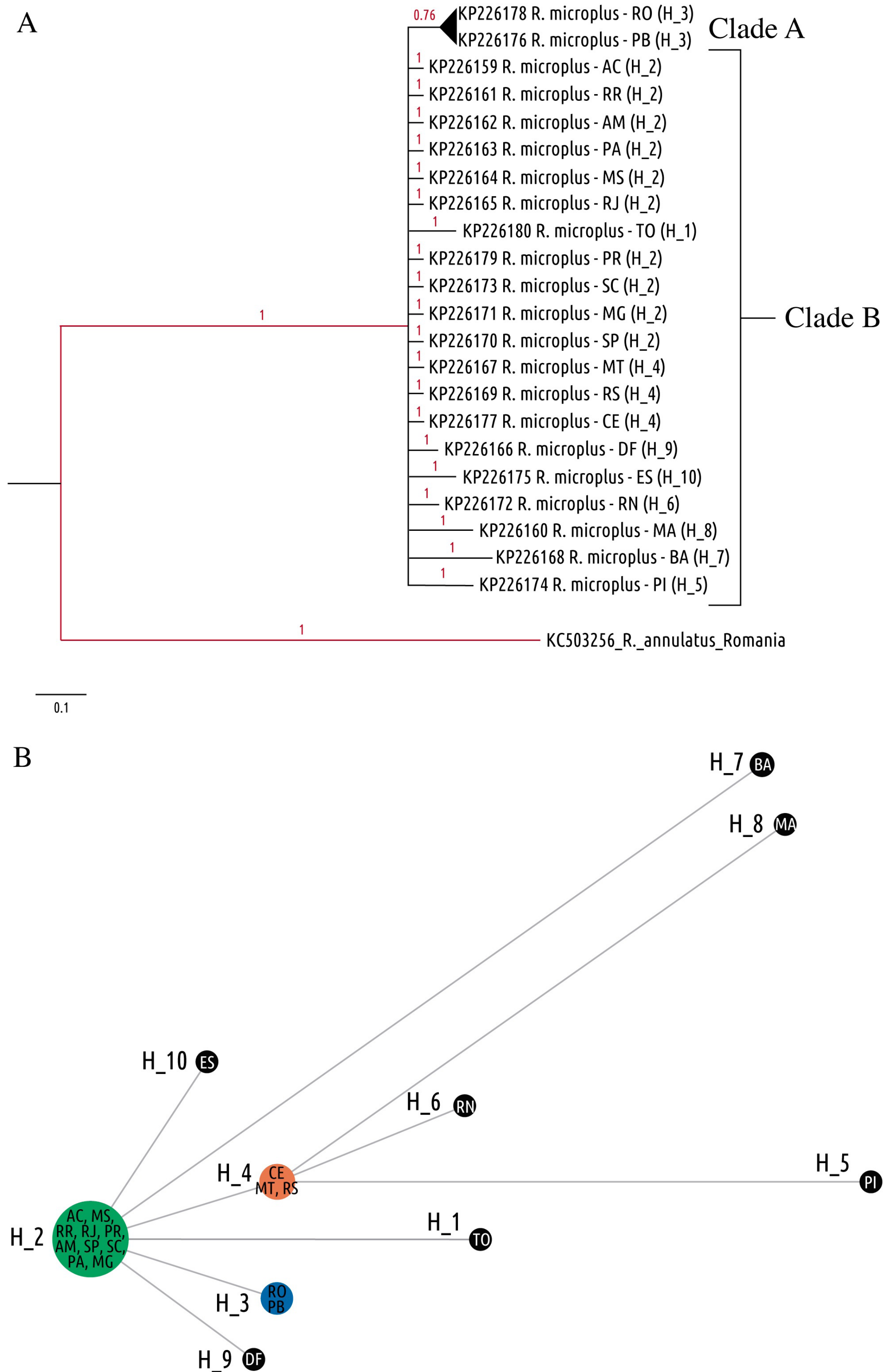

Figure 2. (A) Bayesian phylogenetic tree recovered for cytochrome oxidase subunit 1 gene (COX-I) of 643 nucleotides. *R. annulatus* (GenBank: KC503256) was used as the outgroup (B) median joining network of *R. (B.) microplus* from five different regions populations in Brazil. A circle represents each haplotype. Relative areas of the circles indicate haplotype frequency of each sample.

However, how these haplotypes are associated in other regions is not clear. ITS-2 genotypes indicate a lack of clear separation of tick populations (data no shown).

Genetic distance, genetic differentiation and gene flow

In the uncorrected "p" distance matrix, the COX-I gene indicated stronger resolving power than ITS-2 (1.36–11.00) (Table 2). The genetic distance of the COX-I H-2 haplotype of *R. (B.) microplus* indicated 0.73% of divergence within this population (red value, Table 2). Overall, a high level of significant genetic differentiation was observed among all populations ($P < 0.001$).

Great genetic differentiation (FST = 1.00), revealed by the COX-I gene, was found with in all haplotypes (Table 3). Additionally, genetic differentiation between H-2 haplotypes and others varied between 0.47-0.89 (Table 3). High levels (30.00) of gene flow (Nm) were observed among tick populations as shown by the COX-I (data no shown).

Among the ten haplotypes studied, *R. (B.) microplus* haplotype H-3 from Rondônia (RO) and Paraíba (PB) were genetically diverse as supported by the highest haplotype and nucleotide diversity based on COX-I (Table 3). Higher inter-population levels (82.69%) and (FST = 0.8269) revealed the existence of population structure in all haplotypes analyzed by means of AMOVA (Table 4).

Livestock transition in Brazil is very intense. Whether these regions actually have different populations not found in other regions can only be answered by analyzing a larger number of samples from various municipalities in the States of Rondônia and Paraíba.

All haplotypes are derived from haplotype H-2. However, it is not possible to assert where haplotype H-2 has originated. Additionally, there is no haplotype H-2 in northeastern Brazil.

The fact that the country has cattle production for imported and local breeds and that animals are transported between different regions do not allow us to infer the exact origin of haplotypes. The natural dispersal ability of ticks might occur in parallel with human-mediated dispersal, causing genetic admixture and high gene flow in Brazilian populations.

Phylogenetic analyses

Bayesian analyses produced phylogenetic trees with high valued probabilities (Figure 2 and Figure 3). The 10 haplotypes (H1-H10) generated from the COX-I sequences of Brazilian *R. (B.) microplus* were subjected to phylogenetic analyses. By the topology of the phylogenetic trees generated, the *R. (B.) microplus* Brazilian population comprises two clades: clade A (H3) and clade B (H1, H2 and H4 to H10) (Figure 2A). Different than results revealed by the haplotype network analysis (Figure 2B), according to Bayesian phylogenetic tree, haplotypes H5, H6 and H8 were associated with ticks from the North region of the country and are derived from haplotype H-4. The tree also indicates that all haplotypes derive from H-2.

Table 2. Percentage of uncorrected "p" distance matrix among the ten haplotypes of COX-1 of *R. (B.) microplus* in Brazil.

	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	H_9	H_10
H_1	-									
H_2	3.18	**0.73**								
H_3	4.00	1.36	-							
H_4	4.00	1.36	2.00	-						
H_5	7.00	4.36	5.00	3.00	-					
H_6	5.00	2.36	3.00	1.00	4.00	-				
H_7	7.00	4.36	5.00	5.00	8.00	6.00	-			
H_8	8.00	6.82	8.00	6.00	9.00	7.00	11.00	-		
H_9	4.00	1.36	2.00	2.00	5.00	3.00	5.00	8.00	-	
H_10	5.00	2.27	3.00	3.00	6.00	4.00	6.00	8.00	3.00	-

Table 3. Genetic differentiation (FST) based on COX-1, in all the haplotypes found of *R. (B.) microplus* populations in Brazil.

	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	H_9	H_10
H_1	-									
H_2	0.77143	-								
H_3	1.00000	0.5589	-							
H_4	1.00000	0.59878	1.00000	-						
H_5	1.00000	0.83333	1.00000	1.00000	-					
H_6	1.00000	0.69231	1.00000	1.00000	1.00000	-				
H_7	1.00000	0.83333	1.00000	1.00000	1.00000	1.00000	-			
H_8	1.00000	0.89333	1.00000	1.00000	1.00000	1.00000	1.00000	-		
H_9	1.00000	0.46667	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	-	
H_10	1.00000	0.68000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	1.00000	-

The ITS-2 Bayesian phylogenetic tree revealed one *R. (B.) microplus* genetic clade (Figure 4). The COX-I Bayesian phylogenetic tree revealed two genetic clades of *R. (B.) microplus,* Clade A (Brazil, Texas, Malaysia, *R. australis*) and Clade B (*R. (B.) microplus* China and *R. annulatus*). *Rhipicephalus (B.) microplus* clade A showed a sister relationship with *R. australis*. By contrast, *R. (B.) microplus* clade B is more closely related to *R. annulatus*, with which it forms a sister group relationship, corroborating the data by Burger et al. (2014) (Figure 3).

Table 4. Analysis of molecular variance of molecular marker, gene COX-1.

Source of variation	d.f	Sum of squares	Variance Compo-nents	Percentage of variation
Among populations	9	25.059	1.34_{Va}	82.69
Within populations	13	3.636	0.28_{Vb}	17.31
Total	22	28.696	1.62	
Fixation Index FST:	0.8269	P < 0.001		

The *R. (B.) microplus* populations in Brazil have internal nodes, indicating the occurrence of subpopulations (GenBank: KP226160, KP226167, KP226169, KP226172, KP226174, KP226177). The haplotype H-3 formed another smaller node (GenBank: KP226176, KP226178) (Figure 3).

The intraspecific genetic distance or haplotype frequency, like that of haplotype H2 reported here, are notably higher than previously described for *R. (B.) microplus sensu* Low et al. (2015). Furthermore, the two, and perhaps three, genetic assemblages inferred from Brazilian *R. (B.) microplus* are more genetically diverse than those reported from other regions (Figure 3).

This study sought to obtain high support for the topology of tree and the COX-I molecular marker was found to have the advantage of presenting greater mutation rates than the nuclear marker, corroborating the data presented by Lv et al. (2014). However, the ITS-2 molecular marker failed to resolve discrepancies between the populations of *R. (B.) microplus* and this study corroborates the findings of Burger et al. (2014) in which they showed that the ITS-2 molecular marker has insufficient power to distinguish between very close species.

The comparative analysis in this study shows that *R. (B.) microplus* ticks represent at least two different populations. Moreover, this study also attempted to investigate whether haplotypes are

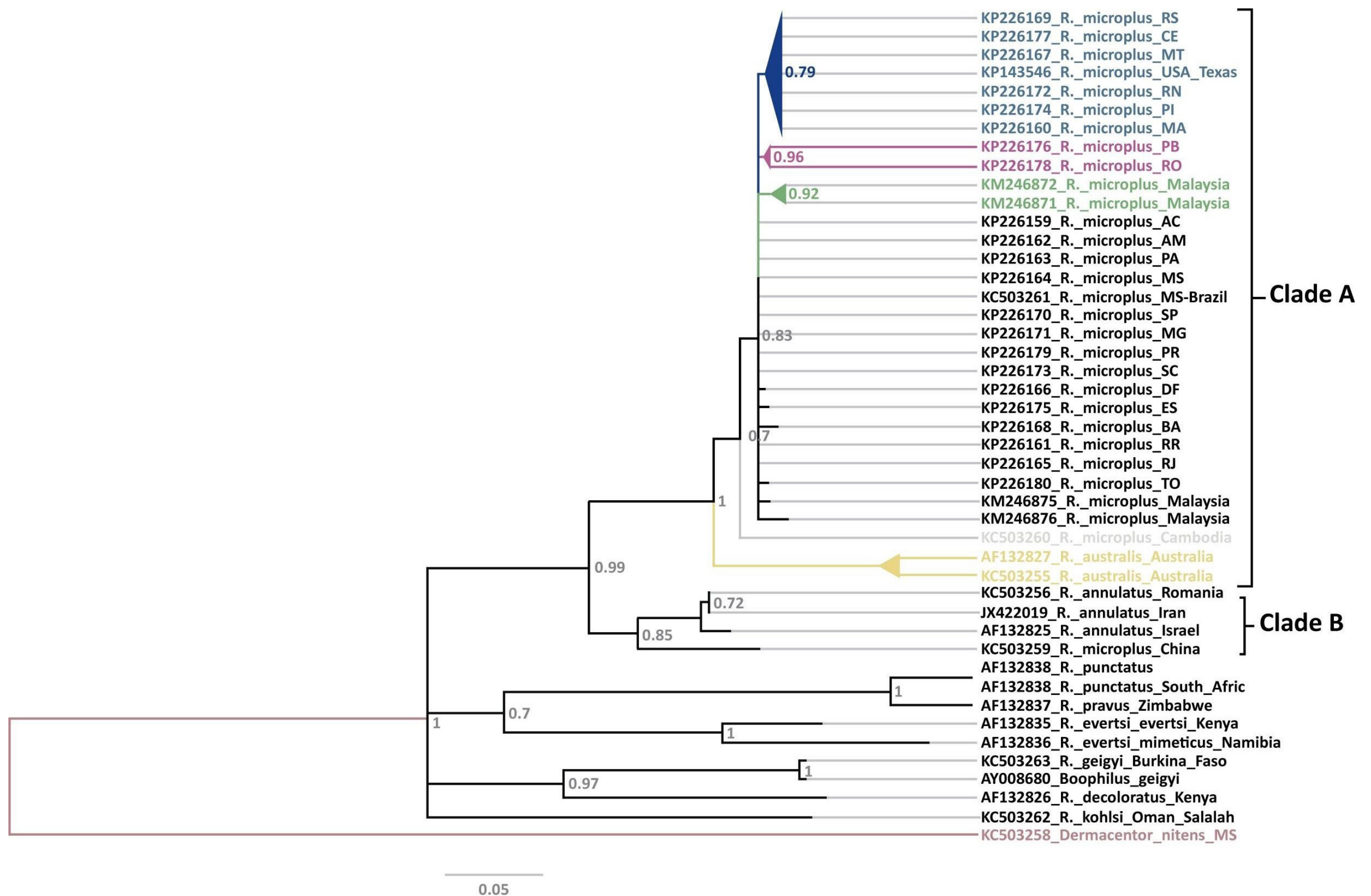

Figure 3. Bayesian phylogenetic tree recovered for cytochrome oxidase subunit 1 gene (COX-I) of 643 nucleotides, for *Rhipicephalus* taxa samples. In blue and green are the smallest nodes formed the Brazilian populations of *R. (B.) microplus. Dermacentor nitens* (GenBank: KC503258) was used as the outgroup.

Figure 4. Bayesian Cladogram recovered for internal transcript spacer 2 (ITS-2) of 580 nucleotides, for sample Rhipicephalus taxa. *Dermacentor nitens* KC503275 was used as the outgroup.

distributed according to the phylogeographical areas. It is unknown whether this indicates a cause-effect association of haplotypes and geographical areas.

However, the prevalence of ticks has been typically associated with cattle breeds, the highly invasive and widespread movement of *R. (B.) microplus* may facilitate its occurrence (IBELLI et al., 2012; BUSCH et al., 2014).

Conclusion

This study provides new insights into the distinct genetic assemblages of the tick *R. (B.) microplus* in Brazil and reveals that the Brazilian *R. (B.) microplus* population consists of at least two different populations. In addition, future studies using a large number of samples from the Americas using COX-1 may

be able to show how these populations differ in their inter- and intra- relationships.

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