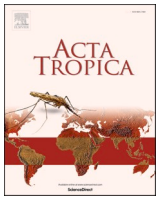
Acta Tropica 238 (2023) 106773

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica



Molecular detection of tick-borne rickettsial pathogens in ticks collected from domestic animals from Cauca, Colombia

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ARTICLE INFO

Keywords:
Molecular identification
Ticks
Rickettsia
Anaplasma
Ehrlichia
Colombia

ABSTRACT

Some hard ticks' species can act as vectors of a wide variety of pathogens of human and animal importance such as *Anaplasma, Ehrlichia* and *Rickettsia* spp. In Colombia, a total of forty-six tick species have been described, and some of them have been implicated as vectors of some infectious agents. The department of Cauca is one of the thirty-two departments of Colombia. Most of its population lives in rural areas and depends on agriculture as the main economic activity, favoring exposure to ticks and tick-borne pathogens. Thus, the present study aimed to determine the tick species and tick-borne pathogens circulating in this region. From August to November 2017, ticks were collected from dogs, horses and cattle from eight rural areas of four municipalities in the department of Cauca. All collected ticks were classified according to taxonomic keys and organized in pools. DNA was extracted from all tick pools for molecular confirmation of tick species and detection of *Anaplasma, Ehrlichia* and *Rickettsia* spp. A total of 2809 ticks were collected which were grouped in 602 pools. Ticks were morphologically identified as *Amblyomma cajennense* sensu lato, *Dermacentor nitens, Rhipicephalus microplus* and *Rhipicephalus sanguineus* sensu lato. The molecular identity of *A. cajennense* s.l. was confirmed as *Amblyomma patinoi*. A total of 95% of the pools scored positive for members of the Anaplasmataceae family, of which, 7.8% and 7.3% were positive to *Anaplasma* and *Ehrlichia* spp., respectively, being identified as *Anaplasma marginale, Ehrlichia mina-sensis* and *Ehrlichia canis*; and 16.1% were positive for *Rickettsia* spp. with high identity for *Rickettsia asembo-nensis, Rickettsia felis* and *Candidatus* Rickettsia senegalensis. This is the first report describing the natural infection of ticks with rickettsial pathogens and the occurrence of *A. patinoi* ticks in Cauca department, Colombia.

1. Introduction

Hard ticks (Acari: Ixodida: Ixodidae) are blood-feeding ectoparasites of wild and domestic vertebrates (Guglielmone et al., 2014). They are widely distributed throughout the world and some species act as vectors of a wide variety of pathogenic microorganisms for humans and different animal species (Parola et al., 2013; Madison-Antenucci et al., 2020).

Some of these microorganisms are members of the Order Rickettsiales, which are vector-borne bacteria mainly transmitted by different hard tick species. This order includes two families: Anaplasmataceae and Rickettsiaceae; both comprise different bacterial genera that include several pathogenic species of human and animal importance such as *Anaplasma, Ehrlichia, Neorickettsia, Rickettsia* and *Orientia* spp. (Dumler et al., 2001; Lu et al., 2019).

Anaplasma, Ehrlichia (Anaplasmataceae family) and *Rickettsia* spp.

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https://doi.org/10.1016/j.actatropica.2022.106773
Received 10 August 2022; Received in revised form 26 October 2022; Accepted 19 November 2022
Available online 20 November 2022

Fig. 1. Map with the location of the four municipalities where the sampling was done in the present study, department of Cauca, Colombia.

(Rickettsiaceae family) are obligate intracellular Gram-negative bacteria that infect a wide range of arthropods and vertebrates (Perlman et al., 2006; Parola et al., 2013; Dumler et al., 2015). In Colombia, several pathogenic *Anaplasma, Ehrlichia* and *Rickettsia* species have been detected, of which, *Rickettsia rickettsii* is the most important due to the extremely severe and potentially fatal disease it can cause in humans and a wide range of domestic animals (Paddock et al., 2002; Hidalgo et al., 2007; Labruna et al., 2009).

Currently, a total of forty-six tick species of the Ixodidae family have been reported in Colombia, including *Amblyomma, Ixodes, Dermacentor, Haemaphysalis* and *Rhipicephalus* spp. (Acevedo-Gutiérrez et al., 2020), some of them being recognized vectors of several pathogenic *Anaplasma, Ehrlichia* and *Rickettsia* species for humans and animals, which are also circulating in the country (Acevedo-Gutiérrez et al., 2020; Charles et al., 2021).

The department of Cauca is one of the thirty-two departments of Colombia and is located in the southwestern part of the country. Its population represents approximately 3% of the total population of the country and the majority lives in rural areas and depends on agriculture as the main economic activity. The climate in the department of Cauca is variable and depends on the geographical location since it has different thermal floors that go from the coast to the mountains; however, most part of the department is characterized by a tropical climate and warm temperatures with two seasons of high rainfall (Gobernación del Cauca, Oficina Asesora de Planeación, 2019). All these characteristics support the presence of some tick's species and associated pathogens that may be circulating in the Cauca department.

To date, tick species *Amblyomma cajennense* sensu lato, *Dermacentor nitens, Ixodes auritulus, Ixodes montoyanus* and *Rhipicephalus microplus* have been found in different regions of the department of Cauca (Acevedo-Gutiérrez et al., 2020). Although some of them are known vectors of several pathogenic microorganisms, information on the circulation of these infectious agents in the region is still scarce due to many reasons related mainly to inaccessibility and the armed conflicts which occurred and still occur in several parts of the department (Tose Vergara and Ortiz Ruiz 2019). However, a 2015 report confirmed that active cases of rickettsiosis in humans are occurring among the population of Cauca department (Peña-R et al., 2015). Thus, the aim of the present study is to

Table 1
Weather and geographical conditions from the municipalities included in the study.

Municipality	Rural areas	Geographical coordinates	Altitude (m.a.s.l.)	Average temperature of the municipality
La Sierra	Juana Castaña	N 2°1423.022″ / O 76° 54' 1.139″	773	20 °C
		N 2°14' 22.212″ / O 76° 5352.835″	755	
El Tambo	El Zarzal	N 2°27' 18.226″ / O 76° 4354.571″	1677	20 °C
	El Placer	N 2°25' 4.381″ / O 76° 4653.959″	1789	
	Betania	N 2°28' 41.673″ / O 76° 4836.351″	1723	
Santander de Quilichao	Lomitas Arriba	N 3°2' 15.223″ / O 76° 3437.983″	1024	26 °C
		N 3°3' 39.535″ / O 76° 3341.36″	991	
	Lomitas Abajo	N 3°4' 28.979″ / O 76° 3323.671″	985	
		N 3°5' 19.195″ / O 76° 33' 24.057″	976	
Caloto	El Credo	N 3° 2' 1.45″ / O 76°17' 57,865″	1606	18 °C
	Huellas	N 2°59' 35.156″ / O 76°2246.918″	1723	

update the knowledge on which tick and *Anaplasma, Ehrlichia* and *Rickettsia* species are circulating in the Cauca region.

2. Materials and methods

2.1. Ethics statement and environmental regulations

All procedures involving animals followed ethical regulations and were approved on May 11th, 2016, by the Ethics and Research Committee from the Faculty of Sciences of "Pontificia Universidad Javeriana". Permits for capture and sampling were regulated by the Framework Permission for the Collection of Specimens from Wild Species of Biological Diversity for the Purpose of Non-Commercial Scientific Research, license no. 01364 of August 21th, 2018, obtained from the "Ministerio de Ambiente y Desarrollo Sostenible" and the "Autoridad Nacional de Licencias Ambientales (ANLA)", Colombia.

2.2. Tick collection and processing

From August to November 2017, ticks were collected from dogs (*Canis lupus familiaris*), horses (*Equus caballus*), and cattle (*Bos taurus* and *Bos indicus)* from eight rural areas of four different municipalities (Caloto, El Tambo, La Sierra and Santander de Quilichao) in the department of Cauca, Colombia (Fig. 1 and Table 1). These areas were chosen due to the reports of tick bites in the inhabitants and the presence of ticks ectoparasitizing domestic animals in these areas (unpublished data), added to a previous report of three active cases of human rickettsiosis confirmed by seroconversion found in La Sierra municipality during a serological and entomological study carried out in Cauca department previously (Peña-R et al., 2015).

Ticks were manually collected from animals after informed consent was signed by their owners and placed directly into 96% ethanol solution at room temperature for further processing by DNA extraction and molecular identification. All collected ticks were taxonomically classified according to their morphology using standard taxonomic keys (Barros et al., 2006; Jones et al., 1972; Nava et al., 2014), and then organized in pools by collection site, tick species, host, and life stage.

DNA from tick pools was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) following manufacturer instructions and modified with an overnight digestion in DNAzol at 56 °C, and stored at −20 °C until further use for PCR amplification procedures (Ramírez-Hernández et al., 2013). The DNA quality was assessed by establishing the A260/A280 ratio and the DNA concentration was determined with the help of a spectrophotometer (West Tune NanoGenius series, Hangzhou, China).

2.3. Molecular identification of ticks

After morphological identification, all tick pools were confirmed by molecular identification using a conventional PCR targeting a fragment of the mitochondrial *16S rRNA* gene (Mangold et al., 1998). Identity of larvae and nymphs of *Amblyomma cajennense* s.l. were molecularly confirmed by two conventional PCR protocols: the first targeting a ≈ 700 bp fragment of the cytochrome oxidase subunit I (*COI-I*) (Folmer et al., 1994) and the second targeting a ≈ 1000-bp fragment of the ribosomal second internal transcribed spacer (*ITS-2*) (Mclain et al., 1995; Zahler et al., 1995) (Table 2). All PCR protocols for the amplification of the *16S rRNA, COI-I* and *ITS-2* were performed by electrophoresis on a 2% agarose gel stained with SYBR™ Safe DNA gel Stain (Invitrogen, Waltham, MA, USA) including a positive and a negative control corresponding to *Amblyomma mixtum* DNA (stored in the special bacteriology laboratory from Pontificia Universidad Javeriana) and nuclease-free water, respectively. All PCR amplicons were visualized through an

Table 2
Primer sequences used for the present study and their target genes.

Organism to identify	Refs.	Target gene	Amplicon size (bp)	Primers	Sequence (5′−3′)
Rickettsia spp.	(Labruna et al., 2004b)	*gltA*	401	CS-78	GCAAGTATCGGTGAGGATGTAAT
				CS-323	GCTTCCTTAAAATTCAATAAATCAGGAT
	(Roux and Raoult, 2000)	*sca5*	816	120–3599	TACTTCCGGTTACAGCAAAGT
				120–2788	AAACAATAATCAAGGTACTGT
	(Webb et al., 1990)	*htrA*	434	17kD1	GCTCTTGCAACTTCTATGTT
				17kD2	CATTGTTCGTCAGGTTGGCG
Anaplasmataceae	(Mustapha Dahmani *et al.*, 2017)	*23S rRNA*	169	TtAna-F	TGACAGCGTACCTTTTGCAT
				TtAna-R	TGGAGGACCGAACCTGTTAC
Anaplasma spp.	(M Dahmani *et al.*, 2017)	*rpoB*	525	Ana-rpoB-F	GCTGTTCCTAGGCTYTCTTACGCGA
				Ana-rpoB-R	AATCRAGCCAVGAGCCCCTRTAWGG
Ehrlichia spp.	(M Dahmani *et al.*, 2017)	*groEL*	590	Ehr-groEL-F	GTTGAAAARACTGATGGTATGCA
				Ehr-groEL-R	ACACGRTCTTTACGYTCYTTAAC
Ticks	Mangold et al., 1998	*16S-rRNA*	460	16S-F	CCGGTCTGAACTCAGATCAAGT
				16S-R	GCTCAATGATTTTTTAAATTGCTGT
	(Folmer et al., 1994)	*COI-1*	700	LCO1490-F	GGTCAACAAATCATAAAGATATTGG
				HCO2198-R	TAAACTTCAGGGTGACCAAAAAATCA
	(Zahler et al., 1995) (Mclain et al., 1995).	*ITS-2*	1000	ITS2-F	CCATCGATGTGAAYTGCAGGACA
				MCLN-R	GTGAATTCTATGCTTAAATTCAGGGGGT

ultraviolet trans-illuminator (Gel Doc XR Plus, Bio-Rad Laboratories, Inc.).

Subsequently, amplicons obtained from each tick pool of all species and stages were randomly selected for bidirectional sequencing, except for *A. cajennense* s.l. amplicons, which were all sent for sequencing. All selected amplicons were sequenced by Sanger method using a 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Both forward and reverse sequences were edited with the free software BioEdit Sequence Alignment Editor v 7.0.5.3 (Hall, 1999). The alignment of all sequences was performed using the Clustal W algorithm (Larkin et al., 2007), and all sequences obtained were compared with reference sequences available in GenBank.

2.4. Molecular identification of Anaplasma, Ehrlichia and Rickettsia spp

All tick pools were initially screened using a real-time PCR targeting the *htrA* gene for the detection of *Rickettsia* spp. (Webb et al., 1990) and the *23S rRNA* gene for the detection of members of the *Anaplasmataceae* family (Dahmani et al., 2017a). Positive samples for *Rickettsia* spp. *htrA* gene were further screened by conventional PCR for two additional genes: *gltA* and *sca5* (Roux and Raoult, 2000; Labruna et al., 2004b); positive samples for *Rickettsia* spp. were considered if at least one of the additional genes was successfully amplified.

For the identification of *Anaplasma* and *Ehrlichia* spp., *Anaplasmataceae 23S rRNA* positive samples were screened targeting the *rpoB* and *groEL* genes of *Anaplasma* and *Ehrlichia* spp., respectively (Dahmani et al., 2017b) by conventional PCR (Table 2). All PCR protocols included a positive and a negative control: *Rickettsia vini* DNA (kindly provided by Marcelo B. Labruna from the Universidade de São Paulo, São Paulo, Brazil) was used as positive control for the *htrA, gltA* and *sca5* protocols, *Anaplasma marginale* DNA for the *23S rRNA* and *rpoB* protocol, and *Ehrlichia canis* DNA for the *groEL* protocol, both kindly provided by Byron Hernández from Corporación Colombiana de Investigación AGROSAVIA, Bogotá D.C., Colombia; nuclease-free water was used as negative control in all protocols.

Additionally, the minimum infection rate (MIR) was calculated considering all tick species and all life stages using to following formula: the ratio of the number of positive tick pools divided by the total number of collected ticks of the same species, all multiplied by a hundred (Labruna et al., 2004a).

All PCR products for *gltA, sca5, rpoB* and *groEL* genes were purified using the commercial kit Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA). All positive samples were further sent for sequencing process using the same methodology described in the molecular identification of ticks' section, as well as forward and reverse sequence editing and alignment of all sequences. Reference sequences of all available *Anaplasma, Ehrlichia* and *Rickettsia* spp. were obtained from the GenBank database to compare them with the sequences obtained in the present study.

2.5. Phylogenetic analyses

For those successfully sequenced positive samples, the percentage of nucleotide identity has been established by Blast analysis, and were further analyzed by phylogenetic analysis using the maximum likelihood (ML) method based on the best predicted models. The evolutionary distances were computed using the Tamura 3-parameter (Tamura, 1992) for the *16S rRNA* tick gene, and the *Rickettsia gltA* and *sca5* gene; the Generalized Time Reversible model (Thomas, 2001) for the *COI-I* gene; the Kimura two-parameter model (Kimura, 1980) for the *ITS-2* gene; and the Tamura-Nei model (Tamura and Nei, 1993) for the *Anaplasma rpoB* gene and the *Ehrlichia groEL* gene. All of them with 1000 bootstrap replicates. All positions containing gaps and missing data were eliminated, and analyses were conducted in MEGA X Software (Kumar et al., 2018). All obtained sequences in this study were deposited in GenBank.

Table 3
Total number of tick pools collected based on species, life stage, host and site of collection.

Municipality	Species	Life Stage	Total Number of pools	Host
El Tambo	*Rhipicephalus microplus*	Larva	3	*Bos taurus*
		Nymph	7	
		Female	48	*Bos indicus* (2) and *Bos taurus* (46)
		Male	12	*Bos taurus*
	Rhipicephalus sanguineus s.l.	Larva	1	*Canis lupus familiaris*
		Nymph	6	
		Female	60	
		Male	36	*Bos taurus* (1) and *Canis lupus familiaris* (35)
	Dermacentor nitens	Larva	18	*Equus caballus*
		Nymph	44	
		Female	62	
		Male	29	
La Sierra	*Amblyomma cajennense* s.l.	Larva	1	*Equus caballus*
		Nymph	12	*Equus caballus* (9) and *Canis lupus familiaris* (3)
	Dermacentor nitens	Female	3	*Equus caballus*
		Male	1	
	Rhipicephalus sanguineus s.l.	Nymph	2	*Canis lupus familiaris*
		Female	11	
		Male	6	
Santander de Quilichao	*Rhipicephalus (Boophilus) microplus*	Nymph	3	*Bos taurus* (2) and *Equus caballus* (1)
		Female	47	*Bos indicus* (4), *Bos taurus* (40) and *Equus caballus* (3)
		Male	10	*Bos indicus* (1), *Bos taurus* (8) and *Equus caballus* (1)
	Rhipicephalus sanguineus	Larva	2	*Canis lupus familiaris*
		Nymph	4	
		Female	42	
		Male	26	
	Dermacentor nitens	Larva	10	*Equus caballus*
		Nymph	27	
		Female	23	
		Male	11	
Caloto	*Rhipicephalus sanguineus* s.l.	Nymph	1	*Canis lupus familiaris*
		Female	1	
		Male	4	
	Dermacentor nitens	Larva	4	*Equus caballus*
		Nymph	4	
		Female	16	
		Male	5	

3. Results

3.1. Tick sampling

A total of 2809 partially-fed ticks were collected from domestic animals in the four municipalities selected for the present study. All ticks were organized in pools of six to seven males, one to two females, eight to nine nymphs and sixteen to seventeen larvae. A total of 602 tick pools were obtained: 204 pools from 56 dogs, 272 pools from 45 horses and 126 pools from 26 cattle, Ticks collected from dogs were morphologically identified as *R. sanguineus* s.l. and *A. cajennense* s.l.; while the ticks collected from horses were identified as *D. nitens, R. microplus,* and *A. cajennense* s.l.; and those from cattle were identified as *R. microplus* and *R. sanguineus* s.l. (Table 3).

3.2. Molecular identification of ticks

All 602 tick pools amplified successfully for the *16S rRNA* gene. The

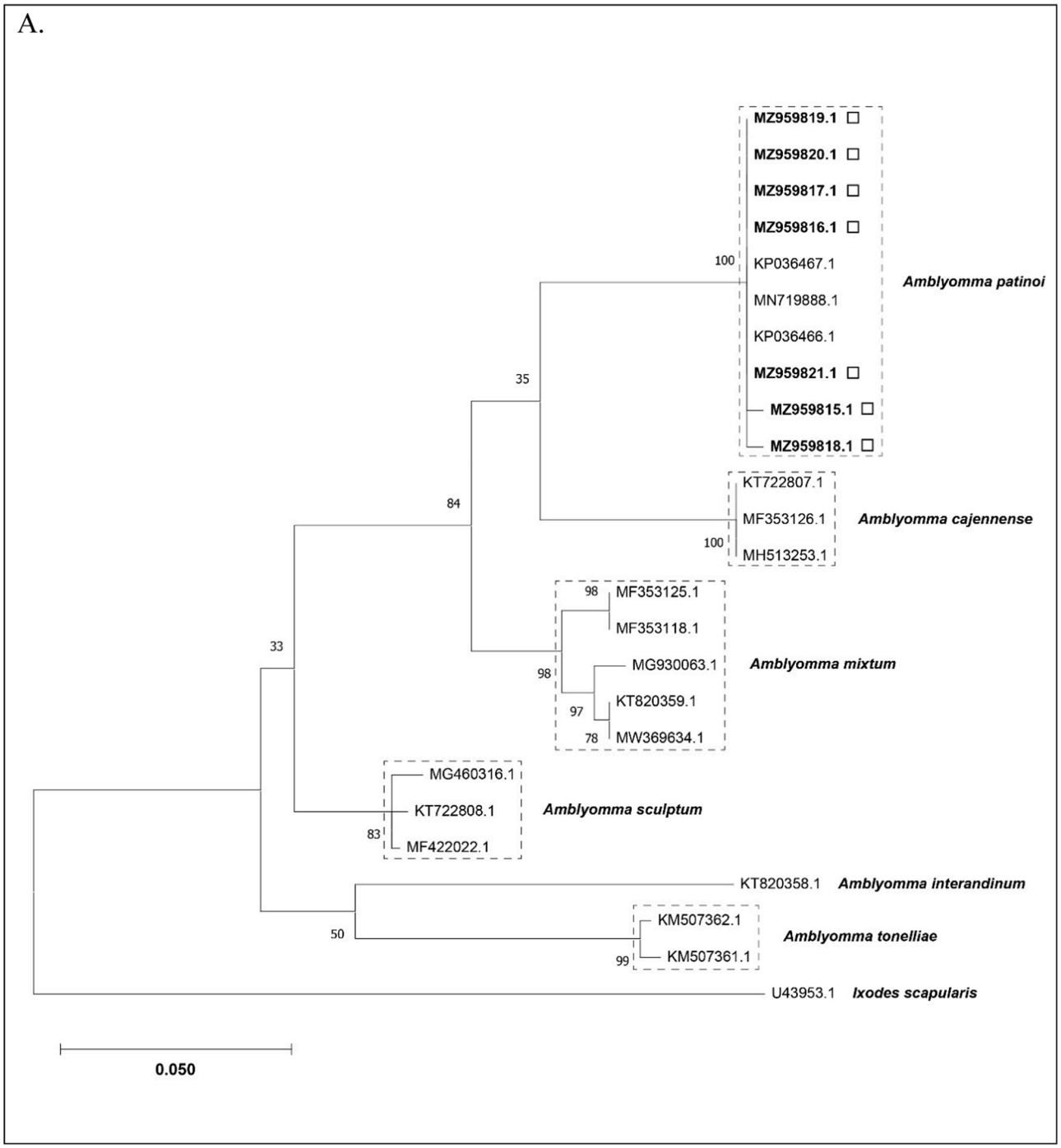

Fig. 2. Phylogenetic trees based on (A) *Amblyomma cajennense* complex *16S rRNA* gene sequences, (B) *Amblyomma* spp. *COI-I* gene sequences, and (C) *Amblyomma* spp. *ITS-2* gene sequences, in which all retrieved sequences were identified as *Amblyomma patinoi*. The sequences retrieved in the present study are in bold and marked with white squares. GenBank numbers from retrieved and reference sequences are indicated in all cases.

identity of all *A. cajennense* s.l. morphologically identified was molecularly confirmed as *A. patinoi* by the amplification of three genes (*16S rRNA, COI-I*, and *ITS-2*) from all sampled stages. The identification of the other morphologically characterized tick species was confirmed by amplification of the *16S rRNA* gene of all stages.

The phylogenetic analysis performed with the *16S rRNA* and *ITS-2* genes showed that the sequences obtained in the present study formed a single clade with *A. patinoi* sequences from other regions of Colombia (bootstrap support: 100% for *16S rRNA* gene and 99% for *ITS-2* gene). The obtained sequences of *16S rRNA* gene showed an identity of 100% with sequences of *A. patinoi* from Villeta and Antioquia, Colombia (GenBank accession numbers: KP036467.1; KP036466.1; MN719888.1); and the obtained sequences of *ITS-2* gene showed results of identity ranging from 96.05 to 100% with *A. cajennense* and *A. patinoi* from Colombia, respectively (GenBank accession numbers: JN866881.1 and KF527298.1). There were no *COI-I* gene reference sequences for *A. patinoi* available in the GenBank database; however, all the *COI-I* sequences obtained in the present study formed a unique group different from other *Amblyomma* species (Fig. 2).

Before performing the phylogenetic analysis for *D. nitens, R.*

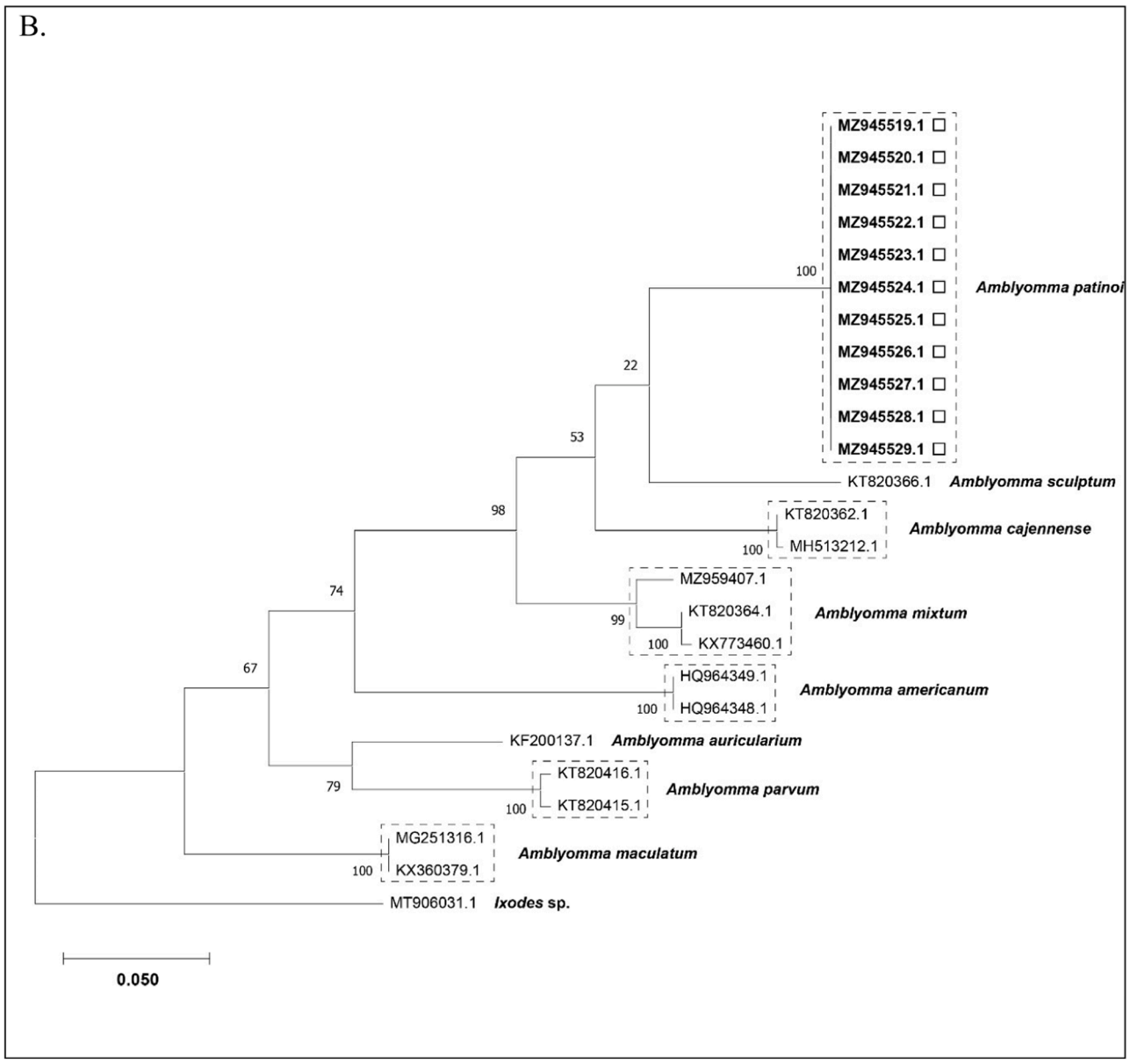

Fig. 2. (*continued*).

microplus and *R. sanguineus* s.l., an identity matrix was performed to reduce the number of sequences added to the phylogenetic tree. Sequences with 100% identity to each other were randomly selected and those with <99.9% identity were included. As a result, it was observed that *D. nitens* was grouped into three clusters (bootstrap support: 98%): one with sequences reported from Colombia and other Latin-American countries, a second which did not cluster with reference sequences and a third that grouped with MF353108 Leticia-Colombia sequence. The obtained sequences showed an identity ranging from 96.69 to 99.10% with sequences of *D. nitens* from Cuba, Brazil and Colombia (GenBank accession numbers: MN880496; KY020994; MF353108.1).

R. microplus showed a single cluster with different reference sequences from other countries (bootstrap support: 100%). The obtained sequences showed an identity of 100% with *R. microplus* from Japan, Argentina and Colombia (GenBank accession numbers: AB819268.1; EU918176.1; MN650729.1). Finally, *R. sanguineus* s.l. also formed an individual group with the tropical lineage (bootstrap support: 62%). The obtained sequences showed an identity ranging from 98.34 to 98.57% with *R. sanguineus* from Cuba, Costa Rica and Chile (GenBank accession numbers: KP830114.1; KT382449.1; KX632154.1) (Fig. 3).

Tick sequences obtained in the present study were deposited in the GenBank database with the following access numbers: *16S rRNA* gene for *A. patinoi* [MZ959815 to MZ959821], *D. nitens* [MZ959833 to MZ959853], *R. microplus* [MZ959859 to MZ959868] and *R. sanguineus* s. l. [MZ960023 to MZ960055], and *COI-1* and *ITS-2* genes for *A. patinoi* [MZ945519 to MZ945529] and [MZ962663 to MZ962671], respectively.

3.3. Molecular detection of *Anaplasma* and *Ehrlichia* spp

From the total of tick pools evaluated in the present study, 95% (576/602) were positive to Anaplasmataceae infection. These positive samples were further evaluated targeting the *rpoB* and *groEL* genes of *Anaplasma* spp. and *Ehrlichia* spp., respectively, showing a positivity of 7.8% (45/576) and 7.3% (42/576), respectively, with *rpoB* and *groEL* partial sequences' amplification (Table 4).

For *Anaplasma* spp., the highest MIR was found in *R. microplus* with 8.4% (35/417), followed by 0.6% (3/518) and 0.4% (7/1828) in

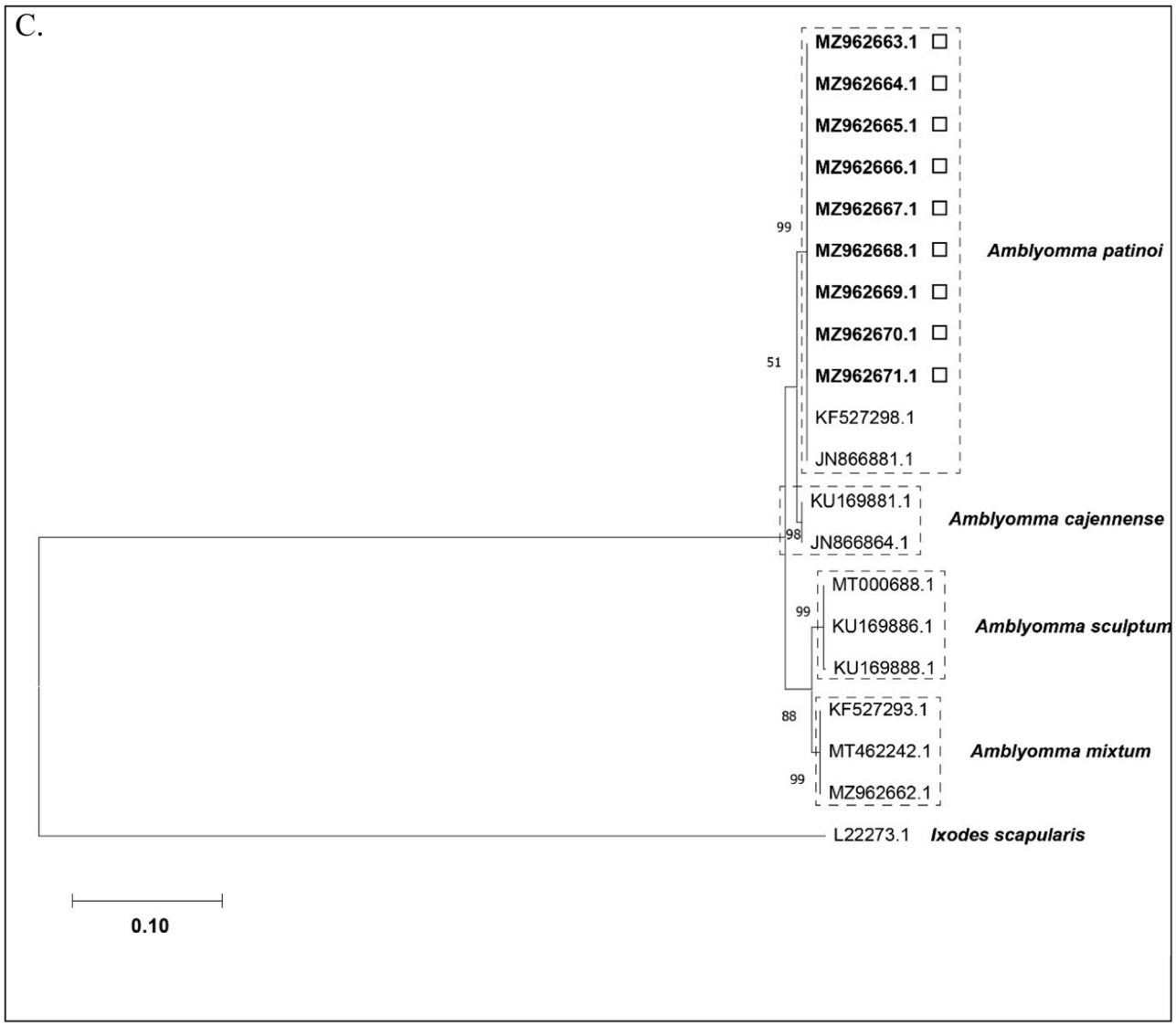

Fig. 2. (*continued*).

R. sanguineus s.l. and *D. nitens*, respectively. However, no *Anaplasma* spp. infection was found among *A. cajennense* s.l. ticks. Considering *Ehrlichia* spp. infection, the highest MIR was also found in *R. microplus* with 6.2% (26/417), followed by *R. sanguineus* s.l. and *D. nitens* with 2.9% (15/518) and 0.05% (1/1828), respectively (Table 5).

A total of seven of the forty-five *rpoB* positive tick pools were successfully sequenced. All these pools were identified as *R. microplus* collected from cattle from El Tambo municipality. All obtained sequences showed an identity of 99.62 to 100% with reference sequences of *Anaplasma marginale* (GenBank accession numbers: CP023731, CP023730, MH651041, CP001079). Phylogenetic tree based on *Anaplasma* spp. *rpoB* partial sequences showed that the seven sequences obtained in the present study clustered in a clade with reference sequences of *A. marginale* (Fig. 4). Sequences obtained in the present study for *rpoB* gene of *Anaplasma* spp. were deposited in Genbank database under the following accession numbers: [OK322365 to OK322371].

For *Ehrlichia* spp., a total of sixteen of the forty-two *groEL* positive samples were successfully sequenced. Ten of these tick pools were identified previously as *R. sanguineus* s.l., all of them were collected from dogs in different regions of the four municipalities. The ten obtained sequences showed an identity percentage of 99% to 100% with reference sequences of *Ehrlichia canis* (GenBank accession numbers: MN216188, MG953295, CP025749 and MN216187). The remaining six positive tick pools were identified as *R. microplus* which were collected from cattle in the four studied regions, and showed an identity ranging from 99.44% to 100% with *Ehrlichia minasensis* and related uncultured *Ehrlichia* strains (GenBank accession numbers: JX629806, KY046305, KJ930195, JX402611, MH500006, MH675614 and KX987387). The phylogenetic analyses based on *Ehrlichia* spp. *groEL* gene showed that some of the sequences obtained in the present study (MW548518 to MW548522 and MW548507 to MW548511) clustered in the *E. canis* clade, while others (MW548512 to MW548517) grouped within the clade of *E. minasensis* (Fig. 5). Sequences obtained in the present study for *groEL* gene of *Ehrlichia* spp. were deposited in Genbank database under the following accession numbers: [MW548507 to MW548522].

3.4. Molecular detection of Rickettsia spp

From all tick pools positive for *Rickettsia* spp., 16.1% (97/602) were infected by using at least two genes used in the present study. A total of 92% (555/602) of the tick pools were positive for the *htrA* gene, of which, 9.9% (55/555) were also positive by using *gltA* partial sequence amplification, 6.6% (37/555) were also positive by using *sca5* partial sequence amplification, and only 0.4% (2/555) were positive by using the three different genes (Table 4). Considering the MIR for *Rickettsia* spp., an infection rate estimated at 9.6% was found in *R. microplus*,

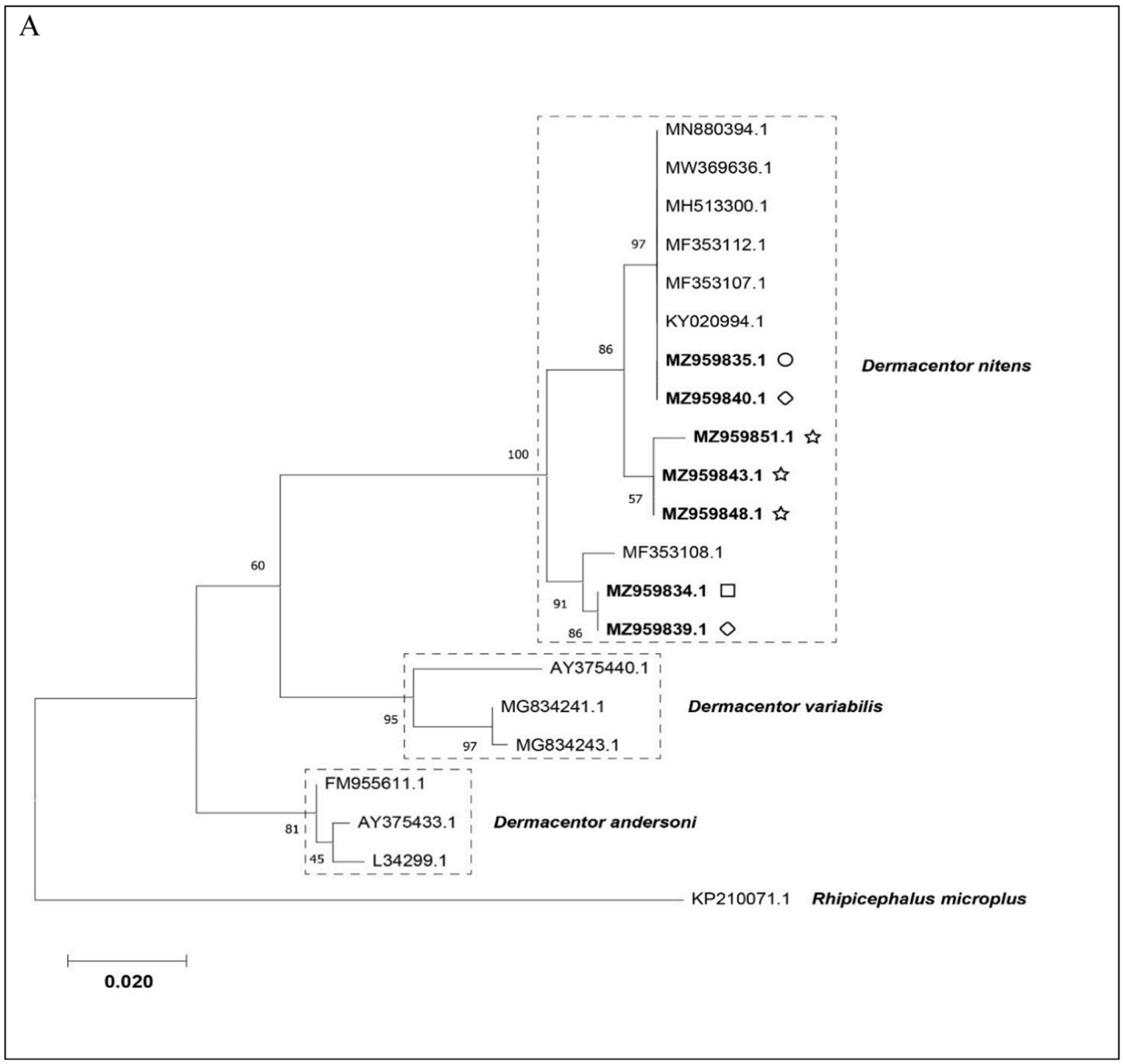

Fig. 3. Phylogenetic trees based on (A) *Dermacentor* spp. 16 s *rRNA* gene sequences in which all retrieved sequences were identified as *Dermacentor nitens*, and (B) *Rhipicephalus* spp. 16 s *rRNA* gene sequences in which retrieved sequences were identified as *Rhipicephalus sanguineus* and *Rhipicephalus microplus*. The sequences retrieved in the present study are bolded and marked with symbols: white circles (retrieved from El Tambo municipality), white diamonds (retrieved from Santander de Quilichao), white stars (retrieved from Caloto municipality), and white squares (retrieved from La Sierra municipality). GenBank numbers from retrieved and reference sequences are indicated in all cases.

followed by 6.5% in *A. cajennense* s.l., 3.3% in *R. sanguineus* s.l. and 2% in *D. nitens* ticks (Table 5).

Only five of the thirty-seven tick pools positive for *sca5* gene were successfully sequenced. These tick pools were collected from horses of El Tambo and La Sierra municipalities, and were identified as *D. nitens* and *A. cajennense* s.l. Three sequences showed an identity of 98.99 to 100% with *Rickettsia asembonensis* (GenBank accession numbers: KY650699, JN315972, MK923741), one pool was 98.9 to 100% similar to *Rickettsia felis* (GenBank accession numbers: AF182279.9, GQ385243), and one tick pool showed an identity of 98.9% to 100% with *Candidatus* Rickettsia senegalensis (GenBank accession numbers: MK548198, KT304219, KF666470, KU167060). The phylogenetic tree built with *Rickettsia* spp. *sca5* sequences obtained in the present study and reference sequences obtained from GenBank showed that three sequences (MZ997335, MZ997336 and MZ997337) clustered with *R. asembonensis* in a separate clade, while two sequences (OK052998 and OK052999) clustered with *R. felis* and *Candidatus* R. senegalensis clades, respectively (Fig. 6). Sequences obtained in the present study for *sca5* gene of *Rickettsia* spp. were deposited in Genbank database under the following accession numbers: MZ997335-MZ997337 for *R. asembonensis*, OK052998 for *R. felis* and OK052999 for *Candidatus* R. senegalensis.

None of the *gltA* positive samples were successfully sequenced, and due to difficulties occurred during the tick DNA sample storage, all DNA samples were partially degraded. Thus, re-amplifications of *gltA* and other *sca5* positive samples were not possible, preventing the ability to perform sequencing and phylogenetic analysis necessary to identify the infecting species.

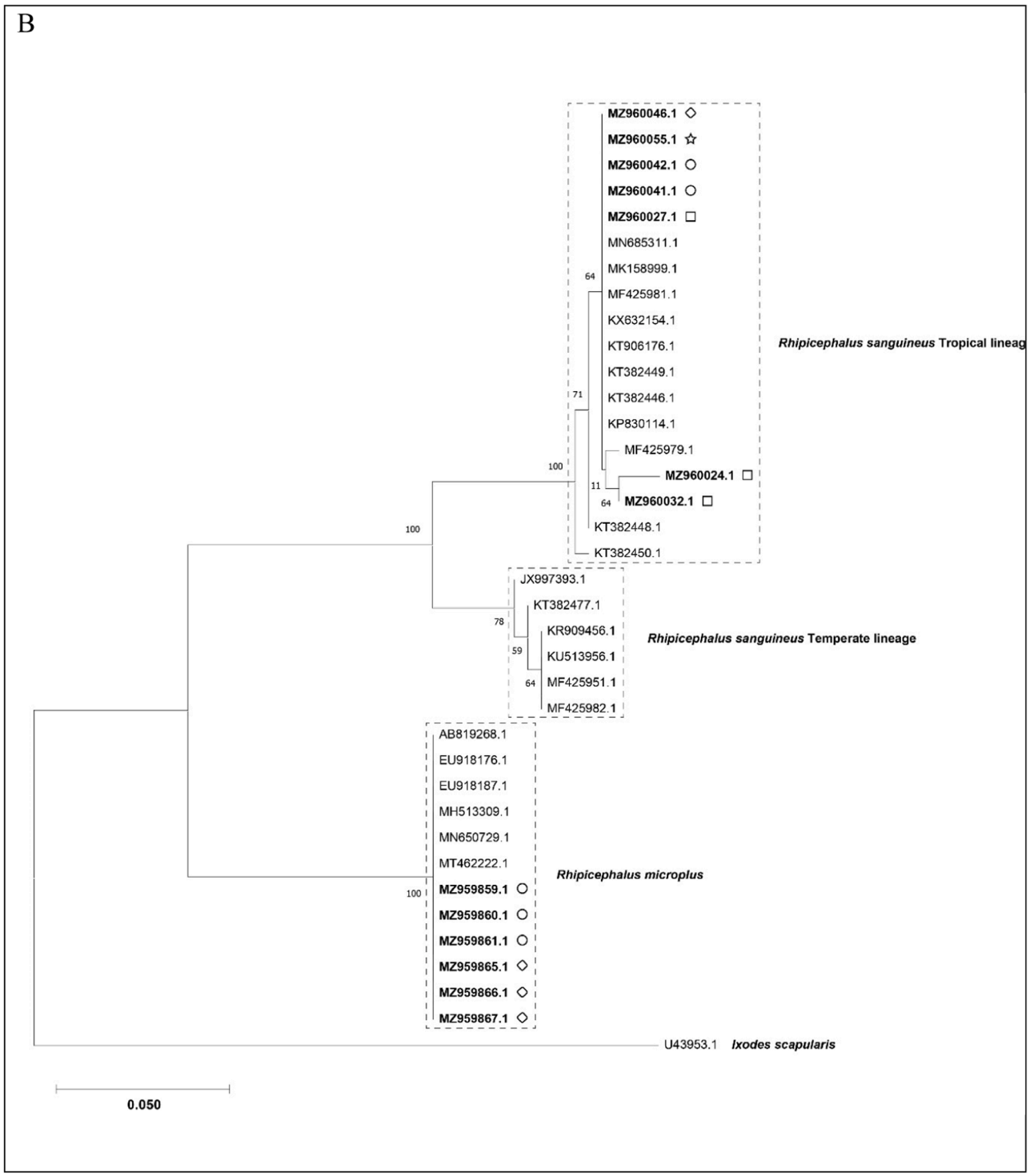

Fig. 3. (*continued*).

4. Discussion

The present study reports the presence of *A. patinoi*, one of the species that makes up the *A. cajennense* s.l. complex, as well as the presence of *R. microplus, R. sanguineus* s.l. and *D. nitens* ectoparasitizing different species of domestic animals in four regions of the department of Cauca. In addition to reporting the detection of *E. minasensis* for the first time in Cauca department, as well as the detection of *R. asembonensis, R. felis, Candidatus* R. senegalensis, *A. marginale* and *E. canis* in different species of ticks collected in the same region.

In Colombia, two studies have identified the presence of *Rickettsia* spp. in *A. cajennense* s.l.; the first one was carried on in Villeta municipality, Cundinamarca department, in which *Candidatus* Rickettsia amblyommii was detected from a nymph of *A. cajennense* s.l. collected from humans (Faccini-Martínez et al., 2016); and the second one found the presence of different *Rickettsia* species among larvae and adults of *A. cajennense* s.l. collected from horses and vegetation in the Tayrona National Park, Magdalena department (Santodomingo et al., 2019). *A. patinoi* is one of the members of the *A. cajennense* s.l. complex and it is considered the main vector of *R. rickettsii* in some areas of Central and

Table 4
Tick species hosts and number of positive tick pools for rickettsial agents from each one of the municipalities.

Municipality	Tick Species	No. Infected/ No. Tested (%) *Rickettsia* sp.	No. Infected/ No. Tested (%) *Anaplasma* sp.	No. Infected/ No. Tested (%) *Ehrlichia* sp.
El Tambo	*Rhipicephalus microplus*	17/70 (24.3%)	30/70 (42.9%)	20/70 (28.6%)
	Rhipicephalus sanguineus s.l.	10/103 (9.7%)	1/103 (1%)	9/103 (8.7%)
	Dermacentor nitens	18/153 (11.8%)	6/153 (3.9%)	1/153 (0.7%)
Santander de Quilichao	*Rhipicephalus microplus*	23/60 (38.3%)	5/60 (8.3%)	6/60 (10.0%)
	Rhipicephalus sanguineus s.l.	4/74 (5.4%)	2/74 (2.7%)	4/74 (5.4%)
	Dermacentor nitens	8/71 (11.3%)	1/71 (1.4%)	0/71 (0%)
La Sierra	*Amblyomma cajennense* s.l.	3/13 (23.1%)	0/13 (0%)	0/13 (0%)
	Dermacentor nitens	1/4 (25.0%)	0/4 (0%)	0/4 (0%)
	Rhipicephalus sanguineus s.l.	0/19 (0%)	0/19 (0%)	2/19 (%)
Caloto	*Rhipicephalus sanguineus* s.l.	3/6 (50.0%)	0/6 (0%)	0/6 (0%)
	Dermacentor nitens	10/29 (34.5%)	0/29 (0%)	0/29 (0%)
	Total	97/602 (16.1%)	45/602 (7.5%)	42/602 (7.0%)

South America (Guedes et al., 2005; Faccini-Martínez et al., 2015). However, a study found that *A. patinoi* might not be able to sustain *R. rickettsii* infection by transovarial transmission for successive tick generations without the horizontal transmission among animal hosts, and that this tick species has a low *R. rickettsii* infection rate under natural conditions (Martínez-Diaz et al., 2021). In Colombia, *R. rickettsii* has been detected from *A. patinoi* in an endemic area for Rocky Mountain Spotted Fever (RMSF) (Faccini-Martínez et al., 2015), thus its importance cannot be underestimated. The presence of *A. patinoi* is a very important finding, and a reason to continue doing more research in order to establish the role of this tick species in the epidemiology of rickettsiosis in these areas, considering the active cases of rickettsiosis have already been detected in the department of Cauca (Peña-R et al., 2015).

The second tick species found in Cauca department, Colombia, was *R. microplus*, one of the most important ectoparasites of cattle worldwide, that can also ectoparasite other host species including buffaloes, horses and dogs (Tan et al., 2021) was also found in Cauca department, Colombia, which has been found infected with the three screened microorganisms. Molecular evidence of *R. microplus* with several *Rickettsia* species have been reported in different regions in the American continent including Panama (Bermúdez et al., 2009), Ecuador (Pesquera et al., 2015) and Brazil (Moura-Martiniano et al., 2014). *R. microplus* is a recognized vector of babesiosis due to *Babesia bigemina* and *Babesia bovis*, but it also acts as a vector of anaplasmosis due to *Anaplasma marginale* and some studies have also reported its relationship with some *Ehrlichia* spp. (Scoles et al., 2007; Cruz et al., 2012; Gray et al., 2019). In Colombia, detection of *Anaplasma* spp. and *Ehrlichia* spp. in *R. microplus* has already been done with a MIR of 1% and 1.2%, respectively (Miranda and Mattar, 2015), which are lower than the MIR found in the present study for both microorganisms (8.4% and 6.2%, respectively) which might relay in the amount of samples obtained, the initial screening methodology or may highlight the high infection of ticks from Cauca region and the importance that it may represent for human and animal health. Additionally, DNA of *E. minasensis*, a species which infects cattle and cause ehrlichiosis in infected calf (Aguiar et al., 2019; Cabezas-Cruz et al., 2019), was found among *R. microplus* ticks collected from cattle in El Tambo municipality. *E. minasensis* is a relatively novel *Ehrlichia* species that was isolated from the hemolymph of engorged *R. microplus* ticks from Minas Gerais, Brazil (Cruz et al., 2012; Cabezas-Cruz et al., 2016). In Colombia, this species has already been detected in *R. sanguineus* s.l. (Miranda and Mattar, 2015) and the present study confirms that this *Ehrlichia* species is also circulating in Cauca department, therefore, further studies should consider this pathogen in cattle population.

Another tick species found in the present study was *R. sanguineus* s.l., commonly known as the brown dog tick, which was identified as a vector of RMSF in Eastern Arizona, United States (Demma et al., 2005). In Latin America, DNA of *R. rickettsii* has been previously detected from *R. sanguineus* s.l. in Mexico (Castillo-Martínez et al., 2017), Panamá (Martínez-Caballero et al., 2018) and Brazil (Cunha et al., 2010). Although in the present study, *R. rickettsii* was not detected from *R. sanguineus* s.l., the presence of this tick in Cauca department, should aware the local authorities for a possible new endemic area for RMSF in Colombia. Furthermore, *R. sanguineus* s.l. is also recognized as vector of

Table 5
Minimum Infection Rate (MIR) for each tick species and rickettsial agents.

Tick Species	Life Stage	Total No. Ticks	No. Pools	*No. of positive (MIR%) Rickettsia* sp.	*No. of positive (MIR%) Anaplasma* sp.	*No. of positive (MIR%) Ehrlichia* sp.
Rhipicephalus microplus	Larva	3	3	0	1 (33,3)	2 (66,6)
	Nymph	21	10	3 (14,3)	1 (4,8)	2 (9,5)
	Adult Female	317	95	26 (8,2)	26 (8,2)	19 (6,0)
	Adult Male	76	22	11 (14,5)	7 (9,2)	3 (3,9)
	Total	417	130	40 (9,6)	35 (8,4)	26 (6,2)
Rhipicephalus sanguineus	Larva	11	3	0	0	0
	Nymph	28	13	2 (7,1)	0	0
	Adult Female	207	114	7 (3,4)	2 (1,0)	9 (4,3)
	Adult Male	272	72	8 (2,9)	1 (0,4)	6 (2,2)
	Total	518	202	17 (3,3)	3 (0,6)	15 (2,9)
Dermacentor nitens	Larva	338	32	1 (0,3)	1 (0,3)	0
	Nymph	746	75	17 (2,3)	0	0
	Adult Female	450	104	11 (2,4)	2 (0,4)	0
	Adult Male	294	46	8 (2,7)	4 (1,4)	0
	Total	1828	257	37 (2,0)	7 (0,4)	1 (0.05)
Amblyomma cajennense s.l.	Larva	11	1	0	0	0
	Nymph	35	12	3 (8,6)	0	0
	Total	46	13	3 (6,5%)	0	0

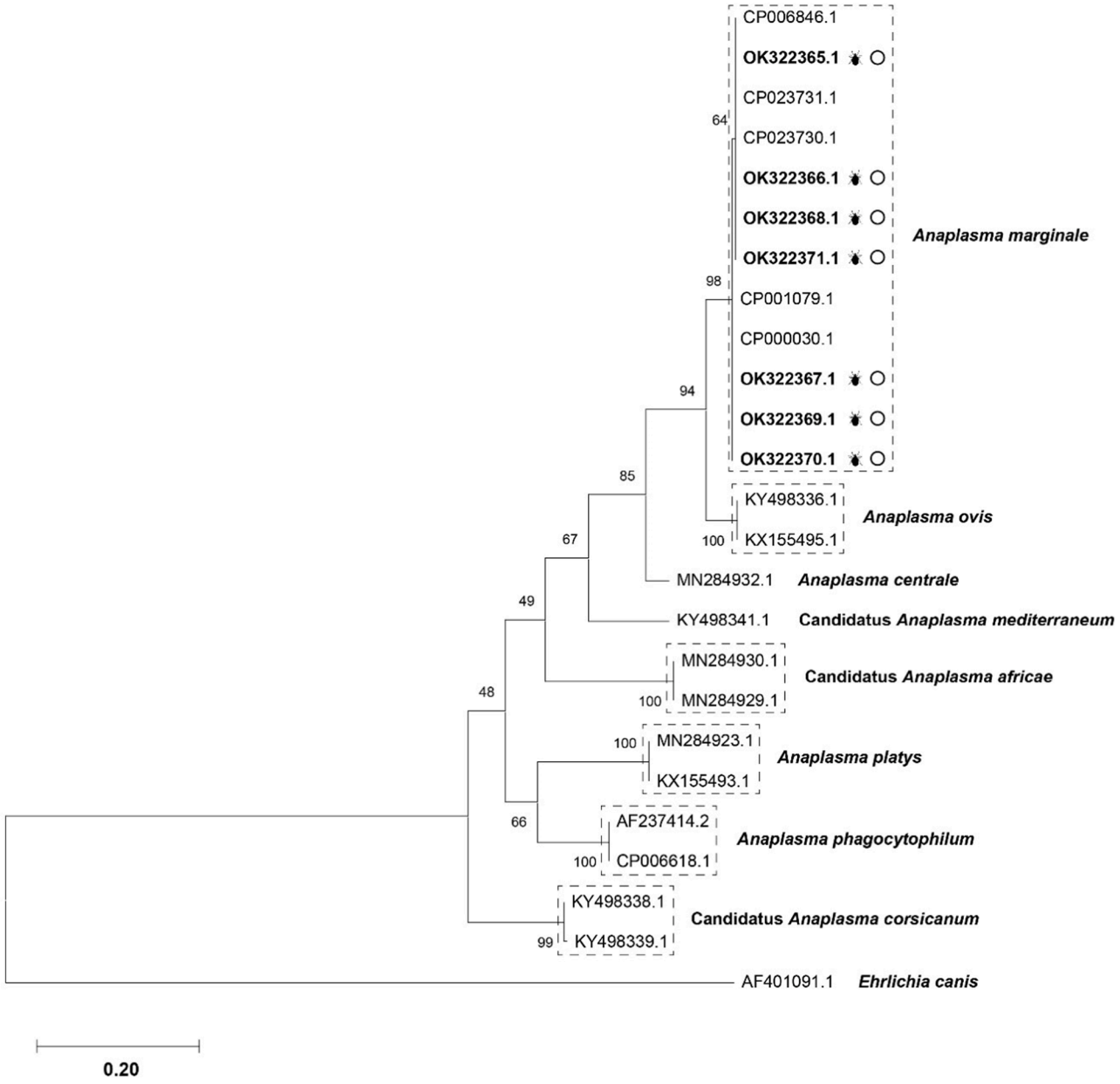

Fig. 4. *Anaplasma* spp. *rpoB* gene sequence-based phylogenetic tree in which all sequences retrieved in the present study clustered in clade within *Anaplasma marginale* reference sequences. The sequences retrieved in this study are in bold and marked with a tick figure and white circles. GenBank numbers from reference sequences are indicated in all cases.

other microorganisms like *Anaplasma platys* and *Ehrlichia canis*. Due to the canine host preference of this tick species (Dantas-Torres 2010), the detection of *A. platys* and *E. canis* in *R. sanguineus* s.l. in a specific area must aware veterinarians to take the necessary measures on time when a dog is highly suspicious of having the disease. In Colombia, 3.4% and 11.8% of *R. sanguineus* s.l. sampled in Medellín, Antioquia department, were found infected with *A. platys* and *E. canis*, respectively (Arroyave et al., 2020). In the present study even though no evidence of *A. platys* was detected, some of the obtained sequences obtained clustered within the *E. canis* clade, which must highlight the importance for animal health in this region.

Finally, the last tick species identified during the course of the present study was *D. nitens*, a tick species that ectoparasites mainly horses, but other mammals like cattle and dogs can also be infected with this tick species (Guzmán-Cornejo et al., 2016). Studies concerning *D. nitens* in Colombia are scarce and limited. Detection of microorganisms have only been performed in two studies: one of them detected DNA of *Rickettsia* spp. and *Anaplasma* spp. in 26% (5/19) and 5.3% (1/19) of *D. nitens* collected from horses and pastures in the Tayrona National Park, Magdalena department (Santodomingo et al., 2019) and the second one detected the presence of *Anaplasma* spp. in 5.2% (5/19) of this tick species collected from horses in several regions of Cordoba department (Miranda and Mattar 2015). The present study contributes to the knowledge of this tick species and the microorganisms that they carry in Colombia. Unfortunately, neither *Ehrlichia* spp. nor *Anaplasma* spp. positive pools could be successfully sequenced. However, positive samples for *Rickettsia* spp. detected from *D. nitens* were successfully sequenced and corresponded to *R. asembonensis, R. felis* and *Candidatus* R. senegalensis. Although in Colombia several species of *Rickettsia* spp. have been reported. To date *R. asembonensis* and *Candidatus* R. senegalensis, two *R. felis* like organisms (RFLO), have only been reported from fleas' samples (Faccini-Martínez et al., 2016; Betancourt-Ruiz

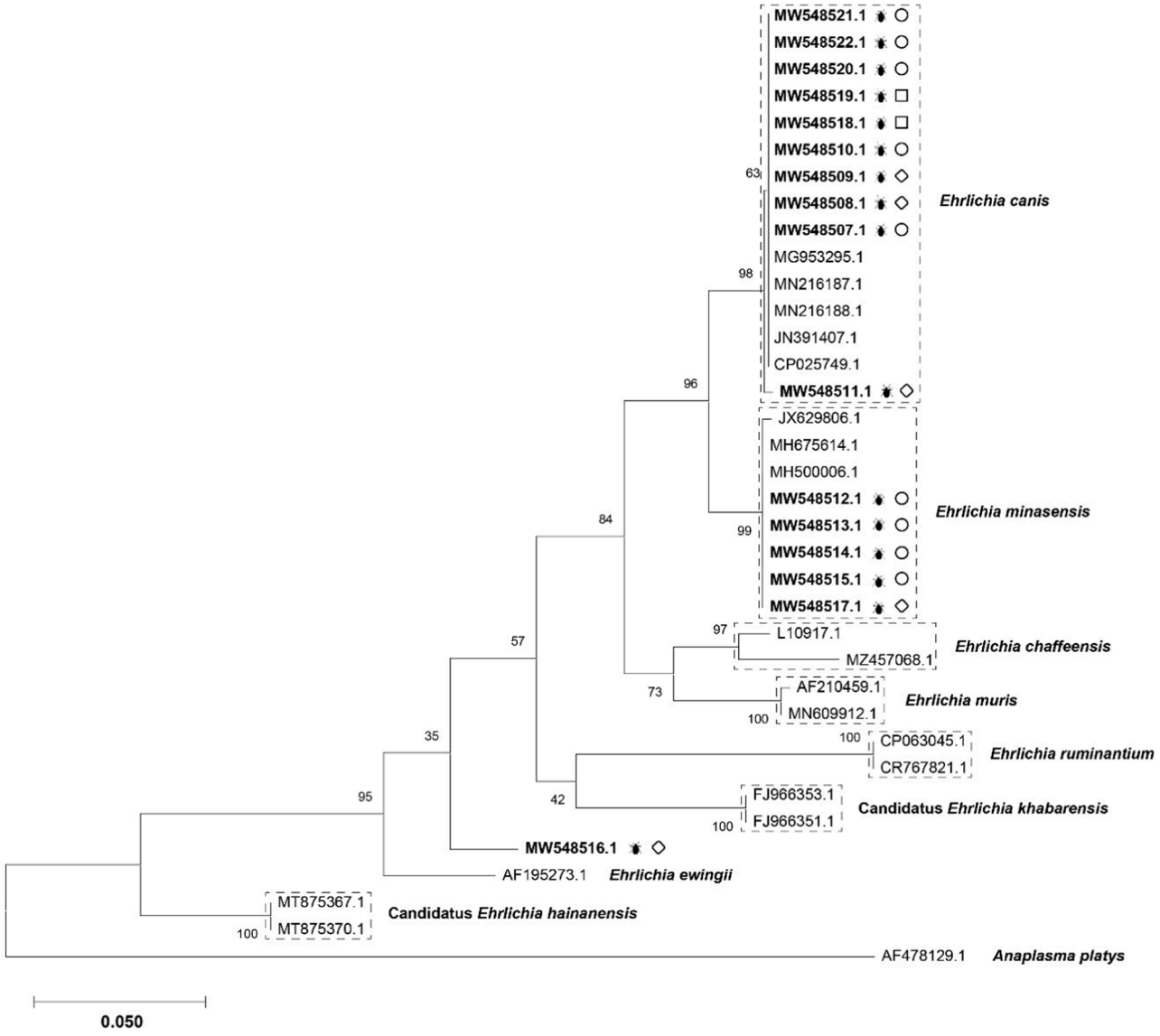

Fig. 5. *Ehrlichia* spp. *groEL* gene sequence-based phylogenetic tree in which sequences retrieved in the present study clustered in two different clades within *Ehrlichia canis* and *Ehrlichia minasensis* reference sequences. The sequences retrieved in this study are in bold, marked with a tick figure and symbols: white circles (retrieved from El Tambo municipality), white diamonds (retrieved from Santander de Quilichao), and white squares (retrieved from La Sierra municipality). GenBank numbers from reference sequences are indicated in all cases.

et al., 2020). However, worldwide both *Rickettsia* species were found infecting some tick species: *R. asembonensis* has been detected from *R. sanguineus* s.l. in Brazil (Dall'Agnol et al., 2017), Malaysia (Low et al., 2017) and Peru (Kocher et al., 2016), and from *A. ovale* and *R. microplus* in Costa Rica (Troyo et al., 2016); and *Candidatus* R. senegalensis has been detected from *R. microplus* ticks in United States (Cleveland et al., 2019). Both species actually are gaining more importance as they might act as emerging pathogens in the future due to its close relationship with *R. felis*, which has been already recognized as a pathogenic *Rickettsia* species (Labruna et al., 2007; Brown and Macaluso, 2016). To date, the importance for human and animal health of *Candidatus* R. senegalensis is not clear yet, however, in Peru a report of an acute febrile illness case possibly due to *R. asembonensis* already been reported (Palacios-Salvatierra et al., 2018) which highlights the importance of RFLO as pathogenic species of new emerging infectious diseases.

5. Conclusions

This is the first report of rickettsial agents from ticks and the detection of *A. patinoi* tick species in Cauca department, Colombia, where data concerning these pathogens is yet limited. Current data supports the relevance of tick-borne diseases, which are often neglected by other vector-borne diseases. The species found in the present study are rarely reported in Colombia and provide information about the ecoepidemiology of rickettsial pathogens and their associated tick vectors in the country. Hence, our study demonstrates the need for further research that can enhance the knowledge acquired for tick-borne diseases in Cauca.

Funding

This study was supported by Colciencias (code 120374455209) from the call No. 744 of 2016 for projects of Science, Technology, and Innovation in Health and the call No. 727 of 2015 for national doctorates.

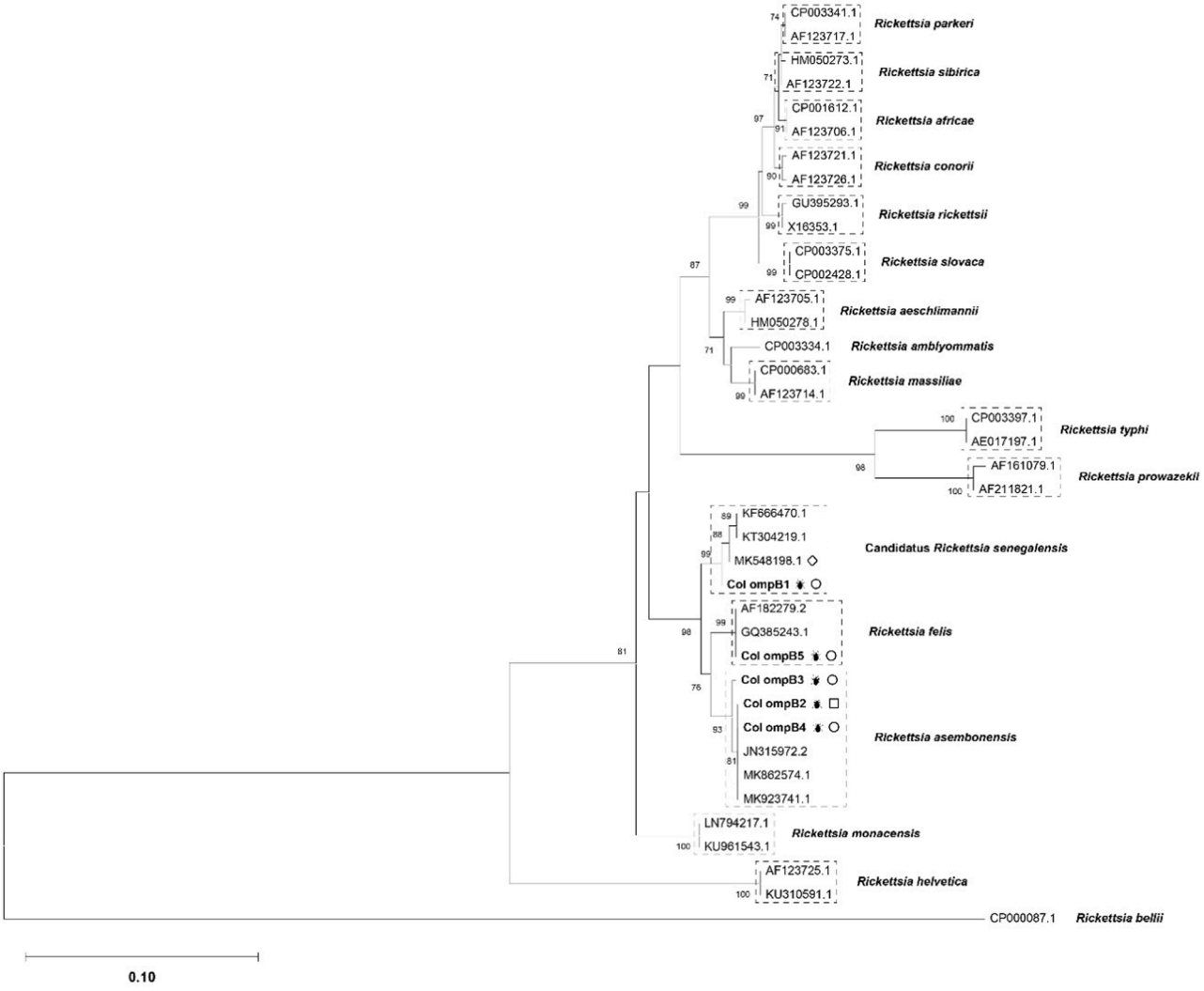

Fig. 6. *Rickettsia* spp. *sca5* gene sequence-based phylogenetic tree in which sequences retrieved in the present study clustered in three different clades within *Candidatus Rickettsia senegalensis, Rickettsia felis* and *Rickettsia asembonensis* reference sequences. The sequences retrieved in this study are in bold, marked with a tick figure and symbols: white circles (retrieved from El Tambo municipality), white diamond (retrieved from Santander de Quilichao), and white squares (retrieved from La Sierra municipality). GenBank numbers from reference sequences are indicated in all cases.

CRediT authorship contribution statement

Heidy-Carolina Martínez Díaz: Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Juliana Gil-Mora:** Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Paola Betancourt-Ruiz:** Investigation, Resources. **Carlos Ramiro Silva-Ramos:** Writing – original draft, Writing – review & editing. **J. Manuel Matiz-González:** Formal analysis, Visualization. **María-Alejandra Villalba-Perez:** Investigation. **María Catalina Ospina-Pinto:** Investigation. **Alejandro Ramirez-Hernández:** Validation, Writing – review & editing. **Luz-Adriana Olaya-M:** Investigation, Resources. **Eliana Bolaños:** Investigation. **Claudia Cuervo:** Formal analysis, Investigation. **Efraín Benavides:** Methodology, Validation. **Marylin Hidalgo:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We acknowledge Pontificia Universidad Javeriana ID: 7277 for physical and human resources provided. We additionally thank Byron Hernandez, innovation manager from the department of biodiversity of the Corporación Colombiana de Investigación AGROSAVIA, who gently provided the *A. marginale* positive control used in this study. We also thank Nelson Fernando Santana Clavijo PhD for the first versions of the phylogenetic trees.

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