UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR E MOLECULAR)

CARRAPATOS DUROS (ACARI: IXODIDAE) ASSOCIADOS A HOSPEDEIROS DOMÉSTICOS EM DIFERENTES REGIÕES DA COLÔMBIA E SUA INTERAÇÃO COM *Rickettsia* spp.

FREDY ARVEY RIVERA PÁEZ

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular).

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TÍTULO DA TESE: CARRAPATOS DUROS (ACARI: IXODIDAE) ASSOCIADOS A HOSPEDEIROS DOMÉSTICOS EM DIFERENTES REGIÕES DA COLÔMBIA E SUA INTERAÇÃO COM Rickettsia spp.

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Rio Claro, 11 de agosto de 2017

Dedicatória

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RESUMO

Os carrapatos de corpo duro (Acari: Ixodidae) contam com 724 espécies descritas, sendo 120 espécies registradas oficialmente na região neotropical. No entanto, na Colômbia o registro e distribuição das espécies da família Ixodidae é escasso e pouco se sabe sobre a interação destes ectoparasitas com as diferentes espécies de *Rickettsia* na região, deixando uma lacuna em estudos e em conhecimento ixodológico e zoonótico no país. Desta forma, o presente trabalho buscou: a) realizar o levantamento e identificação de espécies de carrapatos duros associados a hospedeiros domésticos em regiões da Colômbia, utilizando caracteres morfológicos externos e marcadores moleculares; b) aferir a existência de caracteres morfohistológicos do sistema reprodutor masculino de carrapatos do gênero *Amblyomma* e c) verificar a ocorrência de bactérias *Rickettsia* spp. em carrapatos duros em regiões da Colômbia. Durante o período de agosto de 2014 e maio de 2016, foram coletados 1.745 carrapatos diretamente de hospedeiros domésticos e em ativa alimentação, em 17 municípios de 10 departamentos da Colômbia. Os indivíduos coletados foram identificados com base em sua morfologia externa e preparados para aplicação de técnicas de microscopia, bem como técnicas de análise molecular dos genes ITS2, citocromo oxidase I (COI) e 16S rDNA. Os resultados encontrados permitiram o registro de três gêneros e oito espécies de carrapatos duros nas áreas de estudo, sendo registrada pela primeira vez a espécie *Amblyomma mixtum* para a Colômbia. Outro registro inédito, no presente estudo foi um caso de ginandromorfismo em *A. mixtum*, sendo o primeiro caso para a espécie e o primeiro para carrapatos no país. As análises das populações de *Rhipicephalus sanguineus* sensu lato mostraram que os carrapatos do departamento do Casanare são morfologicamente distintos aos de outras regiões da Colômbia, apesar das análises moleculares evidenciarem tratar-se da mesma linhagem. Embora ainda preliminar, os dados obtidos mostraram também que o sistema reprodutor masculino de carrapatos e suas células germinativas possuem caracteres elegíveis para análises de sistemática de Ixodidae. Confirmou-se também a presença de agentes infecciosos do grupo das rickettsias em espécimes de carrapatos coletados, alertando sobre a importância de medidas governamentais epidemiológicas em alguns dos departamentos colombianos amostrados.

Palavras-chave: *Amblyomma mixtum***,** Ginandromorfo, Marcadores moleculares, Morfohistológicos, Morfológicos, *Rhipicephalus sanguineus* s.l.

ABSTRACT

Hard-bodied ticks (Acari: Ixodidae) comprise 724 known species, with 120 species officially registered for the Neotropical region. However, in Colombia, species registry and distribution for the family Ixodidae is scarce, and little is known of the interaction of these ectoparasites with the different *Rickettsia* species in the region, leading to gaps regarding the ixodological and zoonotic study and knowledge in the country. In this context, this study aimed to: a) establish and identification hard-bodied ticks associated with domestic hosts in various regions of Colombia, using external morphological characters and molecular markers; b) assess the existence of morphohistological characters of the male reproductive system of *Amblyomma* ticks; and c) verify the occurrence of *Rickettsia* spp. in hard-bodied ticks in various regions of Colombia. During August of 2014 and May of 2016, 1745 ticks were collected actively feeding on domestic hosts in 17 municipalities of 10 departments of Colombia. The specimens were identified based on their external morphology and prepared for microscopy, as well as for molecular analyses of genes ITS2, cytochrome oxidase I (COI) and 16S rDNA. The results show the presence of three genera and eight hard-bodied tick species in the municipalities studied, including the first register of the species *Amblyomma mixtum* for Colombia. In addition, we report an unprecedented case of gynandromorphism in *A. mixtum*, which also constitutes the first report for the species and the first for ticks in the country. A population analysis of *Rhipicephalus sanguineus* sensu lato showed that specimens of the department of Casanare are morphologically distinct from those of other regions of Colombia, despite the fact that the molecular analyses showed that these belong to the same lineage. Although preliminary, the data obtained also show that the male tick reproductive system and its germ cells present eligible characters for Ixodidae systematics. We also confirmed the presence of infectious agents of the rickettsial group in the tick specimens collected, which alerts the importance of governmental epidemiological measures in some departments of Colombia.

Keywords: *Amblyomma mixtum*, Gynandromorph, Molecular markers, Morphohistological, Morphological, *Rhipicephalus sanguineus* s.l.

SUMÁRIO

1. Introdução

1.1. Biologia e Sistemática dos Carrapatos

Os carrapatos são artrópodes ectoparasitas de hábito hematófago obrigatório durante todo o seu ciclo ontogenético, na maioria das espécies conhecidas. Pertencem ao Filo Arthropoda, Classe Arachnida, Subclasse Acari, Superordem Parasitiformes, Ordem Ixodida, Superfamília Ixodoidae (KRANTZ; WALTER, 2009). No mundo existem aproximadamente 937 espécies de carrapatos (Acari: Ixodida), representadas em três famílias: Ixodidae, conhecidas como carrapatos duros com 724 espécies; Argasidae, conhecidas como carrapatos moles com 212 espécies; e a Nuttalliellidae com só uma espécie, *Nuttalliella namaqua* Bedford, 1931, restrita à África (NAVA et al., 2017).

Os carrapatos da família Ixodidae são de grande importância para a saúde e a economia mundial, por serem vetores de um grande número de patógenos que afetam o homem, animais domésticos e silvestres, além de diminuir o valor comercial do couro e outros produtos de origem animal, o que provoca consideráveis perdas econômicas (COLWELL; DANTAS-TORRES; OTRANTO, 2011; FLECHTMANN, 1975; SONENSHINE; ROE, 2014).

As espécies de carrapatos que pertencem à família Ixodidae, têm como caraterísticas: capítulo sempre em posição terminal (visível dorsalmente); escudo em todos os estágios ontogenéticos; dimorfismo sexual marcante; fixação profunda do hipostômio na pele do hospedeiro e postura única dos ovos no solo após o completo ingurgitamento (BARROS-BATTESTI et al., 2006; SAMPIERI, 2016).

Na região neotropical, o número registrado para a família Ixodidae é de aproximadamente 120 espécies (NAVA et al., 2017). A sistemática dos carrapatos duros vem sendo discutida há décadas e vários grupos foram revalidados através de novas ferramentas de análises, como a biologia molecular. Algumas espécies como as da subfamília Amblyomminae, foram consideradas polimórficas por alguns pesquisadores (HOOGSTRAAL; AESCHLIMANN, 1982), no entanto, outros estudos como os de Black e Piesman (1994), Burger et al. (2012), Beati et al. (2013) e Nava et al. (2014) demostraram a origem polifilética da subfamília, sua distribuição atual no continente americano e o real nível taxonômico de algumas espécies de grande importância médica e veterinária.

Nas Américas, existem ao menos três complexos de espécies dentro da subfamília Amblyomminae de relevância para a medicina veterinária e para a saúde pública, sendo "complexo *Amblyomma cajennnense*", "complexo *Amblyomma maculatum*" e "complexo *Amblyomma ovale*". O "complexo *A. cajennense*" era então representado por uma só espécie no novo mundo, *Amblyomma cajennense* sensu lato, desde o sul dos Estados Unidos até a Argentina; em estudos recentes de extensa revisão foram identificadas como parte desse complexo seis espécies válidas: *Amblyomma cajennense* sensu stricto (Fabricius, 1787) (restrita à região Amazônica), *Amblyomma mixtum* (Koch, 1844) (desde o Texas até o Equador), *Amblyomma sculptum* (Berlese, 1888) (norte da Argentina, Bolívia, Paraguai e Brasil), *Amblyomma interandinum* (Beati, Nava & Cáceres, 2014) (vale interandino do Peru), *Amblyomma tonelliae* (Nava, Beati & Labruna, 2014) (áreas secas do norte da Argentina, Bolívia e Paraguai), e *Amblyomma patinoi* (Labruna, Nava & Beati, 2014) (cordilheira oriental da Colômbia) (BEATI et al. 2013; NAVA et al. 2014). Na Colômbia, as espécies do complexo estão representadas por *A. patinoi* (NAVA et al. 2014) e *A. mixtum* (RIVERA-PÁEZ et al. 2016).

O "complexo *A. maculatum*" abriga atualmente as espécies: *Amblyomma maculatum* Koch, 1844; *Amblyomma neumanni* Ribaga, 1902; *Amblyomma parvitarsum* Neumann, 1901; *Amblyomma tigrinum* Koch, 1844 e *Amblyomma triste* Koch, 1844 (CAMICAS et al., 1998). *A. maculatum, A. triste,* e *A. tigrinum* foram descritas e validadas por Koch (1844), baseado em espécimes adultos dos Estados Unidos, Uruguai e Brasil, respectivamente. No entanto, Neumann (1899) considera que estas três espécies são uma só, *A. maculatum.* A diferenciação destas três espécies apresenta ainda muita controvérsia, especialmente a distinção entre *A. maculatum* e *A. triste* (ESTRADA-PEÑA et al., 2005; GUGLIELMONE et al., 2013; LADO, 2015; MENDOZA-URIBE; CHAVEZ-CHOROCCO, 2004; MERTINS et al., 2010). Lado (2015) fazendo uso de marcadores moleculares mitocondriais e nucleares sugere que *A. triste* pode ser sinonimizado com *A. maculatum.*

Amblyomma aureolatum Pallas, 1772 e *Amblyomma ovale* Koch, 1844, Aragão e Fonseca (1961), as consideram como parte do "complexo *A. ovale"*, redescreveram o macho e a fêmea de ambas as espécies de carrapatos e discutiram sua sistemática dando suporte ao uso dos nomes *A. aureolatum* e *A. ovale* (GUGLIELMONE et al., 2003a).

Outra espécie de grande importância, cuja sistemática vem sendo discutida, é *Rhipicephalus sanguineus* sensu lato, uma espécie cosmopolita com elevada capacidade reprodutiva e ampla variedade de hospedeiros, a qual parasita várias espécies animais, incluindo o homem (DANTAS-TORRES, 2010), sendo encontrada em regiões tropicais e subtropicais (DANTAS-TORRES, 2010; WALKER, 1994). Segundo vários autores, entre eles Moraes-Filho et al. (2011), Nava et al. (2012) e Dantas-Torres et al. (2013) a posição taxonômica de *R. sanguineus* s.l. é controversa e os estudos morfológicos e moleculares evidenciaram pelo menos dois grandes grupos de *R. sanguineus* s.l., sendo uma linhagem temperada e outra tropical.

Na Colômbia, informações taxonômicas e biogeográficas de carrapatos são escassas, tendo início com Osorno-Mesa (1940), em sua publicação "*Las Garrapatas de la República de Colombia*", na qual foi realizada a compilação dos registros oficiais de carrapatos Ixodidae e Argasidae no país, apresentando chaves taxonômicas para identificação da espécie. Segundo Mattar; López-Valencia (1998), Guglielmone et al. (2003b), Nava et al. (2014), Rivera-Páez et al. (2016), Apanaskevich; Bermudez (2017) a Colômbia presenta aproximadamente 56 espécies (15 Argasidae e 41 Ixodidae). O maior número de artigos na Colômbia versa geralmente nas mesmas espécies como os estudos de Cortes-Vecino et al. (2010) com *Rhipicephalus* (*Boophilus*) *microplus* Canestrini, 1888 os quais confirmaram a presença desta espécie no Altiplano Cundiboyacense, em altitudes superiores aos 2600 m.s.n.m.

Diante dessa exposição, o registro aproximado existente para o país é de 56 espécies, o que se mostra como um baixo número dado as dimensões e diversidade do território colombiano. Existem indícios que ainda existem muitas espécies de carrapatos sem identificação na Colômbia, bem como sua distribuição, o que evidência a necessidade de propostas de projetos de pesquisa com uma abordagem moderna que permitam a obtenção precisa de um mapa sobre a identificação e distribuição de espécies de carrapatos tanto em animais domésticos como silvestres em toda a extensão e diversidade do território colombiano.

Assim sendo, fica claro que existem ainda muitos problemas de natureza taxonômica, assim como um grande número de espécies crípticas que necessitam de estudos utilizando

ferramentas integradoras, como o são os estudos da morfologia externa, a morfologia interna e a utilização de marcadores moleculares nucleares e mitocondriais, num país como a Colômbia, onde estas informações são escassas e fragmentadas.

1.2. Sistema Reprodutor Masculino do gênero *Amblyomma*

Os espermatozóides ou células germinativas masculinas apresentam morfologia e ultraestrutura diversas e muitos autores assumem que essa diversidade é espécie-específica. E seria possível através da investigação de sua forma definir desde o filo até a espécie do macho que as produziu (BIRKHEAD; HOSKEN; PITNICK, 2009; JAMIESON; ROUSE, 1989; SAMPIERI, 2016; SONENSHINE; ROE, 2014).

Os testículos são os órgãos onde as células germinativas masculinas têm origem. Em carrapatos, esses ficam posicionados dorso lateralmente ao corpo do animal e seu tamanho e estágio de desenvolvimento dependem do estado nutricional do macho. Os testículos são pequenos nos animais jovens em jejum, contrariamente ao que se observa nos animais em alimentação e naqueles que ou estão ou estiveram em contato com as fêmeas, que é quando as gônadas aumentam de tamanho (SAMPIERI, 2016).

As células germinativas encontram-se envoltas por um epitélio pavimentoso simples, que formam os espermatocistos até estágios avançados de desenvolvimento. As células germinativas ocorrem basicamente em duas etapas: a) a espermatogênese, divisões mitóticas e meióticas das espermatogônias e dos espermatócitos, originando as espermátides e b) a espermiogênese, desenvolvimento final (diferenciação) das espermátides, dando origem aos espermatozóides (SAMPIERI, 2016). Da mesma forma, as fases que compreendem a espermiogênese são dependentes de outros processos fisiológicos, como o avanço do processo alimentar do adulto e das secreções ejaculatórias produzidas pelo macho, o que é estimulado via contato mecânico com a fêmea e é finalizado quando o espermatóforo despeja o conteúdo espermático na abertura genital feminina (útero) (FELDMAN-MUHSAM; BORUT, 1978, 1983; REGER, 1963; SAMPIERI 2016; SONENSHINE; ROE, 2014).

Neste contexto, a espermiotaxonomia mostra-se como uma boa ferramenta na solução de controvérsias de ordem sistemática, o que pode auxiliar nas análises moleculares e de morfologia externa em carrapatos de maneira integrada, proporcionando resultados mais

robustos. Para tanto, faz-se necessário estudos de novas espécies, como as implicadas na transmissão de rickettsias inseridas em complexos (*A. maculatum, A ovale* e *A. cajennense*), de modo a gerar um pool de informações relevantes para análises comparadas, compilando os resultados até agora obtidos nas descrições realizadas por Sampieri et al. (2014; 2016a; 2016b) sobre a anatomia e a morfologia do sistema reprodutor masculino destes ectoparasitas, principalmente os do gênero *Amblyomma* de ocorrência no Brasil e Colômbia.

1.3. Gênero *Rickettsi*a

As rickettsioses, ou doenças provocadas por *Rickettsia* spp., compreendem quatro grupos, os dois principais grupos são aquelas conhecidas como febre maculosa ou febre manchada (exantemáticas ou petequiais) e grupo tifo (epidêmico e murino). Apresentam síndrome febril aguda que pode chegar a ser fatal, caso não seja tratada com antibiótico adequado (HIDALGO, 2010; PAROLA; PADDOCK; RAOULT, 2005; WALKER, 1982). As rickettsioses são de distribuição global e transmitidas por artrópodes vetores de característica emergente ou reemergente em algumas regiões. Howard T. Ricketts descobriu, entre 1906 e 1909, que o agente etiologico da febre manchada das montanhas rochosas (*RMSF*), *Rickettsia rickettsii*, era transmitido por carrapatos (OTEO et al., 2014).

O gênero *Rickettsia* abriga as bactérias do filo Proteobacteria, subclasse a-1, ordem Rickettsiales, as quais são pequenos cocobacilos (0,3 a 1 µm) com genomas entre 1,1 e 1,6 Mb, Gram-negativas e intracelulares obrigatórias que sobrevivem pouco tempo fora do hospedeiro (OTEO et al., 2014). As rickettsias são bactérias intracelulares estritas, transmitidas por artrópodes que infectam principalmente as células endoteliais (WALKER, 1982). A maioria das espécies do gênero *Rickettsia* são classificadas no grupo tifo (GT) que incluem *Rickettsia typhi* e *Rickettsia prowazekii* sendo seus vetores pulgas e piolhos, respectivamente; e o grupo das febres manchadas (GFM) que incluem mais de 20 espécies, tendo como principais vetores os carrapatos duros (Ixodidae) (OTEO et al., 2014). Os principais sintomas das rickettsioses são: febre, dor de cabeça, mal-estar geral, algumas vezes sintomas gastrintestinais e erupção cutânea depois de 2-7 dias do início da infecção, a qual se apresenta em aproximadamente 50% dos pacientes (HIDALGO, 2010; WALKER, 1982).

Os reservatórios das rickettsias são principalmente roedores silvestres, além dos próprios carrapatos. A contaminação do carrapato pelas rickettsias pode se dar por: a) ingestão da mesma durante o repasto sanguíneo em hospedeiro infectado e riquetsêmico e b) aquisição por transmissão transovariana ou transestadial (SOARES et al., 2012). As rickettisas disseminam-se nos carrapatos ixodideos, multiplicando-se no citoplasma de células intestinais, ovarianas, das glândulas salivares e dos túbulos de Malpighi, sendo encontradas também na própria hemolinfa. A presença generalizada da bactéria nos órgãos do carrapato explicaria a transmissão transestadial e a infecção dos ovários a transmissão transovariana. Nos ixodideos em diapausa, as rickettsias entram em estágio de "dormência celular" e, após o repasto sanguíneo, quando há um aumento no metabolismo do carrapato, as mesmas tornam-se ativas e se multiplicam, culminando no sucesso da transmissão (HAYES; BURGDORFER, 1982).

As técnicas de biologia molecular e o desenvolvimento de cultura de indivíduos do gênero *Rickettsia* em tubos fechados (*shell-vial*) vêm produzindo nas últimas décadas um aumento na descrição de novas espécies e de candidatos a novas espécies (OTEO et al., 2014). Algumas espécies, previamente consideradas não patogênicas, vêm demostrando sua patogenicidade em humanos, como registrado para as Américas com *Rickettsia parkeri*, um membro já conhecido do GFM, descrito pela primeira vez em 1939 (OTEO et al., 2014). Neste contexto, fica demostrado que qualquer espécie nova de *Rickettsia* descrita em carrapatos deve ser considerada potencialmente patogênica para os humanos e a atenção deve estar concentrada nas espécies e candidatas a espécies que estão ligadas a vetores cosmopolitas antrópicos (LABRUNA et al., 2011; OTEO et al., 2014).

Na América Latina e no Caribe, as espécies de carrapatos, implicadas como vetores comprovados ou potenciais de rickettsias, estão incluídas dentro dos gêneros *Amblyomma*, *Rhipicephalus, Haemaphysalis* e *Dermacentor*, todos pertencentes a família Ixodidae na qual as espécies *A. cajennense* s.l., *A. triste, A. ovale, A. aureolatum* e *R. sanguineus* s.l. são as principais envolvidas (OTEO et al., 2014).

Na Colômbia, os estudos com rickettsias iniciaram no Município de Villeta (Cundinamarca), região considerada na atualidade como endêmica para febre manchada das montanhas rochosas (*RMSF*) ou febre maculosa brasileira (FMB) ou "febre de tobia" para a

Colômbia. Na população de Tobia, município de Cundinamarca, Colômbia, a "febre de tobia" foi o primeiro registro de rickettsioses na Colômbia, causada por *R. rickettsii* e afetou cerca de 20% da população (PATINO; AFANADOR; PAUL, 1937). Depois de um prolongado silêncio epidemiológico (por carência de estudos) entre 2003 e 2004, Hidalgo et al. (2007a) confirmaram *R. rickettsii* como agente causador da morte de dois pacientes locais.

Mais recentemente, alguns surtos de rickettsioses do GFM ocorreram nos municípios de Necocli e Turbo, no departamento de Antioquia, em 2006 e 2008, respectivamente, e um terceiro no município dos Cordobas, no departamento de Córdoba, em 2007 (ACOSTA et al., 2006; HIDALGO et al., 2007b; PACHECO et al., 2008). No departamento de Cundinamarca, Faccini-Martinez et al. (2015), conseguiram isolar *R. rickettsii* no carrapato *A. patinoi* e detectaram molecularmente *Rickettsia amblyommii* (FACCINI-MARTINEZ et al., 2016). Londoño et al. (2014) encontraram espécimes de *A. ovale* infectados por *Rickettsia* sp. strain Atlantic rainforest, uma cepa patogênica para humanos, em cães e roedores em municípios do Departamento de Antioquia e Córdoba.

Em adição, estudos sorológicos em humanos e animais, realizados na Orinoquía da Colômbia (departamentos de Arauca, Casanare, Guaviare, Meta e Vichada), mostraram uma grande soroprevalência contra "*RMSF*" (MIRANDA et al., 2011; RIVEROS-PINILLA et al., 2015). Gómez-Quintero et al. (2017) registraram um provável caso de infecção rickettsial por um membro do GFM numa paciente de 50 anos, procedente da mesma região da Orinoquía Colombiana, onde Rivera et al. (2016) relataram a presença de *A. mixtum*, vetor de *R. rickettsi*.

2. Objetivos

2.1. Objetivo Geral

Diante das informações expostas, o presente trabalho teve como objetivo contribuir para o conhecimento de carrapatos duros (Acari: Ixodidae) associados a hospedeiros domésticos em diferentes regiões da Colômbia, fazendo uso de ferramentas morfológicas, assim como de marcadores moleculares ITS2, COI e 16S, separando e determinando as espécies, assim como fornecendo dados de sua variabilidade genética e da presença de espécies do gênero *Rickettsia*.

2.2. Objetivos Específicos

A) realizar o levantamento das espécies de carrapatos duros associados a hospedeiros domésticos em diferentes localidades da Colômbia.

B) validar por meio da morfologia externa as espécies de carrapatos duros presentes no território colombiano;

C) determinar por meio do uso de marcadores moleculares a variabilidade genética das espécies de carrapatos duros destas mesmas regiões;

D) estudar através da aplicação de técnicas histológicas no sistema reprodutor masculino, as espécies *A. ovale* e *A. maculatum* coletadas na Colômbia, e comparar seu morfologia com dados do gênero *Amblyomma* disponíveis na literatura;

E) realizar pesquisa da infeção por *Rickettsia* spp. nos carrapatos duros do território colombiano, determinando as espécies de rickettsias envolvidas.

3. Materiais e Métodos

O presente trabalho foi realizado em 17 cidades pertencentes a 10 departamentos da Colômbia durante o período de Agosto de 2014 e Maio de 2016. Foram coletados carrapatos duros diretamente de hospedeiros domésticos em cada um dos pontos de amostragem. O estudo contou com a permissão de todos os proprietários responsáveis pelos locais visitados (fazendas), autorizando a visitação técnica nas propriedades privadas, quando era o caso, além de amparo através da Permissão de Pesquisa concedida por órgão competente do país (*Resolución No. 1166 ANLA "Autoridad Nacional De Licencias Ambientales"* Colômbia).

3.1. Obtenção dos carrapatos

Os carrapatos foram coletados diretamente de hospedeiros domésticos em e em ativa alimentação em 40 pontos de amostragem de 17 cidades pertencentes a 10 departamentos da Colômbia (Tabela 1). Os carrapatos foram coletados em fase parasitária fixados aos animais domésticos e eventualmente em humanos, ou ativamente em fase de vida livre (no meio ambiente). Os carrapatos foram coletados manualmente e com auxílio de pinças apropriadas (oftálmicas).

Depois de coletados, os carrapatos foram acondicionados e preservados em criotubos com álcool absoluto, devidamente identificados (data, coordenadas geográficas e hospedeiro) e levados para o "*Laboratório de Biologia Molecular, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad de Caldas*" Colômbia, onde foram realizados as análises moleculares, uma vez que aquelas morfológicos foram totalmente realizadas no Brasil.

Tabela 1. Localidades na Colômbia onde os carrapatos foram coletados

Pontos de amostragem	Departamento	Município	Coordenadas geográficas
1-11	Amazonas	Leticia	4°6'31"S, 69°58'5"W; 4°8'42"S, 69°56'28"W; 4°6'18"S, 69°56'23"W; 4°4'24"S, 70°0'10W; 4°4'17"S, 69°59'57"W; 4°4'38"S, 70°0'6"W; 4°7'27"S, 69°56'59"W; 4°8'39"S,69°56'20"W; 4°9'15"S, 69°56'1"W; 4°9'59"S, 69°57'41"W; 4°6'16"S, 69°58'24"W
12	Antioquia	Medellín	14'41"N, 75°34'29"W
13-18	Arauca	Arauca	06°55´43"N, 70°27'36"W; 06º02'N, 69º25'W; 06°56'24"N, 70°32'0"W; 07°1'48"N,70°43'39"W; 07°3'55"N, 70°44'2"W; 07°4'34"N, 70°42'37"W
19	Arauca	Fortul	06°47'39"N, 71°46'15"W
20,21	Arauca	Saravena	06°57'26"N, 71°52'31"W; 06°57'21"N, 71°52'21"W
22	Bolívar	San Jacinto	9°84'53"N, 75°11'88"W
23	Caldas	Dorada	5°27´14"N, 74°39'53"W
24	Caldas	Neira	5°11'50"N, 75°38'05"W
25	Caldas	Norcasia	5°34'27"N, 74°53'20"W
26-28	Casanare	Nunchia	5°21'1"N, 72°4'53"W; 5°21'13"N, 72°5'50"W; 5°21'40"N, 72°6'7"W
29-31	Casanare	Yopal	5°19' 27"N, 72°24'31"W; 5°25'53"N,72°14'36"W; 5°25' 26"N, 72°14' 17"W
32	Cundinamarca	Puerto Salgar	5°27'55"N, 74°39'11"W
33	Meta	S. J. de Arama	3°20'47"N, 73°53'21"W
34-36	Tolima	Ibagué	04°24'6"N, 75°4'21"W; 04°23'50"N, 75°8'12"W; 04°49'30"N, 75°28'83"W
37,38	Tolima	Saldaña	3°55'36"N, 74°58'33"W; 3°93'47"N, 75°02'W
39	V. del Cauca	Cali	3°24'21"N, 76°32'47"W
40	V. del Cauca	Restrepo	3°51'12"N, 76°30'43"W

3.2. Análises morfológicas

As análises morfológicas para identificação do táxon foram realizadas no Laboratório de Microscopia Eletrônica, do Departamento de Biologia, do Instituto de Biociências da UNESP de Rio Claro (SP, Brasil), fazendo uso das técnicas de microscopia eletrônica de varredura (MEV) em MEV Hitachi TM3000 e de estereomicroscopia em estereomicroscópio Leica M205C. A confirmação do táxon dos espécimes foi realizada no Laboratório de Doenças Parasitárias do Departamento de Medicina Veterinária Preventiva e Saúde Animal – VPS, da Faculdade de Medicina Veterinária e Zootecnia da USP de São Paulo (SP).

Os carrapatos foram separados primeiramente de acordo com o estágio de desenvolvimento, sendo que os adultos foram identificados ao nível de espécie sob lupa estereoscópica, fazendo uso de chaves dicotômicas de Aragão e Fonseca (1961), Jones et al. (1972), Estrada Peña et al. (2005), Barros-Battesti et al. (2006), Martins et al. (2010) e Nava et al. (2014) (Tabela 2).

Para a realização da MEV, pelo menos um macho e uma fêmea de cada espécie e localidade foram submetidos a banhos de 5 minutos cada, em série crescente de acetona (50%, 75%, 90%, 95% e 100%), repetindo-se duas vezes a passagem na última concentração. Após a dessecação em *Critical Point Drying*, os exemplares foram colados com fita adesiva dupla face em suportes de alumínio, foram metalizados com ouro em "*Sputtering*", examinados e fotografados em microscópio eletrônico de varredura.

Tabela 2. Carrapatos coletados de diferentes hospedeiros durante 2014-2016 nas diferentes localidades da Colômbia.

Espécie de carrapato	**Hospedeiro**	**Localidade***
Amblyomma dissimile	*Bos taurus*	35
A. dissimile	vegetation	29
Amblyomma maculatum	*Canis lupus familiaris*	25,35,37
Amblyomma mixtum	*Bos taurus*	14,17,24,27,33
A. mixtum	*Equus asinus*	31
A. mixtum	*Equus caballus*	15-18,26,27,29,31
A. mixtum	vegetation	28,29
Amblyomma ovale	*C. lupus familiaris*	35,37
Amblyomma varium	*C. lupus familiaris*	35
Dermacentor nitens	*B. taurus*	8,18
D. nitens	*E. asinus*	22,31,35
D. nitens	*E. caballus*	2,13,15-18,22,26, 29-31,35
D. nitens	*Sus scrofa*	18
Rhipicephalus microplus	*B. taurus*	1,3-9,11,14,19-21,33-36,40
R. microplus	*C. lupus familiaris*	21
R. microplus	*E. asinus*	22
Rhipicephalus sanguineus	*B. taurus*	1,35
R. sanguineus	*C. lupus familiaris*	10,12,13,18,20,23,25,32,35-40
R. sanguineus	*E. caballus*	18,29

*** O número da Localidade está indicado na Tabela 1.**

3.3. Análise Molecular

3.3.1. Extração do DNA para as análises de carrapatos e *Rickettsias*

Após a identificação morfológica, machos e fêmeas de cada espécie de carrapato e de cada localidade, foram processados individualmente para as análises moleculares com o objetivo de se determinar a espécie de carrapato e identificar a possível presença e qual espécie de *Rickettsia* spp (Tabela 3). A extração do DNA foi feita com o kit DNeasy Blood and Tissue (Qiagen), segundo protocolo sugerido pelo fabricante. A quantidade e qualidade do DNA foram determinadas mediante espectrofotometria, utilizando para isso o espectrofotômetro Nanovue Plus.

3.3.2. Amplificação por PCR do DNA de carrapatos

O DNA extraído de cada carrapato foi submetido à reação em cadeia de polimerase (PCR), amplificando um fragmento do segundo espaço transcrito interno (ITS2) do DNA ribossômico nuclear, presente em todas as espécies de carrapatos. Para a PCR, foi utilizado um par de "*primers*" ITS2 (F) 5'-CCATCGATGTGAAYTGCAGGACA-3' (ZAHLER et al., 1995) e MCLN (R) 5'-GTGAATTCTATGCTTAAATTCAGGGGGT-3' (MCLAIN et al., 1995), os quais correspondem a sequências da região 5.8S e 28S, respectivamente, portanto amplificando um fragmento de DNA contendo a sequência completa do ITS2 do rDNA, possuindo em torno de 1100 pb em carrapatos do gênero *Amblyomma*, com algumas pequenas variações intra e interespecíficas (MARRELLI et al., 2007). Além disso, foram amplificados 2 genes mitocondriais: um DNA *barcoding*, da região 5' do gene mitocondrial Citocromo C oxidase I (COI), utilizando os *primers* standard para invertebrados LCO1490 (F) 5'-GGTCAACAAATCATAAAGATATTGG-3' e HCO2198 (R) 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (FOLMER et al., 1994) que amplificam um fragmento aproximado de 700pb para carrapatos; assim como um fragmento aproximado de 460pb do Gene mtDNA 16S rDNA, utilizando os *primers* F 5'–CTGCTCAATGATTTTTTAAATTGCTGTGG–3' and R 5'–CCGGTCTGAACTCAGATCAAGT-3' (NORRIS et al., 1996).

A reação de amplificação foi feita em microtubos de 200 µL e volume final de 50 µL contendo 28.4 µL de água ultrapura, 10 µL de buffer 5X, 3 µL de $MgCl_2$ (25mM), 4 µL da

mescla (10 mM) de dNTP's, 0.6 µL de cada primer (25 µM), 2 unidades de GoTaq Flexi DNA polimerase (Promega), 3 µL de DNA do carrapato (aproximadamente 300 ng do DNA).

A amplificação dos genes foi realizada em termociclador (Techne -*TC-Plus*), considerando-se as seguintes condições: **a)** para o gene COI mtDNA: desnaturação a 94ºC por 5 min, seguido por 5 ciclos de 94°C por 5 min, 46°C por 1 min 30s e 72°C por 1 min e 30 s, seguido de 35 ciclos de 94°C por 1 min, 53°C por 1 min e 72°C por 1 min, concluindo a reação com uma extensão final a 72°C por 5 min; **b)** para o gene ITS2 foi feita com desnaturação a 95ºC por 5 min, seguido por 36 ciclos de 95°C por 45s, 57°C por 1 min, e 72°C por 1 min e 30 s, concluindo a reação com uma extensão final a 72°C por 5 min; **c)** para o gene 16S a desnaturação deu-se a 94ºC por 2 min, seguido por 7 ciclos de 94°C por 30 s, 45°C por 30s e 72°C por 45 s, seguido de 28 ciclos de 94°C por 30 s, 48°C e 72°C por 45 s, concluindo a reação com uma extensão final a 72°C por 7 min.

3.3.3. Detecção e determinação de *Rickettsia* spp. em carrapatos

Para a detecção e identificação molecular da *Rickettsia* spp. foram utilizados 204 espécimes de carrapatos, pertencentes a 7 espécies da família Ixodiade (Tabela 3).

Tabela 3. Espécies e número de espécimes de carrapatos duros analisados

Espécie de carrapato	Número de espécimene/Estágio	Município
Amblyomma dissimile	1 Ninfa	Yopal.
Amblyomma maculatum	19 Adultos	Ibagué; Saldaña; Norcasia.
Amblyomma mixtum	51 Adultos/2 Ninfas	Arauca; Neira; Nunchía; Yopal; S. J. de Arama
Amblyomma ovale	10 Adultos	Ibagué; Saldaña.
Dermacentor nitens	33 Adultos	Leticia; Arauca; San Jacinto; Nunchía; Yopal; Ibagué.
Rhipicephalus microplus	34 Adultos/2 Ninfas	Leticia; Arauca; Fortul; Saravena; San Jacinto; S. J. de Arama; Ibagué; Restrepo.
Rhipicephalus sanguineus s.l.	49 Adultos/3 Ninfas	Leticia; Medellín; Arauca; Saravena; Dorada; Yopal; Puerto Salgar; Ibagué; Saldaña; Cali; Restrepo.
	Total: 204	

Inicialmente foi amplificado o gene Citrato Sintetasa ***"gltA"*** com os iniciadores CS-78 e CS-323 (LABRUNA et al., 2004) utilizados para a detecção de todas as espécies de

Rickettsia com tamanho aproximado de 400pb. Sendo o carrapato positivo para *"gltA"*, realizava-se então a PCR para o gene de proteínas maiores de superfície *"ompA"* com os iniciadores Rr.190-70 e Rr.190-602 (REGNERY; SPRUILL; PLIKAYTIS, 1991; ROUX; FOURNIER; RAOULT, 1996), específicos para o grupo da febre manchada (GFM), possuindo um fragmento aproximado de 530pb. No entanto, para evitar a ocorrência de falsos negativos por inibição ou sensibilidade diferencial dos *primers*, a totalidade das mostras foi amplificada para os dois genes, tanto o gene "*gltA*" e "*ompA*", utilizando diluições seriadas, além de quantidades maiores do DNA (até 3 vezes mais). Quando a PCR para *"ompA"* foi positiva, seu produto foi sequenciado para estabelecer a espécie de *Rickettsia*. Quando a PCR para *"ompA"* foi negativa indicando que a bactéria não pertence ao grupo das febres manchadas, então se sequenciou o produto do *"gltA"*, para determinar a espécie.

Faz-se necessário esclarecer que não se sequenciou primeiro os produtos do PCR para o gene "*gltA*" antes de o fazer para "*ompA*", porque as sequências de "*gltA*" entre as espécies do grupo das febres manchadas (ex. *R. rickettsii*, *R. parkeri*, *R. conorii*) são praticamente idênticas entre si o que não seria de utilidade para determinar a espécie dentro do grupo de rickettsias das febres manchadas. O gene "*gltA*" é altamente polimórfico entre as espécies que não pertencem ao grupo das febres manchadas, por isso seu sequenciamento funciona muito bem para determinar e separar estas espécies (OTEO e PORTILLO, 2012; PAROLA et al., 2013). No entanto, no presente trabalho, com o intuito de trazer a luz conhecimento mais detalhado das espécies de rickettsias presentes na Colômbia, a totalidade de produtos de PCR positivas foi sequenciado. Em cada grupo de reações, se utilizou controle negativo (água) e positivo (DNA de *Rickettsia parkeri* strain NOD).

A reação de amplificação deu-se em microtubos de 200 µL e volume final de 50 µL contendo 26.8 µL de água ultrapura, 10 µL de buffer 5X, 3 µL de $MgCl_2$ (25mM), 4 µL da mescla (10 mM) de dNTP's, 0.4 µL de cada primer (25 µM), 2 unidades de GoTaq Flexi DNA polimerase (Promega), 5 µL de DNA do carrapato (aproximadamente 500 ng do DNA). A amplificação dos genes *gltA* e *ompA* foi realizada em termociclador (Techne -*TC-Plus*), considerando-se as seguintes condições: **a)** para o gene *gltA* a desnaturação ocorreu a 95ºC por 3 min, seguido por 40 ciclos de 95°C por 15s, 50°C por 30 s, e 72°C por 30 s, concluindo a reação com uma extensão final a 72°C por 7 min; **b)** gene *ompA* a desnaturação ocorreu a

95ºC por 5 min, seguido por 35 ciclos de 95°C por 40s, 50°C por 30 s, e 72°C por 45s, concluindo a reação com uma extensão final a 72°C por 10 min.

3.3.4. Eletroforeses e visualização dos produtos do PCR

Todos os produtos da PCR foram submetidos à eletroforese horizontal em gel de agarose 1% com tampão de corrida TBE 1X pH 8.0 a 110v/50mA. O gel foi posteriormente corado com SYBR Safe® dye e visualizado e fotodocumentado em GelDoc-It®2 310 Imager (UVP). Os produtos da PCR foram purificados utilizando-se o kit Wizard® SV Gel e PCR Clean-Up System (Promega), segundo as instruções do fabricante. Para se obter as sequências de DNA dos carrapatos o material genético foi enviado a Macrogen Advancing Through Genomics – Coréia do Sul e para as sequências de DNA das rickettsias as amostras foram enviadas ao Laboratorio Secuenciación de ADN da *Universidad de los Andes* – Bogotá D.C., Colômbia. As sequências obtidas foram depositadas no GenBank.

3.3.5. Análises das sequências de DNA de carrapatos e rickettsias

Os fragmentos das sequências do DNA foram testados e editados utilizando-se os programas Geneious Trial v8.14 (DRUMMOND et al., 2009) e Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). Em seguida, as sequências foram submetidas ao MegaBlast contra as bases de dados e depositadas no GenBank e Barcode of Life Data Systems (BOLD). As sequências de cada gene foram alinhadas usando ClustalW (THOMPSON et al., 1997), incluído no programa MEGA versão 7 (TAMURA et al., 2013).

As Divergências nucleotídicas intraespecíficas foram estimadas com o programa MEGA, utilizando o modelo de distância Kimura de parâmetros (K2P; KIMURA, 1980). A confirmação das espécies realizou-se mediante a estimativa de similaridade, utilizando o programa MEGA através dos métodos de distância genética Neighbor-Joining (NJ) e Maximum Likelihood (ML), com o modelo K2P e 1000 réplicas de *bootstrap*. Nas análises foram inclusos exemplares do Brasil (*A. cajennense* s.s. - Rondônia), *A. sculptum* (São Paulo), *R. sanguineus* s.l. (Minas Gerais e São Paulo), *A. triste* e *A. aureolatum* (São Paulo). As sequências do gene (*gltA*) e (*ompA*) foram submetidas a "*Basic Local Alignment Search Tool* (BLAST; Altschul et al., 1990)" para determinar "*closest similarities*" com outras

espécies de *Rickettsia*. Na elaboração dos mapas de distribuição das espécies utilizou-se o sistema Geographic Coordinate System (WGS 1984, Datum: DWGS 1984) e o ESRI® ArcMap.

3.4. Técnicas Morfohistológicas

Inicialmente foram utilizados os caráteres morfológicos do sistema reprodutor masculino de carrapatos descritos por Sampieri et al. (2014, 2016a, 2016b), em espécies de ocorrência no território brasileiro, sendo três representativas do gênero *Amblyomma*, *A. aureolatum*, *A. triste* e *A. sculptum*, e duas como grupo externo, sendo *R. sanguineus* s.l. (linhagem tropical) e *Ornithodoros rostratus*. Somado a isso, foram estudadas, através da aplicação de técnicas histológicas no sistema reprodutor masculino, as espécies *A. ovale* e *A. maculatum*, coletadas na Colômbia.

Desta forma, os caráteres elegíveis com variações relevantes basearam-se na observação da morfologia e ultra morfologia do sistema reprodutor masculino destas espécies e codificados conforme o estado do caráter, como: apomórfico = 1 e plesiomórfico = 0. Posteriormente, foi elaborada uma matriz de caracteres contendo os táxons, os caracteres e o código de estado de cada caractere e espécie (0 o 1).

3.5. Histologia Sistema Reprodutor Masculino

Para a realização da histologia do sistema reprodutor masculino, os carrapatos machos foram dissecados em placas de Petri contendo solução fisiológica tamponada com PBS (NaCl 7.5 g/L, Na2HPO4 2.38 g/L e KH2PO4 2.72 g/L) sob estereomicroscópio, quando tiveram seu sistema reprodutor removido. As amostras coletadas foram fixadas em glutaraldeído 2.5% durante 48 horas. Após a fixação as amostras foram desidratadas em quatro banhos de 15 minutos cada, em série crescente de etanol a 70%, 80%, 90%, 95%. Logo após, foram embebidas e incluídas em historesina Leica no interior de moldes plásticos. Os blocos polimerizados foram colados em suportes de madeira e seccionados com 3 μm em micrótomo Leica, cujas secções foram posteriormente recolhidas em lâminas de vidro e coradas pela hematoxilina e eosina (HE) (JUNQUEIRA; JUNQUEIRA, 1983).

4. Resultados

Os resultados obtidos no presente trabalho estão aqui apresentados sob a forma de cinco capítulos (artigos científicos nos status de publicado, aceito para publicação, submetido ou em preparação para ser submetido em periódicos científicos internacionais especializados na área objeto do estudo).

CAPÍTULO 1

Status: Submetido

RIVERA-PÁEZ, F. A., Labruna, M. B., Martins, T. F., Pérez, J. E., Castaño-Villa, G. J., Ossa-López, P. A., Gil, C. A., O., Sampieri, B. R., Aricapa-Giraldo, H. J., Camargo-Mathias, M. I. Contributions to the knowledge of hard-bodied ticks (Acari: Ixodidae) associated to domestic hosts in various regions of Colombia.

Periódico: *Ticks and Tick-borne Diseases*.

CAPÍTULO 2

Status: Publicado

RIVERA-PÁEZ, F. A., Labruna, M. B., Martins, T. F., Sampieri, B. R., Camargo-Mathias, M. I. *Amblyomma mixtum* Koch, 1844 (Acari: Ixodidae): First record confirmation in Colombia using morphological and molecular analyses.

Periódico: *Ticks and Tick-borne Diseases* 7, 842–848, 2016.

CAPÍTULO 3

Status: Aceito para publicação

RIVERA-PÁEZ, F.A., Labruna, M.B., Martins, T.F., Sampieri, B.R., Camargo-Mathias, M.I. A case of gynandromorphism in *Amblyomma mixtum* (Acari, Ixodidae).
Periódico: *Revista Colombiana de Entomología*, ISSN 0120-0488, 2017.

CAPÍTULO 4

Status: Publicado

RIVERA-PÁEZ, F.A., Sampieri, B.R., Labruna, M.B., Martins, T.F., Matos, R.S., Camargo-Mathias, M.I. Comparative analysis of germ cells and DNA of the genus *Amblyomma:* adding new data on *Amblyomma maculatum* and *Amblyomma ovale* species (Acari: Ixodidae).

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CAPÍTULO 5

Status: Em fase de preparação para ser submetido em periódico científico

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4.1. CAPÍTULO 1

Contributions to the knowledge of hard-bodied ticks (Acari: Ixodidae) associated to domestic hosts in various regions of Colombia.

Contributions to the knowledge of hard ticks (Acari: Ixodidae) associated to domestic animals in various regions of Colombia

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Abstract

Hard ticks are represented by 724 species world widely. There have been 120 species documented in the Neotropics, however, in Colombia, the registry and distribution of species of the family Ixodidae have been scarce, making it necessary to carry out studies that build on the current knowledge and state of the family. During August of 2014 and May of 2016, 1,745 ticks were collected directly from domestic hosts, in 17 municipalities from 10 Departments of Colombia. Morphological and molecular determinations allowed us to register eight species of hard-bodies ticks. The presence of *Amblyomma mixtum*, was

confirmed in the Meta and Caldas Departments, indicating that the distribution of *A. mixtum* in Colombia is much broader than currently known. Morphological analyses of *Rhipicephalus sanguineus* sensu lato showed marked differences between individuals from Casanare compared to other populations of the Colombian territory. Nonetheless, molecular assessments confirmed that all of the samples belonged to the Northern Tropical lineage. *Dermacentor nitens,* note some polymorphism in their 16S rRNA or COI sequences, which in both cases resulted in a clade that separated the sequences of Leticia (Amazonas Department) from the other Departments. *Amblyoma maculatum* from Colombia mixed within a clade composed by *A. maculatum* from the United States and Colombia, and *Amblyomma triste* from Brazil and Argentina. Phylogenetic analyses of specimens from poorly explored sites such as Colombia are crucial for our growing knowledge of tick systematics in the Neotropical region, where ticks associated with domestic animals are vectors of a number of pathogens to either animals or humans.

Keywords: *Amblyomma mixtum*, Neotropics, Pathogens, *Rhipicephalus sanguineus*, vectors.

Introduction

The World's tick fauna is currently composed by 937 species allocated into three families: Ixodidae (724 species), Argasidae (212 species) and Nuttalliellidae (1 species). From these, 220 species (23.5%) are known to occur in the Neotropical region (Barros-Battesti et al., 2006; Labruna et al., 2016; Apanaskevich and Bermúdez, 2017; Muñoz-Leal et al., 2017; Chitimia-Dobler et al., 2017; Nava et al., 2017). Broad-scale studies about ticks occurring in Colombia have been scarce, with emphasis for the studies of Osorno-Mesa (1940) and López and Parra (1985). According to the last review of Neotropical ticks (Guglielmone et al., 2003), the following tick species are known to occur in Colombia: Argasidae (15 species) – *Antricola mexicanus* Hoffmann, 1958, *Argas magnus* Neumann, 1896, *Argas miniatus* Koch, 1844, *Ornithodoros azteci* Matheson, 1935, *Ornithodoros brodyi* Matheson, 1935, *Ornithodoros furcosus* Neumann, 1908, *Ornithodoros hasei* (Schulzei, 1935), *Ornithodoros marinkellei* Kohls, Clifford and Jones, 1969, *Ornithodoros marmosae* Jones and Clifford, 1972, *Ornithodoros peropteryx* Kohls, Clifford and Jones 1969, *Ornithodoros peruvianus* Fox, 1947, *Ornithodoros rossi* Kohls, Sonenshine and

Clifford, 1965, *Ornithodoros rudis* Karsch, 1880, *Ornithodoros talaje* (Guérin-Méneville, 1849), and *Ornithodoros yumatensis* Cooley and Kohls, 1941; Ixodidae (38 species): *Amblyomma auricularium* (Conil, 1878), *Amblyomma cajennense* (Fabricius, 1787), *Amblyomma calcaratum* Neumann, 1899, *Amblyomma coelebs* Neumann, 1899, *Amblyomma crassum* Robinson, 1926, *Amblyomma dissimile* Koch, 1844, *Amblyomma geayi* Neumann, 1899, *Amblyomma humerale* Koch, 1844, *Amblyomma longirostre* (Koch, 1844), *Amblyomma maculatum* Koch, 1844, *Amblyomma multipunctum* Neumann, 1899, *Amblyomma naponense* (Packard, 1869), *Amblyomma neumanni* Ribaga, 1902, *Amblyomma nodosum* Neumann, 1899, *Amblyomma oblongoguttatum* Koch, 1844, *Amblyomma ovale* Koch, 1844, *Amblyomma pacae* Aragão, 1911, *Amblyomma rotundatum* Koch, 184, *Amblyomma sabanerae* Stoll, 1894, *Amblyomma scalpturatum* Neumann, 1906, *Amblyomma tapirellum* Dunn, 1933, *Amblyomma triste* Koch, 1844, *Amblyomma varium* Koch, 1844, *Haemaphysalis juxtakochi* Cooley, 1946, *Haemaphysalis leporispalustris* (Packard, 1869), *Ixodes boliviensis* Neumann, 1904, *Ixodes brunneus* Kock, 1844, *Ixodes fuscipes* Kock, 1844, *Ixodes lasallei* Méndez Arocha and Ortiz, 1958, *Ixodes luciae*, Sénevet, 1940, *Ixodes montoyanus* Cooley, 1944, *Ixodes pararicinus* Keirans and Clifford, 1985, *Ixodes tapirus* Kohls, 1956, *Ixodes tropicalis* Kohls, 1956, *Ixodes venezuelensis* Kohls, 1953, *Dermacentor nitens* Neumann, 1897, *Rhipicephalus microplus* (Canestrini, 1887), and *Rhipicephalus sanguineus* (Latreille, 1806). Besides these reports, there was a report of *Ixodes affinis* in Colombia by Mattar and López-Valencia (1998). More recently, Nava et al. (2014) reevaluated the taxonomic status of *A. cajennense* and concluded that this taxon was actually represented by six valid species from which *A. cajennense* sensu stricto was excluded from Colombia, and only *Amblyomma patinoi* Labruna, Nava and Beati, 2014, and *Amblyomma mixtum* Koch, 1844 were confirmed for Colombia (Nava et al., 2014; Rivera-Páez et al., 2016). Finally, Apanaskevich and Bermudez (2017) reported *Ixodes bocatorensis* Apanaskevich and Bermudez, 2017 for Colombia. Considering the above reports, the tick fauna of Colombia is currently composed by 56 species, 15 Argasidae and 41 ixodidae. The present study evaluated ixodid ticks infesting domestic animals and collected from vegetation, in order to expand our current knowledge of the tick fauna of Colombia.

Materials and Methods

From August 2014 to May 2016, hard ticks (Ixodidae) were collected directly from domestic animals in 10 Colombian departments, encompassing 17 municipalities (Table 1, Fig. 1). Collected ticks were placed into plastic vials containing absolute ethanol and taken to the laboratory, where they were taxonomically identified based on current literature (Kohls, 1956; Jones et al., 1972; Estrada Peña et al., 2005; Barros-Battesti et al., 2006; Martins et al., 2010; Nava et al., 2014, 2015).

In order to confirm morphological identifications, some specimens of each tick species were submitted to molecular taxonomic identification. For this purpose, ticks were individually submitted to DNA extraction by using the DNeasy Blood and Tissue kit (Qiagen, Chatsworth, California) following the manufacturer´s protocol. The extracted DNA was tested by polymerase chain reaction (PCR) protocols to amplify fragments of at least one of the three following genes of the tick genome: primers 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', targeting a ≈700-bp fragment of the mitochondrial cytochrome c oxidase subunit I gene (COI) (Folmer et al., 1994); primers 5'– CTG CTC AAT GAT TTT TTA AAT TGC TGT GG–3' and 5'–CCG GTC TGA ACT CAG ATC AAG T-3, targeting a ≈460-bp fragment of the mitochondrial 16S rDNA gene (Norris et al., 1996); and primers 5'-CCA TCG ATG TGA AYT GCA GGA CA-3' (Zahler et al., 1995) and 5'-GTG AAT TCT ATG CTT AAA TTC AGG GGG T-3', which amplifies a ≈1,100-bp fragment that includes the entire second internal transcribed spacer (ITS2) region of the nuclear rRNA region (Mclainet al., 1995). In addition, specimens of the following species collected in Brazil were processed by the above PCR protocols in order to obtain DNA sequences for inclusion in the phylogenetic analyses: *A. cajennense* s.s. from Governador Jorge Teixeira, Rondônia state, *A. sculptum* from Pirassununga, São Paulo state, *Amblyomma triste* from Paulicéia, São Paulo state, *R. sanguineus* s.l. from Chapada Gaúcha, Minas Gerais state, and *R. sanguineus* s.l. from São Paulo City, São Paulo state.

PCR products were purified with the QIAquick PCR purification kit (Qiagen), and sent to Macrogen Inc. (South Korea) for DNA sequencing. The sequenced fragments were evaluated and edited using Geneious Trial v8.14 (Drummond et al., 2009) and Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan). In addition, the sequences were

searched by MegaBlast against the public databases and deposited in GenBank and Barcode of Life Data Systems (BOLD). The sequences for each gene were aligned using ClustalW (Thompson et al., 1997), included in the program MEGA version 7 (Tamura et al., 2013). Intraspecific nucleotide divergences were estimated with the program MEGA, using the Kimura 2-Parameter distance model (K2P; Kimura, 1980). Species confirmation was carried out through a similarity analysis based on Maximum Likelihood (ML), with the K2P model and 1,000 bootstrap replications, using the program MEGA. Each alignment included different sequences from GenBank, as stated by their accession numbers in the trees.

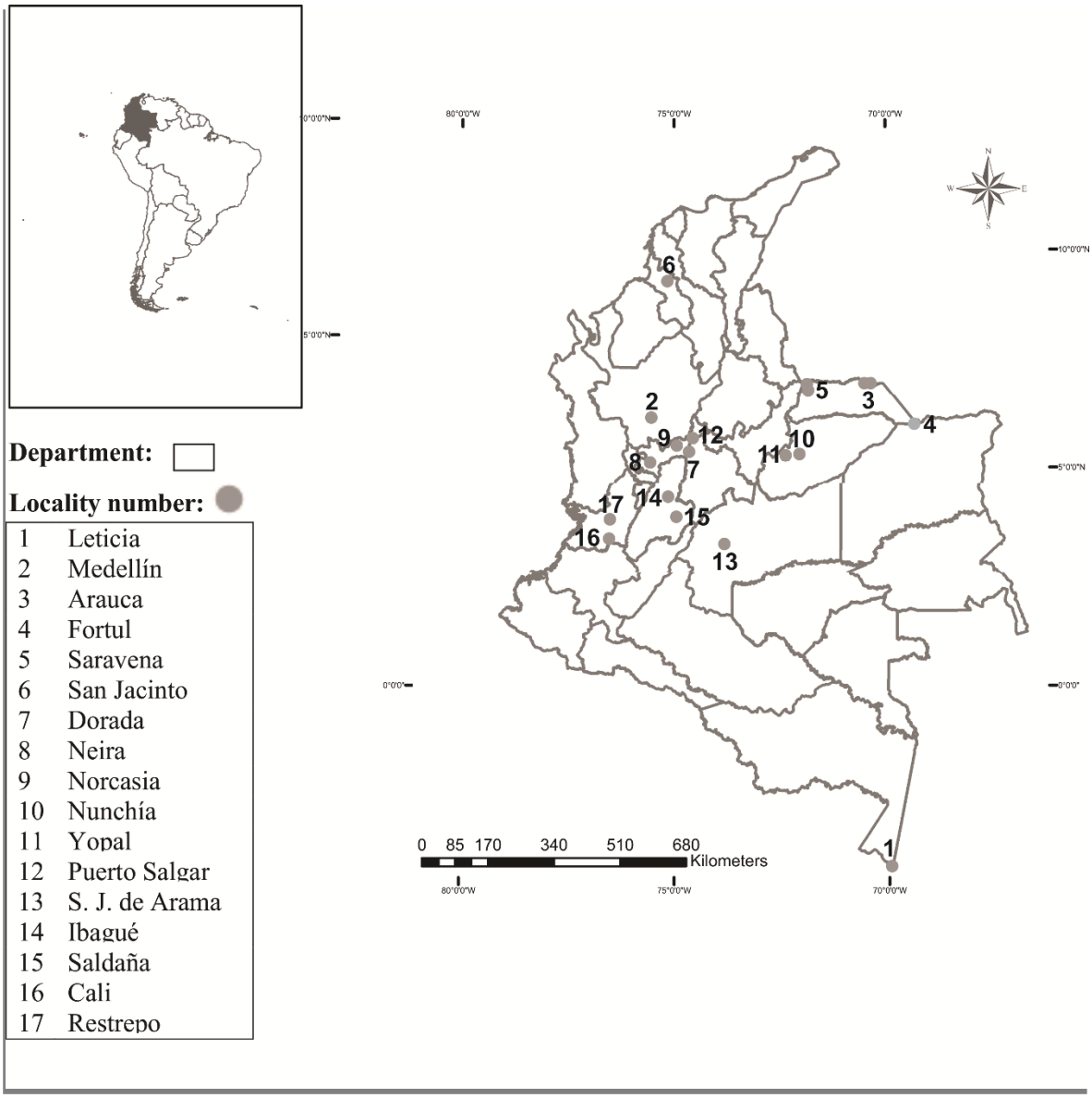

Fig.1. Geopolitical map of Colombia showing the localities where ticks (Acari: Ixodidae) were collected during the present study.

Table 1. Localities in Colombia where ticks were collected in the present study.

Locality number*	Municipality	Department
1	Leticia	Amazonas
2	Medellín	Antioquia
3	Arauca	Arauca
4	Fortul	Arauca
5	Saravena	Arauca
6	San Jacinto	Bolívar
7	Dorada	Caldas
8	Neira	Caldas
9	Norcasia	Caldas
10	Nunchía	Casanare
11	Yopal	Casanare
12	Puerto Salgar	Cundinamarca
13	San Juan de Arama	Meta
14	Ibagué	Tolima
15	Saldaña	Tolima
16	Cali	Valle del Cauca
17	Restrepo	Valle del Cauca

*localities indicated in Fig. 1.

Results

A total of 1,745 tick specimens (1,543 adults, 111 nymphs, 91 larvae) were collected from 85 individual hosts from Colombia, being 28 cattle (*Bos taurus*), 26 domestic dogs (*Canis lupus familiaris*), 25 horses (*Equus caballus*), 5 donkeys (*Equus asinus*), and 1 domestic pig (*Sus scrofa*), plus 3 tick samples collected from vegetation (Table S1). Ticks were morphologically identified into 8 different species: *A. dissimile, A. maculatum* (Fig. 2), *A. mixtum, A. ovale, A. varium, D. nitens, R. microplus*, and *R. sanguineus* sensu lato (s.l.). Hosts and the corresponding localities of each tick species are found in Table 2. Overall, 5 tick species were recorded on cattle (*A. dissimile, A. mixtum, D. nitens, R. microplus*, *R. sanguineus* s.l.), 5 on dogs (*A. maculatum, A. ovale, A. varium, R. microplus*, *R. sanguineus* s.l.), 3 on horses (*A. mixtum, D. nitens, R. sanguineus* s.l.), 3 on donkeys (*A. mixtum, D. nitens, R. microplus*), and 1 on pig (*D. nitens*).

Fragments of the tick 16S rRNA and/or COI and/or ITS2 genes were generated for tick specimens of all collected tick species, except for *A. varium*. DNA sequences of these specimens corroborated morphological identifications, as shown by the BLAST analysis search results (Table 3). Phylogenetically analyses inferred by 16S rRNA and COI partial sequences placed the sequences of *A. maculatum* from Colombia mixed within a clade

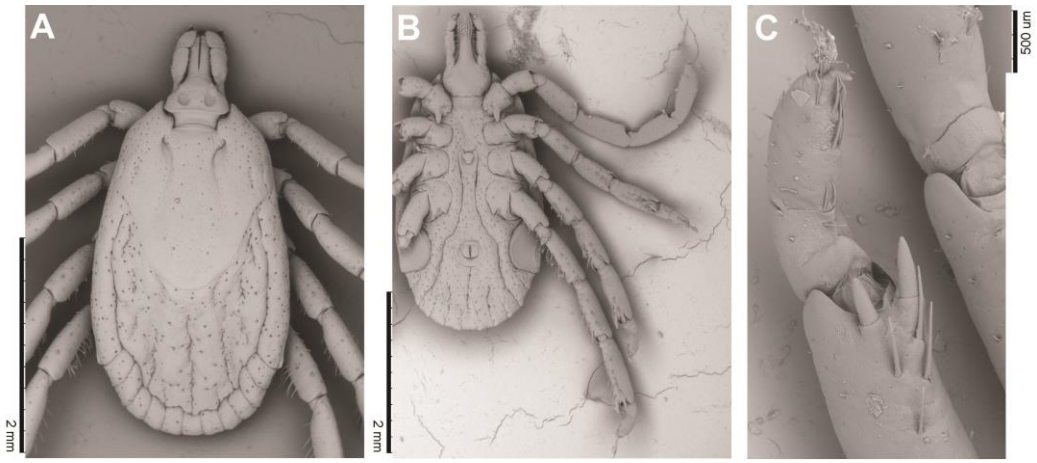

Fig. 2. A female specimen of *Amblyomma maculatum* from Ibagué-Tolima. **(A)** Dorsal view. **(B)** Ventral view. **(C)** Tarsus III showing two spurs.

Table 2. Ticks at different developmental stages (A: adults; N: nymphs; L: larvae) collected from different host species during 2014-2016 at different localities of Colombia.

Tick species	Stage	Host	Localities*
Amblyomma dissimile	A	*Bos taurus*	14
A. dissimile	N, L	vegetation	11
Amblyomma maculatum	A	*Canis lupus familiaris*	9,14,15
Amblyomma mixtum	A, N	*Bos taurus*	3,8,10,13
A. mixtum	A	*Equus asinus*	11
A. mixtum	A, N	*Equus caballus*	3,10,11
A. mixtum	A,N,L	vegetation	10,11
Amblyomma ovale	A	*C. lupus familiaris*	14,15
Amblyomma varium	A	*C. lupus familiaris*	14
Dermacentor nitens	A,N	*B. taurus*	1,3
D. nitens	A,N	*E. asinus*	6,11,14
D. nitens	A,N,L	*E. caballus*	1,3,6,10,11,14
D. nitens	A	*Sus scrofa*	3
Rhipicephalus microplus	A,N,L	*B. taurus*	1,3-5,13,14,17
R. microplus	A	*C. lupus familiaris*	5
R. microplus	A	*E. asinus*	6
Rhipicephalus sanguineus	A	*B. taurus*	1,14
R. sanguineus	A,N	*C. lupus familiaris*	1-3,5,7,9,11,12,14-16,17
R. sanguineus	A	*E. caballus*	3,11

*Locality numbers indicated in Table 1 and Fig. 1.

Table 3. Results of BLAST analysis searches in public data of the DNA sequences generated from ticks collected in the present study.

Tick species (localities*)	Closest identity (%) in GenBank (accession number) according to the tick gene		
	COI	**16S rRNA**	**ITS2**
Amblyomma dissimile (11)	*A. dissimile* [KF200114] 99%	N.A.	N.D.
Amblyomma maculatum (14)	*A. maculatum* [KU302492] 99%	*A. maculatum* [KT037651] 99%	N.D.
Amblyomma maculatum (15)	*A. triste* [KU306582] 99%	*A. triste* [KU284955] 99%	N.D.
Amblyomma maculatum (9)	N.D.	*A. maculatum* [KT037651] 100%	N.D.
Amblyomma mixtum (3)	N.D.	*A. mixtum* [KT820359] 97%	N.D.
Amblyomma mixtum (3,8,10,13)	N.D.	*A. mixtum* [KT820359] 98%	*A. cajennense* [JN866853] 100%
Amblyomma mixtum (8)	*A. mixtum* [KY595138] 99%	N.D.	N.D.
Amblyomma mixtum (11)	*A. mixtum* [KY595138] 99%	N.D.	N.D.
Amblyomma mixtum (13)	*A. mixtum* [KY595138] 99%	N.D.	N.D.
Amblyomma mixtum (10)	*A. mixtum* [KY595139] 100%	N.D.	N.D.
Amblyomma mixtum (11)	*A. mixtum* [KY595136] 99%	N.D.	N.D.
Amblyomma ovale (14,15)	*A. ovale* [KF200080] 99%	*A. ovale* [KU894383] 99%	N.D.
Dermacentor nitens (1)	*D. nitens* [KT906190] 96%	*D. nitens* [KY020994] 96%	N.D.
Dermacentor nitens (3,6,10,11)	*D. nitens* [KT906188] 100%	*D. nitens* [KY020994] 99%	N.D.
Dermacentor nitens (6)	*D. nitens* [KY441487] 99%	N.D.	N.D.
Rhipicephalus microplus (3-5,13,14,17)	*R. microplus* [KT906181] 100%	N.D.	N.D.
Rhipicephalus microplus (5)	*R. microplus* [KT906181] 99%	N.D.	N.D.
Rhipicephalus microplus (1)	*R. microplus* [KY678120] 100%	*R. microplus* [EU918176] 99%	N.D.
Rhipicephalus microplus (1-5,13,14,17)	N.D.	*R. microplus* [EU918176] 100%	N.D.
Rhipicephalus sanguineus (1-3,7,11,12,14-17)	N.D.	*R. sanguineus* [KY413787] 100%	N.D.
Rhipicephalus sanguineus (5)	N.D.	*R. sanguineus* [KY413787] 99%	N.D.
Rhipicephalus sanguineus (1,14,16)	N.D.	N.D.	*R. sanguineus* [KY945496] 99%
Rhipicephalus sanguineus (3,15)	N.D.	N.D.	*R. sanguineus* [JQ625707] 99%
Rhipicephalus sanguineus (11)	N.D.	N.D.	*R. sanguineus* [KF499537] 99%
Rhipicephalus sanguineus (7)	N.D.	N.D.	*R. sanguineus* [AF271283] 99%
Rhipicephalus sanguineus (14)	N.D.	N.D.	*R. sanguineus* [KF499537] 99%
Rhipicephalus sanguineus (2)	N.D.	N.D.	*R. sanguineus* [KF499537] 99%
Rhipicephalus sanguineus (3,7,11,14)	N.D.	N.D.	*R. sanguineus* [KF499537] 99%
Rhipicephalus sanguineus (11)	N.D.	N.D.	*R. turanicus* [KF958425] 99%
Rhipicephalus sanguineus (1)	N.D.	N.D.	*R. turanicus* [KF499532] 99%
Rhipicephalus sanguineus (11)	N.D.	N.D.	*R. turanicus* [KF958425] 99%

*Locality numbers indicated in Table 1 and Fig. 1.
N.D.: not done
N.A.: sequences not available in GenBank

composed by sequences of *A. maculatum* from the United States and Colombia, and *Amblyomma triste* from Brazil and Argentina (Figs. 3, 4); sequences of *A. ovale* grouped with corresponding sequences of *A. ovale* from Brazil and Paraguay (Fig. 3), or Panama (Fig. 4); sequences of *D. nitens* grouped with *D. nitens* from the United States and Brazil (Fig. 3), or Colombia and Panama (Fig. 4); a sequence of *A. dissimile* grouped with *A. dissimile* from Brazil (Fig 4); sequences of *A. mixtum* grouped with *A. mixtum* from Honduras (Fig. 3) or Colombia, Ecuador and Panama (Fig. 4). These results for *A. mixtum* were corroborated by the ITS2 phylogenetic analysis, in which our sequences of *A. mixtum* grouped with *A. mixtum* sequences from Colombia, Costa Rica, Mexico and the United States (Fig. 5).

All 16S rRNA sequences of Colombian *R. sanguineus* s.l. generated in the present study grouped with *R. sanguineus* s.l. from Brazil, Colombia, Thailand, and South Africa (Fig. 6). In the phylogenetic analysis inferred by the ITS2 gene of *R. sanguineus* s.l. ticks, the Colombian specimens grouped in a large clade composed by sequences from tropical countries such as Costa Rica, Honduras, Brazil, Colombia and Thailand, but also by sequences from Australia, Egypt, and India (Fig. 7). Regarding our specimens of *R. microplus*, their 16S rRNA sequences grouped in a clade with *R. microplus* from Argentina (Fig. 6), while their COI sequences grouped with sequences of *R. microplus* from Brazil and Colombia (Fig. 4).

GenBank nucleotide sequence accession numbers for the DNA sequences obtained in the current study are: MF351562-MF351603, MF353094-MF353129 for the mitochondrial rRNA 16S gene; MF363053-MF363093 for the mitochondrial COI gene, and MF353130-MF353154 for nuclear ITS2 gene. Voucher tick specimens were deposited at the tick collection “Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva” (University of São Paulo, São Paulo, Brazil).

Discussion

All tick species collected in the present study have been previously reported in Colombia, as stated by Guglielmone et al. (2003) and Rivera-Páez et al. (2016). Similarly, the tick-host records of our study have been previously reported in the literature (López and Parra 1985; Need et al., 1991; Rivera-Páez et al., 2016; Nava et al., 2017); the only exception is *A. varium* on dog, which is the first confirmed record.

From 7 *A. maculatum* specimens from Colombia, we generated 3 distinct haplotypes for the 16S rRNA gene, and 2 for COI. In each of the trees inferred from these two genes, the Colombian specimens formed a large clade with GenBank sequences of *A. maculatum* and *A. triste*. Noteworthy, while some Colombian specimens grouped with *A. maculatum* from the United States, other Colombian specimens grouped with *A. triste* from Brazil, for both genes. At first sight, these results could indicate that some Colombian specimens could represent the taxon *A. triste*; however, we retained these specimens as *A. maculatum* because their morphology was compatible with *A. maculatum* (two spurs on tarsi II-IV) (Fig. 2) rather than *A. triste* (one spur on tarsi II-IV), as stated in the literature (Kohls, 1956; Estrada Peña et al., 2005). Indeed, further studies are required to elucidate the taxonomic status of *A. maculatum* and *A. triste*, since the taxonomic separation of these two species is yet to be resolved, with the possibility that they might be conspecific (Nava et al., 2017).

From up to 9 *A. mixtum* specimens from Colombia, we generated 2 distinct haplotypes for the 16S rRNA gene, 5 for COI, and 1 for ITS2. In each of the trees inferred from these three genes, the Colombian specimens grouped with GenBank sequences of *A. mixtum*, well separated from all other members of the *A. cajennense* species complex. These results corroborate a recent study that confirmed the presence of *A. mixtum* in Colombia (Rivera-Páez et al., 2016). However, whereas this previous report consisted of specimens from the Departments of Arauca and Casanare (northeastern Colombia), the present records confirm the presence of *A. mixtum* in these two Departments and expand it to two other Departments, Caldas and Meta (central Colombia). These results indicate that the distribution of *A. mixtum* in Colombia is much broader than currently known.

From up to 30 *R. sanguineus* s.l. specimens from Colombia, we generated 2 distinct haplotypes for the 16S rRNA gene, and 10 for ITS2. In the tree inferred from the 16S rRNA gene, all Colombian specimens grouped with *R. sanguineus* specimens from Brazil and Colombia, which represent the so called "tropical species", as reported elsewhere (Burlini et al., 2010; Moraes-Filho et al., 2011, Dantas-Torres et al., 2013). These 30 *R. sanguineus* s.l. specimens were collected in 8 Departments of Colombia (Amazonas, Antioquia, Arauca, Caldas, Casanare, Cundinamarca, Tolima, Valle del Cauca), whereas the previous reports represented specimens from the Departments of Cordoba (Moraes-Filho et al., 2011), Antioquia, and Valle del Cauca (Dantas-Torres et al., 2013). These results indicate that the

taxon *R. sanguineus* s.l. is possibly represented in Colombia solely by the "tropical species", which seems to be widespread in the country. In contrast to the 16S rRNA gene, the tree inferred from the ITS-2 gene did not segregate the Colombian specimens from specimens from other parts of the world. This inconclusive analysis is supported by the study of Latrofa et al. (2013), who demonstrated that the ITS-2 gene is not reliable for identification of ticks within the *Rhipicephalus* genus, especially among specimens of *R. sanguineus* s.l.

From up to 11 *R. microplus* specimens from Colombia, we generated 2 distinct haplotypes for the 16S rRNA gene, and 3 for COI. In the trees inferred from these two genes, the Colombian specimens formed a clade with sequences from Argentina and Brazil, which correspond to bona fide records of *R. microplus* according to the studies of Labruna et al. (2009) and Estrada-Peña et al. (2012), who re-evaluated the taxonomic status of *R. microplus*. Regarding the trees that included *D. nitens* sequences, it was interesting to note some polymorphism in their 16S rRNA or COI sequences, which in both cases resulted in a clade that separated the sequences of Leticia (Amazonas Department) from the other Colombian Departments (Arauca, Bolívar, Casanare, Tolima), although we could not detect significant morphological differences between these specimens (data not shown).

This study provides new records for 8 tick species parasitizing domestic animals in Colombia, with emphasis for species with greater veterinary or/and medical importance in the Neotropical region, such as *R. microplus, R. sanguineus, D. nitens, A. mixtum,* and *A. maculatum* (Guglielmone et al., 2003; Nava et al., 2014, 2017). Most of these species have gone through extensive taxonomic changes or contestations during this decade (Estrada-Peña et al., 2012; Nava et al., 2014, 2015, 2017). For this reason, phylogenetic analyses of specimens from poorly explored sites such as Colombia are crucial for our growing knowledge of tick systematics in the Neotropical region, where ticks associated with domestic animals are vectors of a number of pathogens to either animals or humans (Nava et al., 2017).

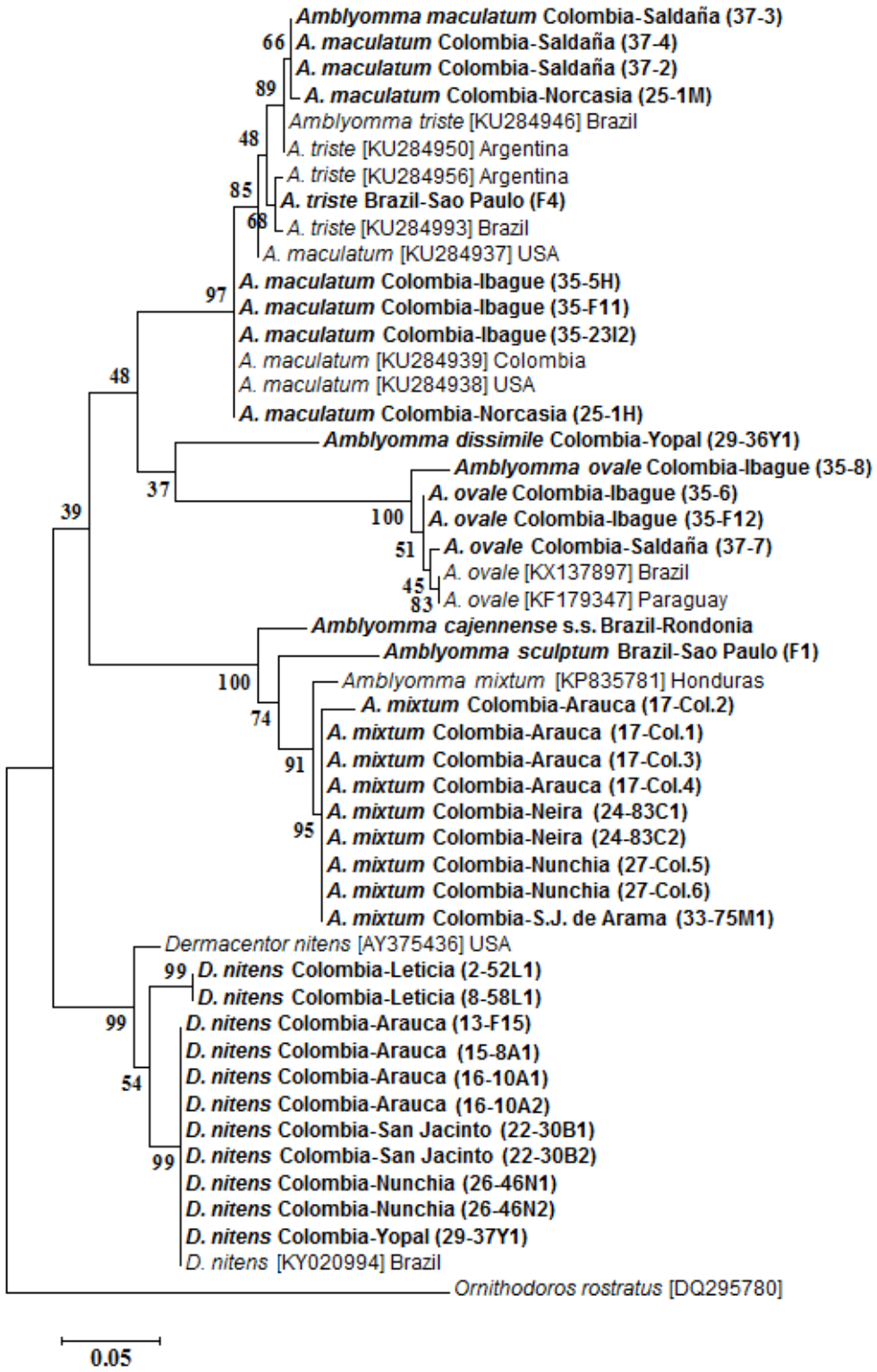

Fig. 3. Maximum Likelihood (ML) tree using sequences of the mitochondrial 16S rDNA gene partial sequences of tick specimens collected in the present study (in bold) and sequences from GenBank (accession numbers in brackets). Numbers at nodes are bootstrap support values. The sequence of *Ornithodoros rostratus* was used as outgroup.

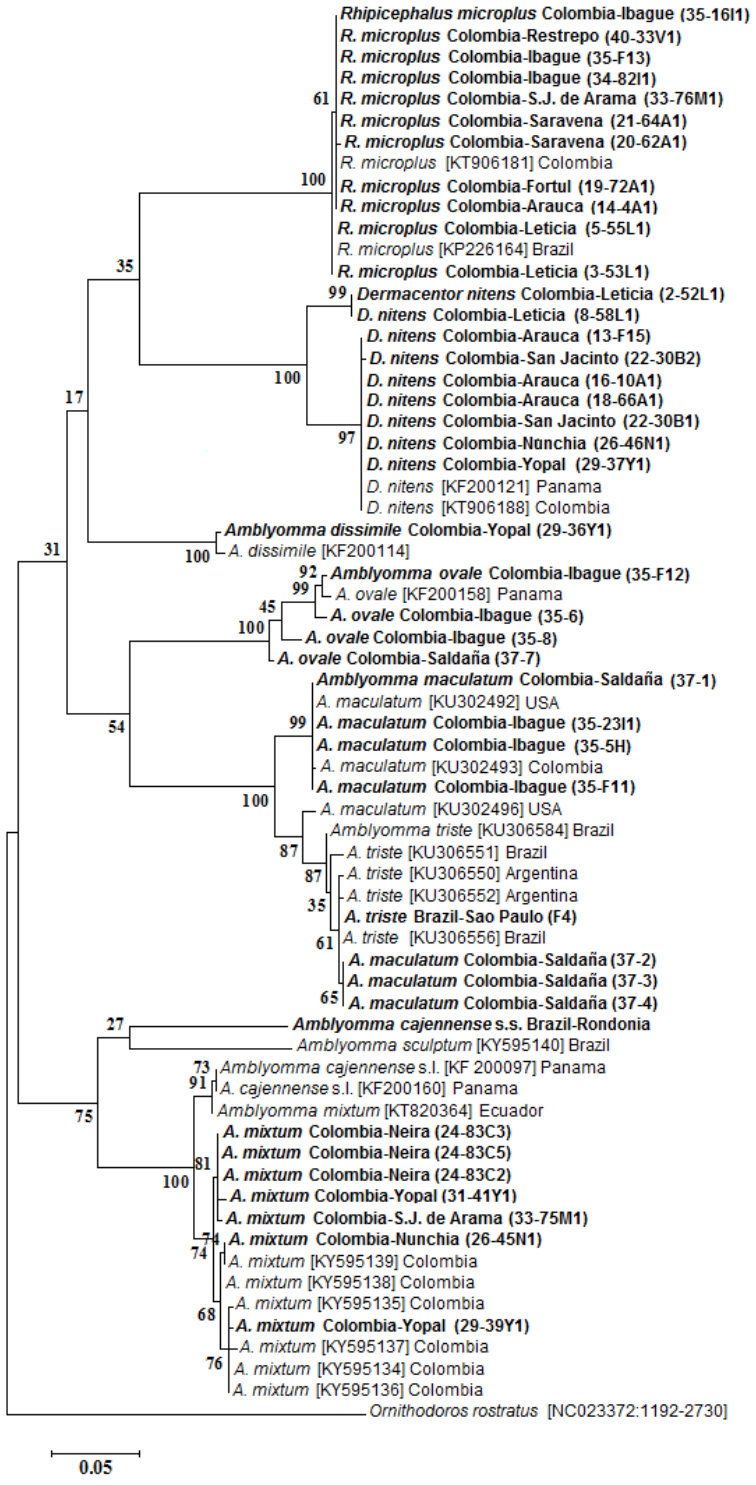

Fig. 4. Maximum Likelihood (ML) tree using sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene partial sequences of tick specimens collected in the present study (in bold) and sequences from GenBank (accession numbers in brackets). Numbers at nodes are bootstrap support values. The sequence of *Ornithodoros rostratus* was used as outgroup.

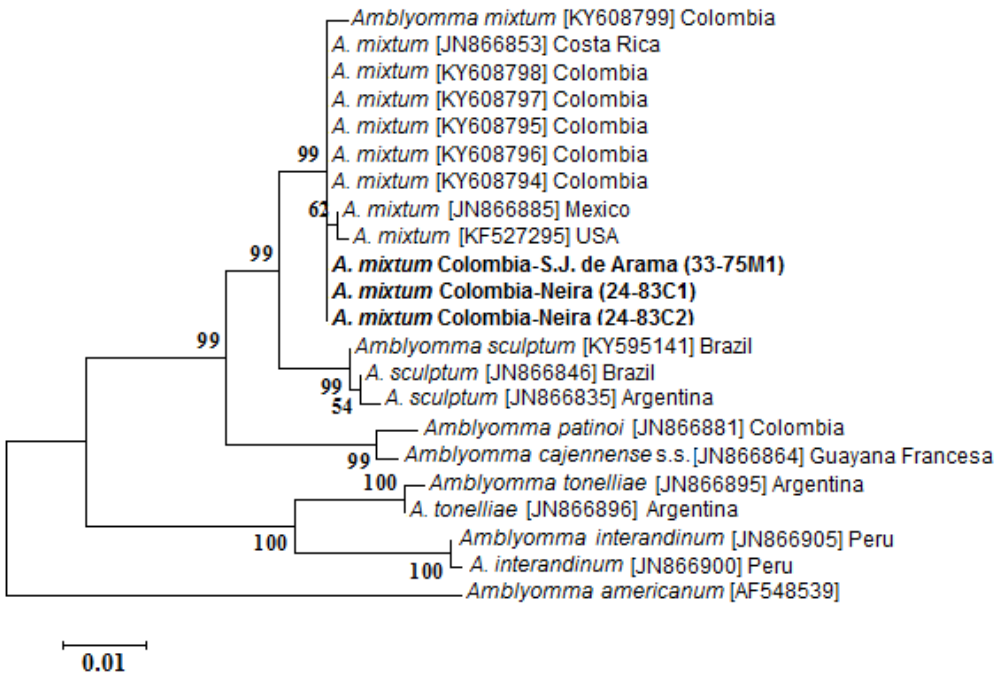

Fig.5. Maximum Likelihood (ML) tree using sequences of the second internal transcribed space (ITS2) gene partial sequences of tick specimens collected in the present study (in bold) and sequences from GenBank (accession numbers in brackets). Numbers at nodes are bootstrap support values. The sequence of *Amblyomma americanum* was used as outgroup.

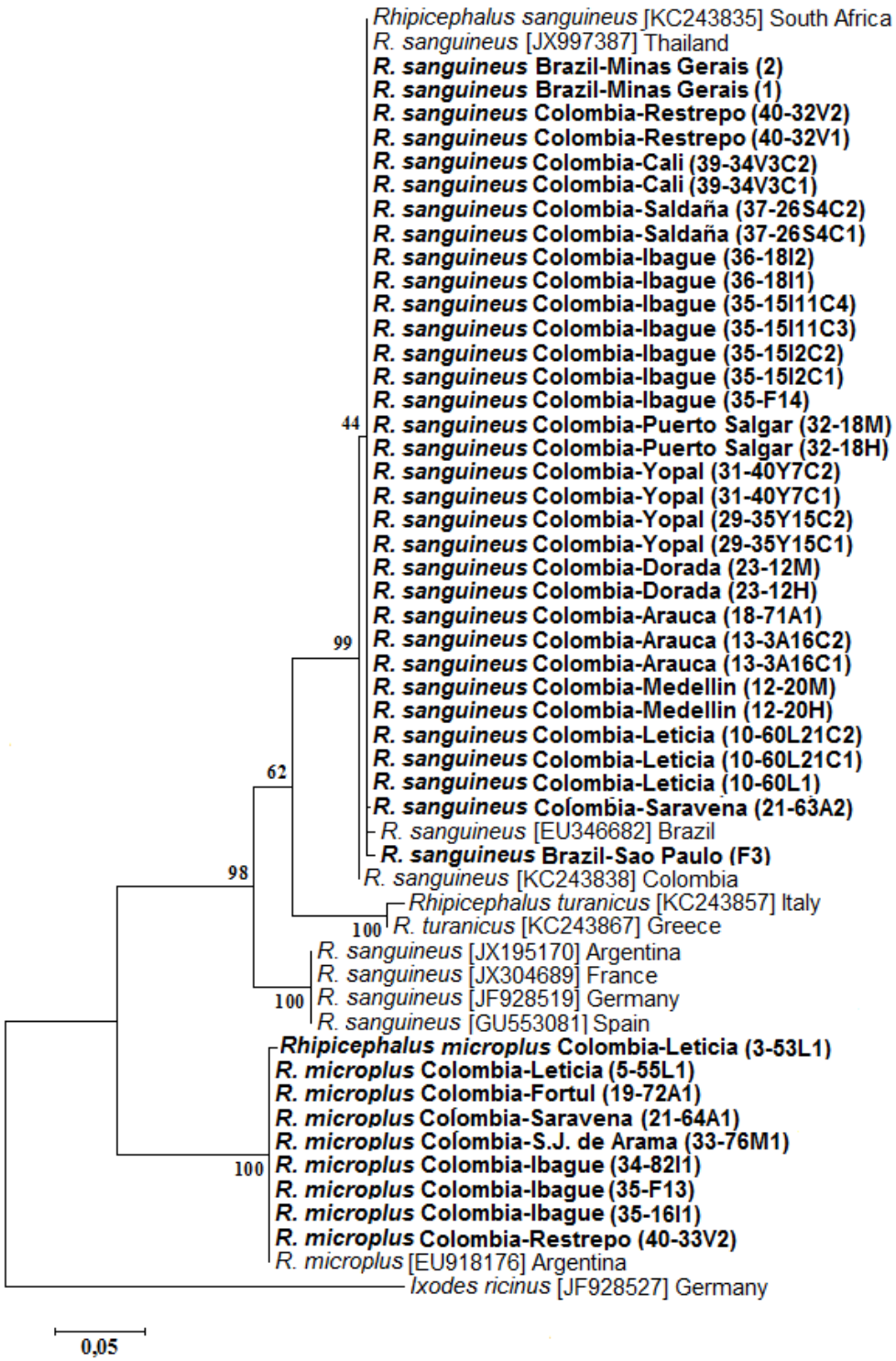

Fig.6. Maximum Likelihood (ML) tree using sequences of the mitochondrial 16S rDNA gene partial sequences of tick specimens collected in the present study (in bold) and sequences from GenBank (accession numbers in brackets). Numbers at nodes are bootstrap support values. The sequence of *Ixodes ricinus* was used as outgroup.

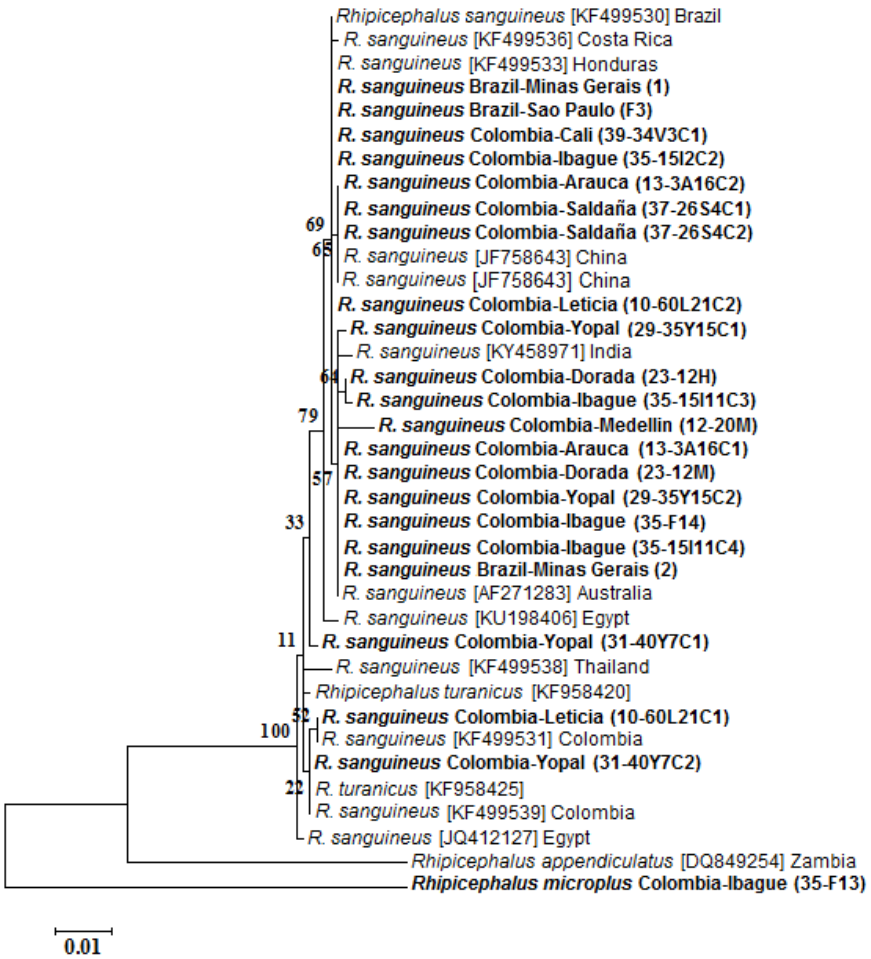

Fig.7. Maximum Likelihood (ML) tree using sequences of the second internal transcribed space (ITS2) gene partial sequences of tick specimens collected in the present study (in bold) and sequences from GenBank (accession numbers in brackets). Numbers at nodes are bootstrap support values. The sequence of *Rhipicephalus microplus* was used as outgroup.

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Table S1. Data on the hard ticks (Acari: Ixodidae) collected from domestic hosts in various regions of Colombia.

Tick Species	Number of ticks* F	M	N	L	Host	State	Municipality	Geographical Coordinates	Altitude masl	Date
R. microplus				8	*Bos taurus*	Amazonas	Leticia	4°6’ 31”S, 69°58’ 5”W	51	08-2015
R. sanguineus			1		*Bos taurus*	Amazonas	Leticia	4°6’ 31”S, 69°58’ 5”W	51	08-2015
D. nitens	17	13	14		*Equus caballus*	Amazonas	Leticia	4°8’ 42”S, 69°56’ 28”W	87	08-2015
R. microplus	5	9	24		*Bos taurus*	Amazonas	Leticia	4°6’ 18”S, 69°56’ 23”W	69	08-2015
R. microplus	6	11	3		*Bos taurus*	Amazonas	Leticia	4°4’ 24”S, 70°0’ 10W	85	08-2015
R. microplus	12	7	7		*Bos taurus*	Amazonas	Leticia	4°4’ 17”S, 69°59’ 57”W	85	08-2015
R. microplus	10	7			*Bos taurus*	Amazonas	Leticia	4°4’ 38”S, 70°0’ 6”W	39	08-2015
R. microplus	14	6	1		*Bos taurus*	Amazonas	Leticia	4°7’ 27”S, 69°56’ 59”W	96	08-2015
D. nitens	5	1			*Bos taurus*	Amazonas	Leticia	4°8’ 39”S, 69°56’ 20”W	88	08-2015
R. microplus	2	2	7		*Bos taurus*	Amazonas	Leticia	4°9’ 15”S, 69°56’ 1”W	40	08-2015
R. sanguineus	5	7			*C. lupus familiaris*	Amazonas	Leticia	4°9’ 59”S, 69°57’ 41”W	40	08-2015
R. microplus	9	3	2		*Bos taurus*	Amazonas	Leticia	4°6’ 16”S, 69°58’ 24”W	78	08-2015
R. sanguineus	10	10			*C. lupus familiaris*	Antioquia	Medellín	06° 14’41”N, 75° 34’29”W	1495	08-2015
D. nitens	12	26	4	3	*Equus caballus*	Arauca	Arauca	06° 55’43”N, 70° 27’36”W	115	09-2014
R. sanguineus	16	21	1		*C. lupus familiaris*	Arauca	Arauca	06° 55’43”N, 70° 27’36”W	115	09-2014
R. microplus	5	1			*Bos taurus*	Arauca	Arauca	06º 02’N, 69º 25’W	125	09-2014
A. mixtum	3	1			*Bos taurus*	Arauca	Arauca	06º 02’N, 69º 25’W	125	09-2014
A. mixtum	8	12			*Equus caballus*	Arauca	Arauca	06° 56’ 24”N, 70° 32’ 0”W	117	09-2014
D. nitens	14	47			*Equus caballus*	Arauca	Arauca	06° 56’ 24”N, 70° 32’ 0”W	117	09-2014
A. mixtum	1	1			*Equus caballus*	Arauca	Arauca	07° 1’ 48”N, 70° 43’ 39”W	132	09-2014
D. nitens	9	33	7	8	*Equus caballus*	Arauca	Arauca	07° 1’ 48”N, 70° 43’ 39”W	132	09-2014
A. mixtum	33	69			*Equus caballus*	Arauca	Arauca	07° 3’ 55”N, 70° 44’ 2”W	129	09-2014
D. nitens	26	66	4		*Equus caballus*	Arauca	Arauca	07° 3’ 55”N, 70° 44’ 2”W	129	09-2014
A. mixtum	23	25	1		*Bos taurus*	Arauca	Arauca	07° 3’ 55”N, 70° 44’ 2”W	129	09-2014
A. mixtum	4				*Equus caballus*	Arauca	Arauca	07° 3’ 55”N, 70° 44’ 2”W	129	09-2015
D. nitens	5	2			*Equus caballus*	Arauca	Arauca	07° 3’ 55”N, 70° 44’ 2”W	129	09-2015
D. nitens	3				*Sus scrofa*	Arauca	Arauca	07° 4’ 34”N, 70° 42’ 37”W	120	08-2015
D. nitens	2		2		*Bos taurus*	Arauca	Arauca	07° 4’ 34”N, 70° 42’ 37”W	120	08-2015
D. nitens	11	5			*Equus caballus*	Arauca	Arauca	07° 4’ 34”N, 70° 42’ 37”W	120	08-2015
A. mixtum	1				*Equus caballus*	Arauca	Arauca	07° 4’ 34”N, 70° 42’ 37”W	120	08-2015
R. sanguineus	3	1			*Equus caballus*	Arauca	Arauca	07° 4’ 34”N, 70° 42’ 37”W	120	08-2015
R. sanguineus	2	5			*C. lupus familiaris*	Arauca	Arauca	07° 4’ 34”N, 70° 42’ 37”W	120	08-2015
R. microplus	3	1	1		*Bos taurus*	Arauca	Fortul	06° 47’ 39”N, 71° 46’ 15”W	240	09-2015
R. microplus	4	2			*Bos taurus*	Arauca	Saravena	06° 57’ 26”N, 71° 52’ 31”W	380	09-2015
R. sanguineus	1	3			*C. lupus familiaris*	Arauca	Saravena	06° 57’ 26”N, 71° 52’ 31”W	380	09-2015
R. microplus	5				*Bos taurus*	Arauca	Saravena	06° 57’ 21”N, 71° 52’ 21”W	390	09-2015
R. microplus	1				*C. lupus familiaris*	Arauca	Saravena	06° 57’ 21”N, 71° 52’ 21”W	390	09-2015
D. nitens	10	14	2		*Equus caballus*	Bolívar	San Jacinto	9°84’ 53”N, 75°11’ 88”W	250	01-2015
D. nitens	4	2	1		*Equus asinus*	Bolívar	San Jacinto	9°84’ 53”N, 75°11’ 88”W	250	01-2015
R. microplus		1			*Equus asinus*	Bolívar	San Jacinto	9°84’ 53”N, 75°11’ 88”W	250	01-2015
R. sanguineus	10	12			*C. lupus familiaris*	Caldas	Dorada	5° 27´ 14”N, 74° 39’ 53”W	180	09-2015
A. mixtum	18	2			*Bos taurus*	Caldas	Neira	5°11´50”N- 75°38’05”W	1919	09-2015
R. sanguineus	10	9			*C. lupus familiaris*	Caldas	Norcasia	5° 34´ 27”N, 74° 53´ 20”W	690	09-2015
A. maculatum	1	1			*C. lupus familiaris*	Caldas	Norcasia	5° 34´ 27”N, 74° 53´ 20”W	690	09-2015
A. mixtum	10	11	4		*Equus caballus*	Casanare	Nunchia	5°21’ 1”N, 72°4’ 53”W	212	02-2015
D. nitens	1	1			*Equus caballus*	Casanare	Nunchia	5°21’ 1”N, 72°4’ 53”W	212	02-2015
A. mixtum	3	5	2		*Equus caballus*	Casanare	Nunchia	5°21’ 13”N, 72°5’ 50”W	214	02-2015
A. mixtum	11	2	1		*Bos taurus*	Casanare	Nunchia	5°21’ 13”N, 72°5’ 50”W	214	02-2015
A. mixtum	3	2			Vegetation	Casanare	Nunchia	5°21’ 40”N, 72°6’ 7”W	183	02-2015
R. sanguineus	1	1			*C. lupus familiaris*	Casanare	Yopal	5°19’ 27”N, 72°24’ 31”W	340	02-2015
A. mixtum			5	40	Vegetation	Casanare	Yopal	5°19’ 27”N, 72°24’ 31”W	340	02-2015
A. dissimile			1	40	Vegetation	Casanare	Yopal	5°19’ 27”N, 72°24’ 31”W	340	02-2015
D. nitens	16	12			*Equus caballus*	Casanare	Yopal	5°19’ 27”N, 72°24’ 31”W	340	02-2015
R. sanguineus	1	4			*Equus caballus*	Casanare	Yopal	5°19’ 27”N, 72°24’ 31”W	340	02-2015
A. mixtum	5	2			*Equus caballus*	Casanare	Yopal	5°19’ 27”N, 72°24’ 31”W	340	02-2015
D. nitens	1		2		*Equus caballus*	Casanare	Yopal	5°25’ 53”N, 72°14’ 36”W	240	02-2015
A. mixtum	2				*Equus asinus*	Casanare	Yopal	5°25’ 26”N, 72°14’ 17”W	193	02-2015
D. nitens	2		1		*Equus asinus*	Casanare	Yopal	5°25’ 26”N, 72°14’ 17”W	193	02-2015
D. nitens	6	8	1		*Equus caballus*	Casanare	Yopal	5°25’ 26”N, 72°14’ 17”W	193	02-2015
A. mixtum	3	6			*Equus caballus*	Casanare	Yopal	5°25’ 26”N, 72°14’ 17”W	193	02-2015
R. sanguineus	10	10			*C. lupus familiaris*	Cundinamarca	Puerto Salgar	5° 27’ 55”N, 74° 39´ 11”W	180	09-2015
A. mixtum	1				*Bos taurus*	Meta	S. J. de Arama	3°20’ 47”N, 73°53’ 21”W	500	09-2015
R. microplus	25	4	5		*Bos taurus*	Meta	S. J. de Arama	3°20’ 47”N, 73°53’ 21”W	500	09-2015
R. microplus	11	1	1		*Bos taurus*	Tolima	Ibagué	04° 24’ 6”N, 75° 4’ 21”W	790	08-2015
A. maculatum	3				*C. lupus familiaris*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2014
R. sanguineus	15	15			*C. lupus familiaris*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2014
A. varium		1			*C. lupus familiaris*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2014
R. microplus	8	11			*Bos taurus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2014
D. nitens	1	2			*Equus caballus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2014
R. microplus	10	10			*Bos taurus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	02-2015
R. sanguineus	5	5			*Bos taurus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	02-2015
A. maculatum	1	5			*C. lupus familiaris*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	02-2015
A. ovale	2	9			*C. lupus familiaris*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	02-2015
A. dissimile	1				*Bos taurus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	02-2015
D. nitens	6	30			*Equus asinus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2015
D. nitens	5	10	2		*Equus caballus*	Tolima	Ibagué	04° 23’ 50”N, 75° 8’ 12”W	924	08-2015
R. sanguineus	9	13			*C. lupus familiaris*	Tolima	Ibagué	04° 49’ 30”N, 75° 28’ 83”W	1850	08-2014
R. microplus	100	100			*Bos taurus*	Tolima	Ibagué	04° 49’ 30”N, 75° 28’ 83”W	1850	08-2014
R. sanguineus	8	12			*C. lupus familiaris*	Tolima	Saldaña	3°55’ 36”N, 74°58’ 33”W	302	01-2015
A. maculatum		31			*C. lupus familiaris*	Tolima	Saldaña	3°55’ 36”N, 74°58’ 33”W	302	01-2015

A. ovale	2	*C. lupus familiaris*	Tolima	Saldaña	3°55’ 36”N, 74°58’ 33”W	302	01-2015
R. sanguineus	15 35	*C. lupus familiaris*	Tolima	Saldaña	3°55’ 36”N, 74°58’ 33”W	302	05-2016
A. maculatum	4	*C. lupus familiaris*	Tolima	Saldaña	3°55’ 36”N, 74°58’ 33”W	302	05-2016
A. ovale	1 1	*C. lupus familiaris*	Tolima	Saldaña	3°55’ 36”N, 74°58’ 33”W	302	05-2016
R. sanguineus	30 30	*C. lupus familiaris*	Tolima	Saldaña	3°93’ 47”N, 75°02’W	300	02-2015
R. sanguineus	7 7 5	*C. lupus familiaris*	V. del Cauca	Cali	3°24’ 21”N, 76°32’ 47”W	982	01-2015
R. sanguineus	3 2	*C. lupus familiaris*	V. del Cauca	Restrepo	3°51’ 12”N, 76°30’ 43”W	1413	01-2015
R. microplus	7	*Bos taurus*	V. del Cauca	Restrepo	3°51’ 12”N, 76°30’ 43”W	1413	01-2015

*** (F) Female, (M) Male, (N) Nymph, (L) Larva**

4.2. CAPÍTULO 2

Amblyomma mixtum Koch, 1844 (Acari: Ixodidae): First record confirmation in Colombia using morphological and molecular analyses.

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Amblyomma mixtum Koch, 1844 (Acari: Ixodidae): First record confirmation in Colombia using morphological and molecular analyses

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ABSTRACT

Up to some years ago, the taxon *Amblyomma cajennense* represented a single tick species in the New World, from southern United States to northern Argentina. Recent studies, based on genetic, reproductive and morphological data reorganized this taxon into a complex of the following 6 valid species: *A. cajennense* sensu stricto, *Amblyomma mixtum*, *Amblyomma sculptum*, *Amblyomma interandinum*, *Amblyomma tonelliae*, and *Amblyomma patinoi*. According to this classification, the *A. cajennense* complex is currently represented in Colombia by only one species, *A. patinoi*. Because the Colombian land is surrounded by confirmed records of *A. mixtum* in Panama and Ecuador, and by *A. cajennense* s.s. in Venezuela and the Brazilian Amazon, it is possible that these two species could also occur in Colombia. This study aimed to determine the occurrence of ticks of the *A. cajennense* complex in the Orinoquía region of Colombia. A total of 246 adult ticks of the *Amblyomma* genus were collected in three sampled regions: 71 females and 110 males in Arauca (Arauca Department), 27 females and 20 males in Nunchía (Casanare Department), and 10 females and 8 males in Yopal (Casanare Department). Based on morphological and molecular analyses, these ticks were identified as *A. mixtum*. Molecular analyses consisted of DNA sequences of two molecular markers, the nuclear second internal transcribed spacer (ITS2) and the mitochondrial cytochrome c oxidase subunit I gene (COI). The presence of *A. mixtum* in Colombia is of medical relevance, since this species is incriminated as a vector of *Rickettsia rickettsii* in Central America.

1. Introduction

Up to some years ago, the taxon *Amblyomma cajennense* represented a single tick species distributed in all tropical and subtropical areas of the New World, from southern United States to northern Argentina (Estrada-Peña et al., 2004). Recent studies, based on genetic, reproductive and morphological data reorganized this taxon into a complex of the following 6 valid species: *A. cajennense* sensu stricto (restricted to the Amazonian region), *Amblyomma mixtum* (from Texas to western Ecuador), *Amblyomma sculptum* (northern Argentina, Bolivia, Paraguay, Brazil), *Amblyomma interandinum* (inter-Andean valley of Peru), *Amblyomma tonelliae* (dry areas of northern Argentina, Bolivia and Paraguay), and *Amblyomma patinoi* (Eastern Cordillera of Colombia) (Beati et al., 2013; Nava et al., 2014). According to this classification, the *A. cajennense* species complex is currently represented in Colombia by only one specie, *A. patinoi* (Nava et al., 2014). However, our current knowledge on the distribution of these species is probably incomplete, and examination of new field-collected material is required for a better definition of species boundaries (Nava et al., 2014). Moreover, *A. cajennense* s.l. constitutes the most important human-biting ticks of South America (Guglielmone et al., 2006), and at least three species of this species complex, namely *A. sculptum*, *A. mixtum*, and *A. patinoi*, are incriminated as important vectors of the bacterium *Rickettsia rickettsii*, the agent of the deadly Rocky Mountain spotted fever (Krawczak et al., 2014; Labruna et al., 2014;

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Faccini-Martínez et al., 2015). Therefore, a precise knowledge of the actual distribution of these species is of highly public relevance.

The six species of the *A. cajennense* species complex are morphologically very similar, making their morphological discrimination sometimes very difficult. The combination of morphological, distributional, and molecular information may sometimes be necessary for the correct determination of problematic specimens (Nava et al., 2014). While *A. patinoi* is the only member of this complex, precisely known to occur in Colombia (Nava et al., 2014; Faccini-Martínez et al., 2015), there have been multiple previous records of *A. cajennense* s.l. from different parts of Colombia (López-Valencia 1989; Estrada-Peña et al., 2004; Miranda et al., 2011). Unfortunately, these specimens are not available for morphological reexamination or molecular analysis. In addition, because the Colombian land is surrounded by confirmed records of *A. mixtum* in Panama and Ecuador, and by *A. cajennense* s.s. in Venezuela and the Brazilian Amazon (Nava et al., 2014), it is possible that these two species could also occur in Colombia.

In view of the above and considering the medical and veterinary importance of *A. cajennense* s.l. in Latin America, associated to the lack of studies on this complex in the Colombian territory, this study aimed to determine the occurrence of ticks of the *A. cajennense* complex in the Orinoquía region of Colombia.

2. Material and methods

Ticks were collected directly from horses (*Equus caballus*), cattle (*Bos taurus*), and a capybara (*Hydrochoerus hydrochaeris*) from different geographical sites of the Orinoquía region (Eastern Plains) of Colombia. The ecosystems of the region are tropical savanna with gallery forests and wetlands along the rivers. Ticks were collected from the following specific sites: Department of Arauca, Arauca municipality (06°55′43″N, 70°27′36″W/06°02′0″N, 69°25′0″W/06°56′24″N, 70°32′0″W/07°1′48″N, 70°43′39″W/07°3′55″N, 70°44′2″W) in September 2014; Department of Casanare, Nunchía municipality (5°21″1″N, 72°4′53″W/5°21′13″N, 72°5′50″W/5°21′40″N, 72°6′7″W) and Yopal municipality (5°19′27″N, 72°24′31″W/5°25′26″N, 72°14′17″W) in February 2015. Collected ticks were submitted to taxonomic identification based on their external morphology following Jones et al. (1972) and Nava et al. (2014) through light microscopy (Leica M205C stereomicroscope). In addition, 2 male and 2 female specimens from each municipality (Arauca, Yopal and Nunchía) were prepared for scanning electron microscopy (SEM) (Hitachi Scanning Electron Microscope, model TM3000) following techniques described by Corwin et al. (1979).

After morphological identification, 2 male and 2 female specimens of each Department were individually processed for molecular analyses. For this purpose, DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen) following manufacturerís protocol, and tested by two PCR protocols, one targeting the ribosomal second internal transcribed spacer (ITS2) region, and the second one targeting the mitochondrial cytochrome c oxidase subunit I gene (COI). For the ITS2 PCR, we used primers ITS2 (F) 5′-CCATCGATGTGAAYTGCAGGACA-3′ (Zahler et al., 1995) and MCLN (R) 5′-GTGAATTCTATGCTTAAATTCAGGGGGT-3′ (Mclain et al., 1995), which correspond to the 5.8S and 28S regions, respectively, thus amplifying a DNA fragment that contains the complete sequence of the ITS2 of the rDNA, which has ≈1000-bp in ticks of the genus *Amblyomma* (Marrelli et al., 2007). For the COI PCR, we used primers LCO1490 (F) 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198 (R) 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′, which amplify a ≈700-bp fragment (Folmer et al., 1994). PCR products were purified using Wizard® SV Gel and PCR Clean-Up System Kit (Promega), according to the manufacturer's instructions, and sent to Macrogen Advancing Through Genomics (South Korea) for DNA sequencing.

DNA sequences were submitted to phylogenetic analyses. For this purpose, the quality analysis of the DNA sequences was performed with the Geneious Trial v8.14 software (Drummond et al., 2009). Sequence alignments were conducted with the ClustalW software (Thompson et al., 1997), included in the Mega 6 software (Tamura et al., 2013). Species identification and confirmation conducted through similarity estimation between sequences obtained from specimens collected in Colombia, in addition to representative sequences of six species of the *A. cajennense* complex derived from GenBank (Beati et al., 2013; Nava et al., 2014). Regarding the COI gene, the studied sequences had their similarities estimated through public sequences from GenBank and Barcode of Life Data Systems (BOLD–<www.barcodinglife.com>), registered as *A. cajennense* s.l. Variation of DNA sequences was estimated using the Kimura 2 parameter-K2 P (Kimura, 1980). A tree was created for o method (Neighbor-Joining–NJ) with 1000 replications in the bootstrap test. The K2 P parameter was selected as the genetic distance model in the Mega 6 software (Tamura et al., 2013).

The localities of the ticks collected in this study were plotted in a map, together with previously published records of *A. patinoi, A. mixtum* and *A. cajennense* s.s. by Nava et al. (2014), using the Geographic Coordinate System (WGS 1984, Datum: DWGS 1984) and the ESRI® ArcMap.

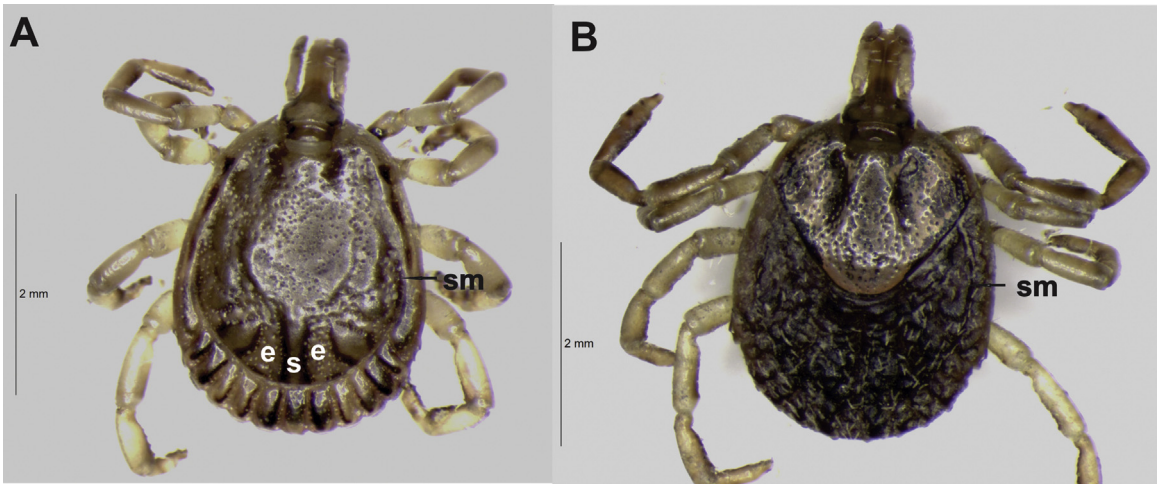

Fig. 1. Dorsal view of *Amblyomma mixtum*. (A) Male; (B) Female. (e) adjacent enamelled stripe, (s) postero-median spot, (sm) marginal groove.

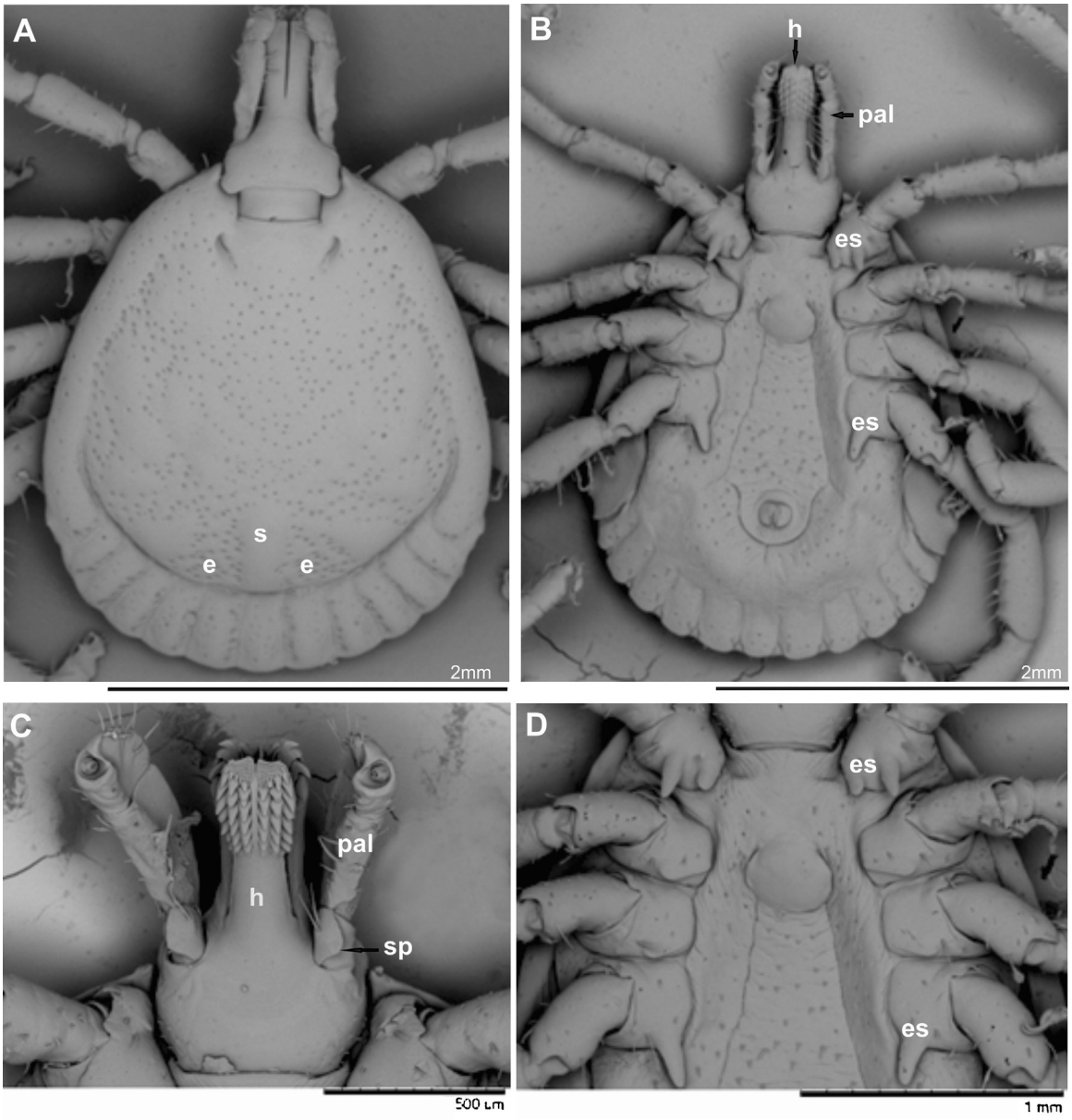

Fig. 2. Scanning Electron Microscopy (SEM) *A. mixtum* male. (A) dorsal view; (B) ventral view; (C) ventral basis capitulum; (D) coxae I–IV. (es) coxal spur, (sp) palpal ventral prolongation, (h) hypostome, (pal) palps, (e) adjacent enamelled stripe, (s) postero-median spot.

3. Results

A total of 246 adult ticks of the genus *Amblyomma* were collected in three sampled regions: 71 females and 110 males in Arauca, 27 females and 20 males in Nunchía, and 10 females and 8 males in Yopal. Initially, all ticks were morphologically identified as *A. cajennense* s.l. (Figs. 1–3). According to Nava et al. (2014), very few external morphological characters could be consistently used to separate species of the *A. cajennense* complex. One of these characters are the female genital opening, which is "V" shaped in *A. cajennense* s.s., *A. tonelliae* and *A. interandinum*, "U" shaped in *A. sculptum* and *A. mixtum,* and with short and bulging lateral flaps in *A. patinoi.* All female specimens of the present study presented an "U" shaped genital opening (Fig. 3D). According to Nava et al. (2014), morphological separation of males *A. sculptum* from *A. mixtum* are easily differentiable by the ornamentation and punctuations of the scutum, and in this case, geographical location should be applied, since the former species seems to be restricted to parts of South America south to the Amazon basin, whereas the later species occurs in regions north of the Amazon basin, from northern South America to southern Texas. Because the Orinoquía region of Colombia is located northern to the Amazon, we supposed that our specimens could be *A. mixtum.*

Attempts to conclusive taxonomic identification were performed through molecular analyses. In this case, fragments of the ITS2 gene were generated for 4 tick specimens of each Department. These sequences were aligned (832-bp) with representative sequences of the six species of the *A. cajennense* complex, in addition to *A. americanum* (outgroup), derived from GenBank. Phylogenetic analysis indicated that the Colombian specimens correspond to *A. mixtum* (Fig. 4). Similarly, fragments of the COI gene were generated from 4 tick specimens of each Department. These sequences were aligned (616-bp) with six sequences from GenBank and Barcode of Life Data Systems (BOLD) of *A. cajennense* s.l. and one of *A. americanum* (outgroup). The Colombian specimens

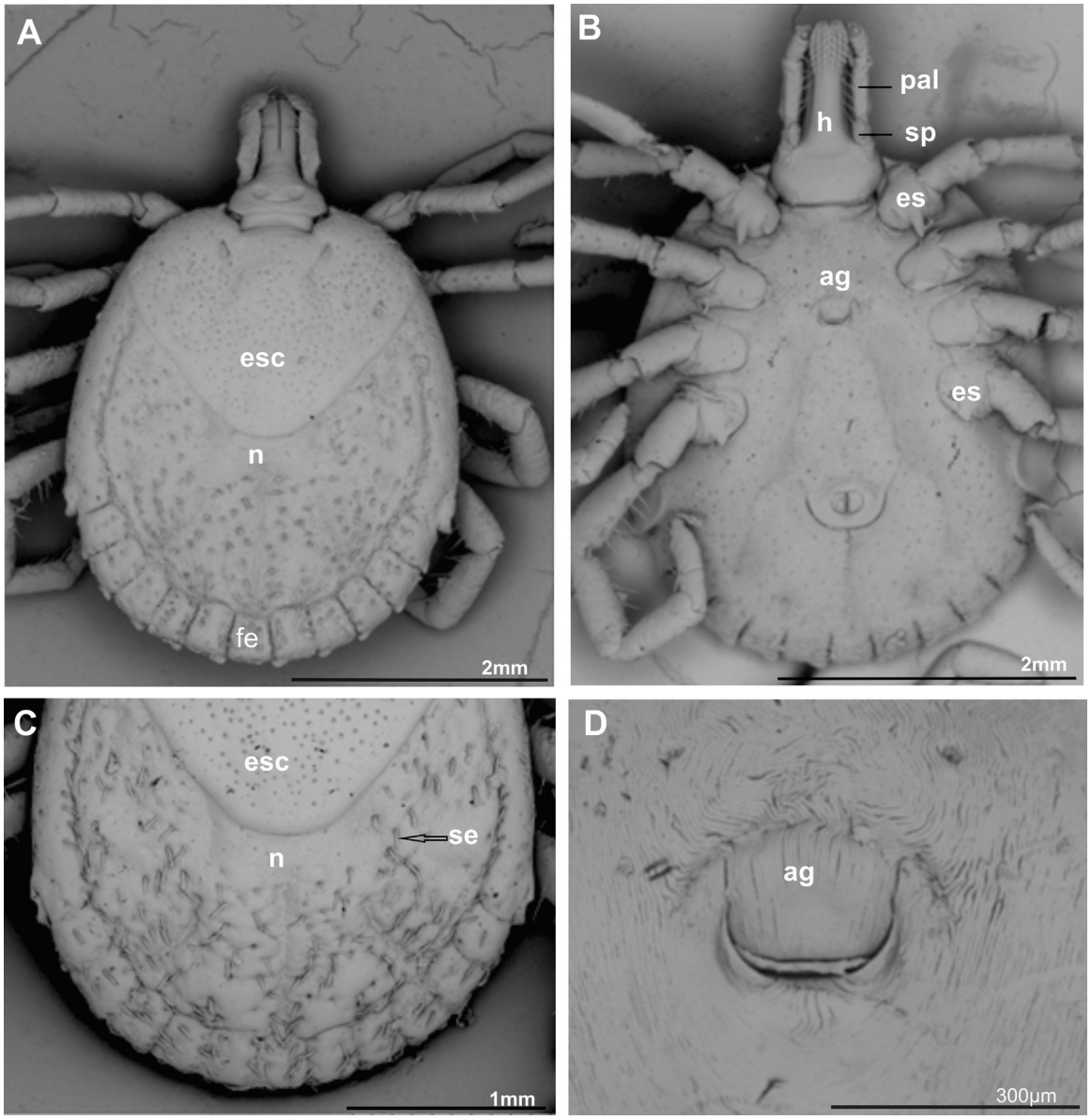

Fig. 3. Scanning Electron Microscopy (SEM) *A. mixtum* female. (A) dorsal view; (B) ventral view; (C) notum; (D) genital aperture. (ag) genital aperture, (esc) scutum, (es) coxal spur, (sp) palpal ventral prolongation, (fe) festoons, (h) hypostome, (n) notum, (pal) palps, (se) setae.

clustered with sequences from Panama (Fig. 5), which correspond to the geographic area of *A. mixtum* according to Nava et al. (2014).

The intraspecific genetic distances between the ITS2 rDNA sequences of *A. mixtum* collected in Arauca and Casanare, in Colombia, and the sequences from GenBank of *A. mixtum* from Mexico, Costa Rica, and Texas revealed 0.5% maximum difference and 0.0% minimum. On the other hand, interspecific differences among the six *A. cajennense* complex-species ranged from 0.9 to 8.3% (Table 1). Regarding the COI sequences, the minimal difference between the Colombian species occurred with an *A. cajennense* s.l. sequence from Panama (Table 2), an area with known occurrence of *A. mixtum,* according to Nava et al. (2014).

4. Discussion

Morphological and molecular analyses of field-collected specimens of *A. cajennense* s.l. in the present study clearly confirm the occurrence of *A. mixtum* in Colombia for the first time. Genetic differences between the ITS2 and COI sequences of Colombian and *A. mixtum* sequences from GenBank are in agreement with Beati et al. (2013), who performed an extensive genetic analysis of all six species of the *A. cajennense* complex. Because there have been previous records of *A. mixtum* in two neighboring countries of Colombia (Panama and Ecuador), ours findings are also corroborated by geographical data, especially because there is apparently no great eco-regional differences between the Orinoquía region of Colombia and other known *A. mixtum* areas (Estrada-Peña et al., 2014). On the other hand, Estrada-Peña et al. (2014) suggested that the Orinoquía region could be an area of sympatry or parapatry between *A. mixtum* and *A. cajennense* s.s. While we did not find any *A. cajennense* s.s. in the present study, we are aware that our convenient tick sample is not representative for the region (Fig. 6). Therefore, further studies are needed to better evaluate the possible occurrence of more species of the *A. cajennense* complex in the Orinoquía region of Colombia.

The presence of *A. mixtum* in Colombia is of medical relevance, since this specie is incriminated as the vector of *R. rickettsii* in Central America (Labruna et al., 2014). On the other hand, there has

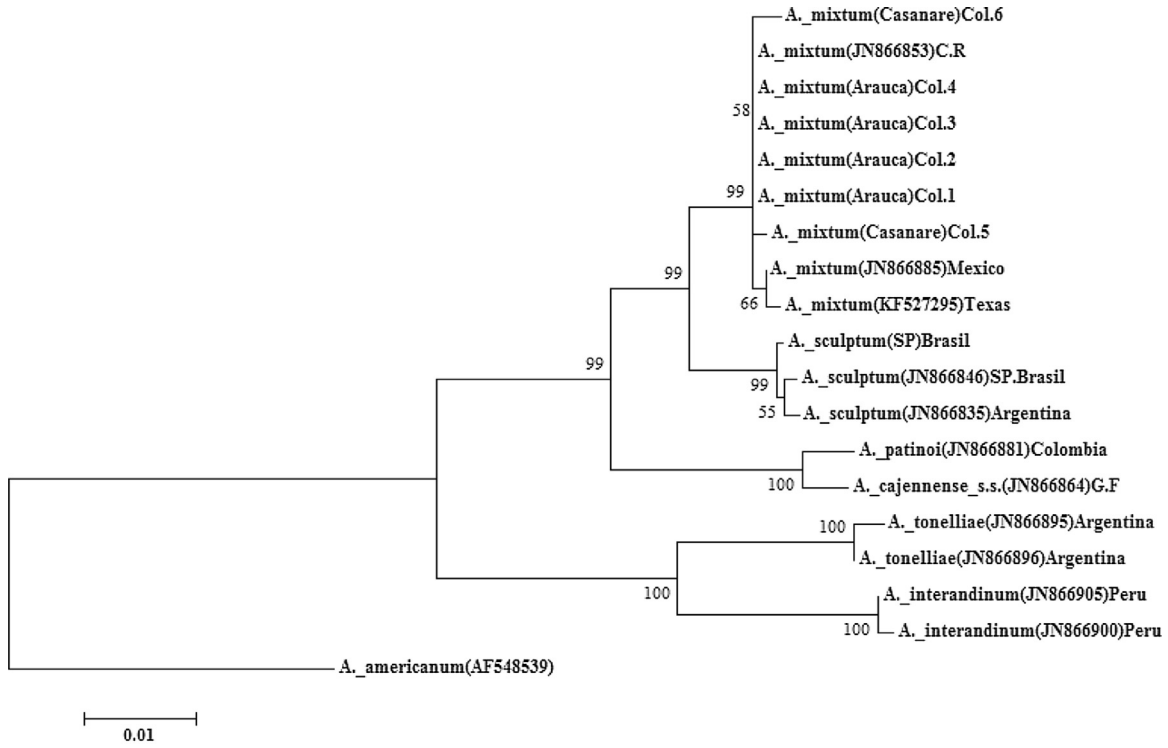

Fig. 4. Neighbor-joining tree using the sequences of the ITS2 rDNA gene.

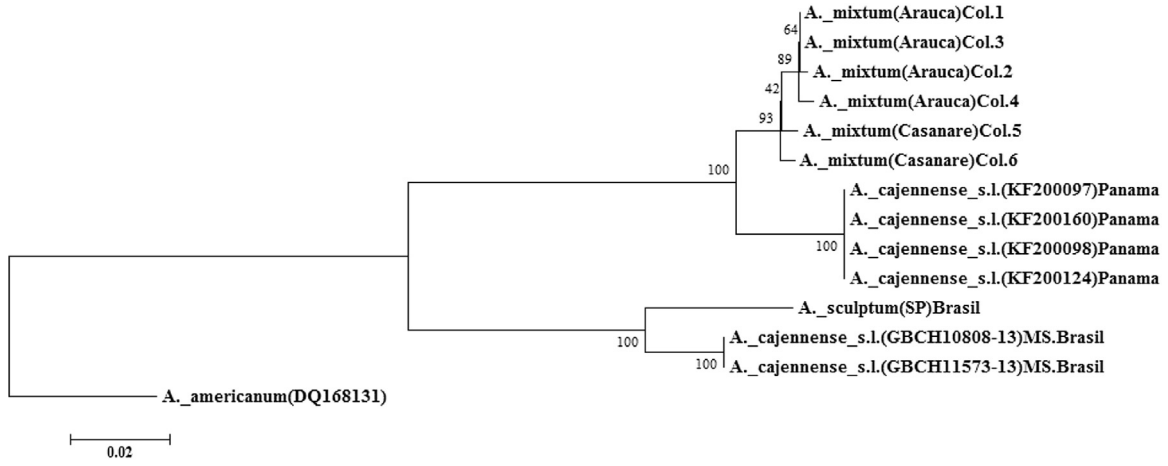

Fig. 5. Neighbor-joining tree using the sequences of the mitochondrial cytochrome c oxidase subunit I gene (COI).

Table 1
Kimura 2 parameter (K2P) distances (in percentage) for the second internal transcribed space (ITS2) sequences of species of the *Amblyomma cajennense* complex, including the *A. mixtum* sequences generated from ticks collected in Colombia in the present study.

Tick species	*A. mixtum*	*A. mixtum* (Colombia)	*A. sculptum*	*A. patinoi*	*A. cajennense*	*A. tonelliae*	*A. interandinum*	*A. americanum*
A. mixtum	0.1–0.3							
A. mixtum (Colombia)	0.0–0.5	0.0–0.4						
A. sculptum	1.4–1.8	1.4–1.8	0.1–0.4					
A. patinoi	3.5–3.8	3.5–3.8	3.8–3.9	–				
A. cajennense	3.4–3.6	3.4–3.6	3.6–3.8	0.9	–			
A. tonelliae	6.5–6.9	6.6–7.2	6.6–7.0	7.7–8.0	7.9–8.2	0.3		
A.interandinum	6.8–7.2	6.8–7.2	7.0–7.3	8.0–8.2	8.2–8.3	3.4–3.8	0.1	
A. americanum	9.6–9.9	9.6–9.9	10.0–10.1	9.7	10.4	10.7–11.0	10.4–10.6	–

been several records *A. mixtum* infection by *Rickettsia amblyommii*, a possible non-human pathogen or an agent of a much milder infectious disease (Bermúdez et al., 2009; Hun et al., 2011; Novakova et al., 2015). Previous studies in Panama and Brazil have reported that natural *R. rickettsii*-infection rates in *A. cajennense* s.l. ticks are usually very low (usually ≤1%) (Sangioni et al., 2005; Krawczak et al., 2014). On the other hand, reported rates for *R. amblyommii*-infected *A. cajennense* s.l. ticks are commonly >25% (Bermúdez et al.,

Table 2
Kimura 2 parameter (K2P) distances (in percentage) for the mitochondrial cytochrome c oxidase subunit I gene (COI) sequences of species of the *Amblyomma cajennense* complex, including the *A. mixtum* sequences generated from ticks collected in Colombia in the present study.

Tick species	*A. mixtum* (Colombia)	*A. sculptum*Brazil	*A. cajennense*. s.l. (Panamá)	*A. cajennense* s.l. Brazil	*A. americanum*
A. mixtum (Colombia)	0.2–1.0				
A. sculptum Brazil	15.6–16.0	–			
A. cajennense s.l. (Panamá)	3.2–3.8	16.2	0.0		
A. cajennense s.l. (Brazil)	14.5–14.7	4.5	14.3	0.0	
A. americanum	18.4–18.9	18.2	20.2	17.8	–

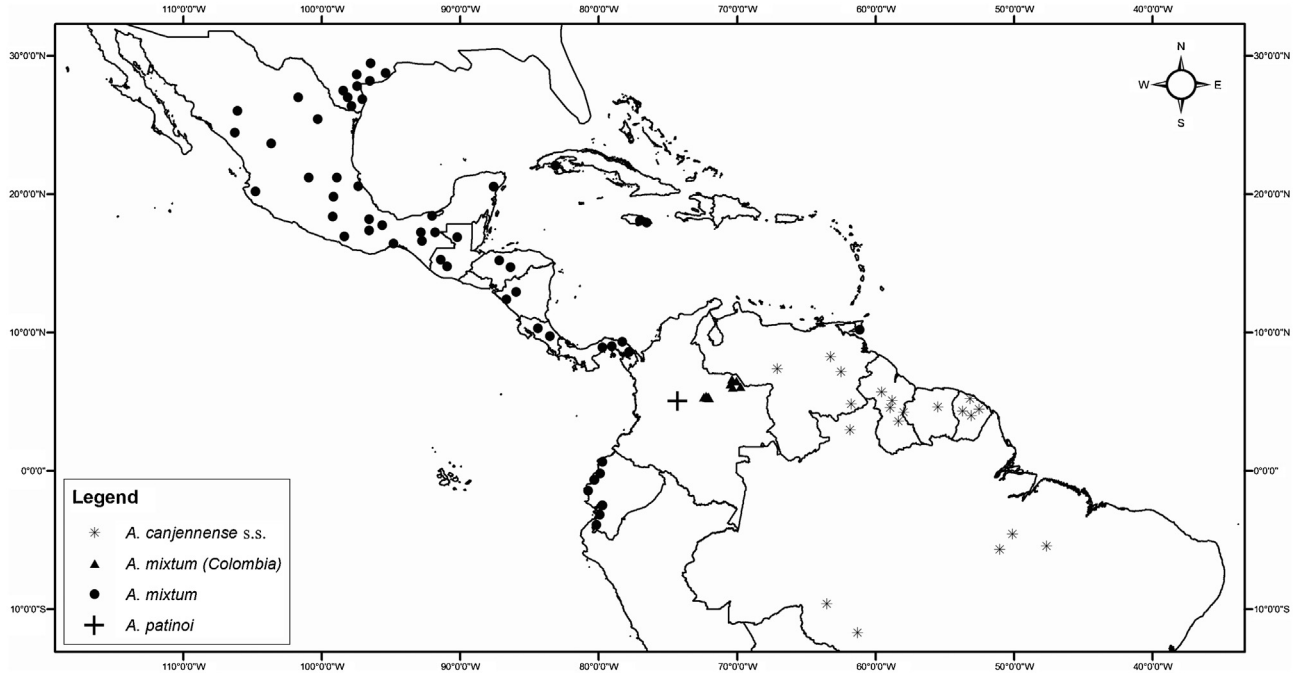

Fig. 6. Distribution of *Amblyomma mixtum*; *Amblyomma cajennense* s.s.; *Amblyomma patinoi* (modified from Nava et al., 2014), with the first records in Colombia of *A. mixtum*▲.

2009; Labruna et al., 2004; Soares et al., 2015). Because laboratory studies have suggested that previous infection by *R. amblyommii* could prevent a severe disease during a subsequent infection by the highly pathogenic *R. rickettsii* (Blanton et al., 2014; Rivas et al., 2015), it is possible that previous human contact with *A. mixtum* ticks could decrease potentially fatal spotted fevers, yet to be reported from the study area.

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4.3. CAPÍTULO 3

A case of gynandromorphism in *Amblyomma mixtum* (Acari, Ixodidae).

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Apreciado profesor Rivera,

Con la presente acuso recibo de su trabajo: ***A case of gynandromorphism in Amblyomma mixtum (Acari, Ixodidae)*** para ser publicado en la Revista Colombiana de Entomología. Su manuscrito ha sido aceptado para la publicación de la RCdeE y le haremos llegar la prueba galeras en cuanto esté lista.

Apreciamos su consideración de someter el trabajo a nuestra revista; lo invitamos a citar este trabajo en sus siguientes publicaciones y a someter nuevos manuscritos en la RCdeE.

Cordialmente,

JAMES MONTOYA LERMA
Editor General
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A case of gynandromorphism in *Amblyomma mixtum* (Acari, Ixodidae)

Un caso de ginandromorfismo en *Amblyomma mixtum* (Acari, Ixodidae)

Short title: Gynandromorphism in *Amblyomma mixtum*

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Resumen

Ginandromorfismo es una condición donde un organismo exhibe simultáneamente características morfológicas de macho y hembra. En Colombia el taxón *Amblyomma cajennense* está representado por las especies *Amblyomma patinoi* y *Amblyomma mixtum*. En septiembre de 2014 en la Orinoquía Colombiana, de infecciones naturales de bovinos y equinos, se colectaron y determinaron garrapatas adultas. En una infección natural de bovino se describió un ginandromorfo clasificado morfológicamente como *A. mixtum.* Éste es el primer registro en la literatura de un ginandromorfo *A. mixtum*, y la primera descripción de un ginandromorfo para una especie de garrapata en Colombia.

Palabras clave: Colombia. Garrapata. Orinoquía.

Abstract

Gynandromorphism is a condition in which an organism simultaneously exhibits male and female morphological characteristics. In Colombia, the taxon *Amblyomma cajennense* is represented by the species *Amblyomma patinoi* and *Amblyomma mixtum*. In September of 2014, in the Colombian Orinoco region, adult ticks were collected and determined from natural infections in bovines and equines. A gynandromorph was described from a natural infestation on a bovine, and morphologically classified as *A. mixtum*. This is the first literature report of a gynandromorph of *A. mixtum*, and the first description of a gynandromorph for a tick species in Colombia.

Keywords: Colombia. Ticks. Orinoco.

Introduction

Until a few years ago, the taxon *Amblyomma cajennense* (Fabricius, 1787) represented a single tick species distributed from southern United States to northern Argentina (Estrada-Peña *et al*. 2004). Recently, the taxon was reorganized into a complex of six valid species: *A. cajennense* sensu stricto (Fabricius, 1787) (restricted to the Amazonian region), *Amblyomma mixtum* (Koch, 1844) (from Texas to western Ecuador), *Amblyomma sculptum* (Berlese, 1888) (northern Argentina, Bolivia, Paraguay, Brazil), *Amblyomma interandinum* (Beati, Nava and Cáceres, 2014) (inter-Andean valley of Peru), *Amblyomma tonelliae* (Nava, Beati and Labruna, 2014) (dry areas of northern Argentina, Bolivia, and Paraguay), and *Amblyomma patinoi* (Labruna, Nava and Beati, 2014) (Eastern Cordillera of Colombia) (Beati *et al*. 2013; Nava *et al*. 2014). In Colombia, this species complex is currently represented by *A. patinoi* (Nava *et al*. 2014) and *A. mixtum* (Rivera-Páez *et al*. 2016). Nevertheless, the current knowledge of the distribution of these species in America is likely incomplete and a species-level definition is necessary (Nava *et al*. 2014). At least three species of the complex, namely *A. sculptum*, *A. mixtum*, and *A. patinoi*, are important vectors of the bacterium *Rickettsia rickettsii*, the causal agent of the Rocky Mountain spotted fever, the deadliest tick-borne bacterial disease of the world (Krawczak *et al*. 2014; Labruna *et al*. 2014; Faccini-Martínez *et al*. 2015).

Gynandromorphs are individuals that possess phenotypic characteristics of males and females, and have been reported in several insect, spider, and tick taxa (Eritja 1996; Labruna *et*

al. 2002). Their maturation begin during embryonic development, due to a loss of or damage to sex chromosomes, binucleated eggs, or infections related to *Wolbachia* species, a common endosymbiont (Narita *et al*. 2010; Keskin *et al*. 2012). Gynandromorphism in ixodid ticks is little known, but the phenomenon has been extensively reviewed and approximately 77 naturally-occurring cases have been documented (Prusinski *et al*. 2015). In the genus *Amblyomma*, over 20 cases have been reported among nine species, including *A. cajennense*, from which two 2 cases have been reported (Labruna *et al*. 2002). Based on the geographical origin (southeastern Brazil) of these two cases in *A. cajennense*, they are likely to correspond to *A. sculptum.* To date, no descriptions or records of gynandromorph presence have been reported in ticks in the Colombian territory.

Material and methods

During a field study on ticks infesting domestic animals in the Colombian Orinoco region, Arauca municipality, Arauca department (07° 3’ 55” N, 70° 44’ 2” W) during September of 2014 (Rivera-Páez *et al*. 2016), the presence of a gynandromorph specimen of *A. mixtum* was noticed and collected from a cow (*Bos taurus*). The gynander was taxonomically evaluated (Jones *et al*. 1972; Nava *et al*. 2014), through a light microscope (Leica M205C stereomicroscope) and a scanning electron microscope (SEM) (Hitachi Scanning Electron Microscope, model TM3000) following techniques described by Corwin *et al*. (1979).

Results and discussion

All male and female specimens in the Orinoquía region of Colombia (Rivera-Páez *et al*. 2016) presented external morphological characters of *A. mixtum*, the males of *A. mixtum* the principal diagnostic character is the tick body outline, round in *A. mixtum* and oval in *A. patinoi* (Fig. 1A, 2A) and females of *A. mixtum* can be differentiated from females belonging to the other species of the group by the combination of notal setae stout and long, more densely distributed on the posterior half of the notum (Fig. 2A), small tubercles (Fig. 2A), and a U-shaped genital aperture with 2 narrow lateral flaps (Nava *et al*. 2014). Dorsally, the gynandromorph of *A. mixtum* showed the left idiosoma with male characteristics and a right idiosoma with typical female characteristics. Conversely, the capitulum possessed female traits at both sides, including a pair of porose areas and equal-sized palps (Fig. 1A, 2A-2B).

A dorsal midline separated the scutum in the male side, where it covered the alloscutum, and the female side showed a reduced scutum, typical of females (Fig. 1A, 2A). The scutum of the specimen showed typical female ornamentation and punctations on the right side, and typical male ornamentation and punctations on the left side as well (Fig. 1A, 2A). The dorsal midline ended at the middle of the sixth festoon, which had male and female halves corresponding to the rest of the dorsal area. A distinct complete lateral groove, typical of *A. mixtum* males, was also present at the male half (Fig. 1A, 2A). Ventrally, the results support those observed at the dorsal view, with a left side that corresponds to the typical male, and a right side corresponding to female, with the ventral midline extending from the capitulum to the sixth festoon (Figure 1B, 2C). The spurs on coxae I–III were typical of the corresponding sex on each side; conversely, coxa IV spur is typically male at both sides, corresponding to a single long, stout, pointed spur (Fig. 1B, 2C-2D).

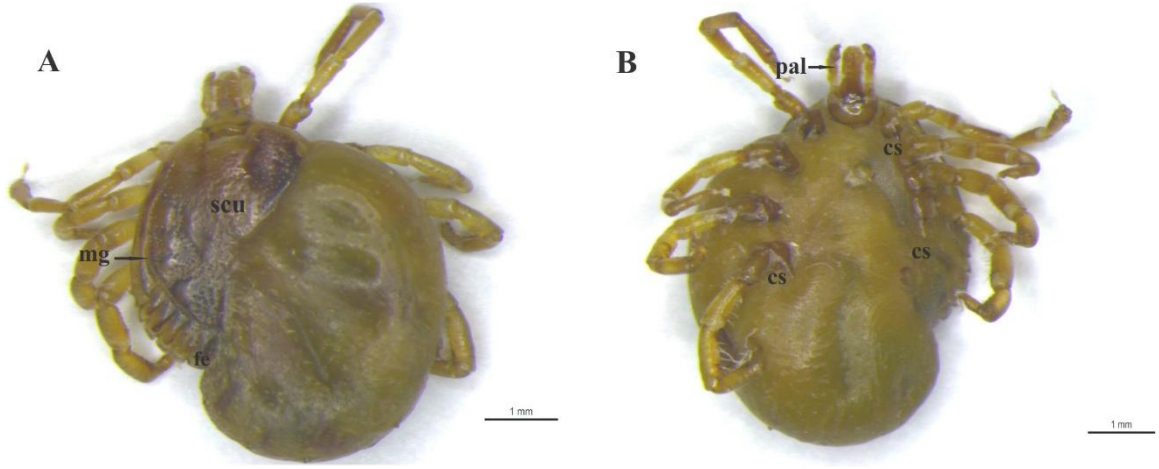

Fig. 1. (A) Dorsal and (B) Ventral view of *Amblyomma mixtum* gynandromorph. (cs) coxal spur, (fe) festoons, (mg) marginal groove, (pal) palps, (scu) scutum.

According to previous definitions of the types of gynandromorphism in ticks (Campana-Rouget 1959), the gynandromorph of *A. mixtum* described in this study is classified as a gynander intriqué of a protogynander, which means that the external sex-linked features are equally represented, except for ‘‘islands’’ of male or female chitin embedded in areas of the opposite sex. In the present specimen, these “islands” are present in the capitulum (mostly of the female type) at the male side, and at coxa IV of the female side. The most common type of gynandromorphism is a bipartite protogynander, whereas gynander intriqué is very rare in ticks

(Labruna *et al*. 2002; Keskin *et al*. 2012). Among the genus *Amblyomma,* the bipartite protogynander is indeed the most common type of gynandromorphism (Labruna *et al*. 2002; Campana-Rouget 1959).

This research represents the first record of a tick gynandromorph in Colombia, and the first for *A. mixtum*.

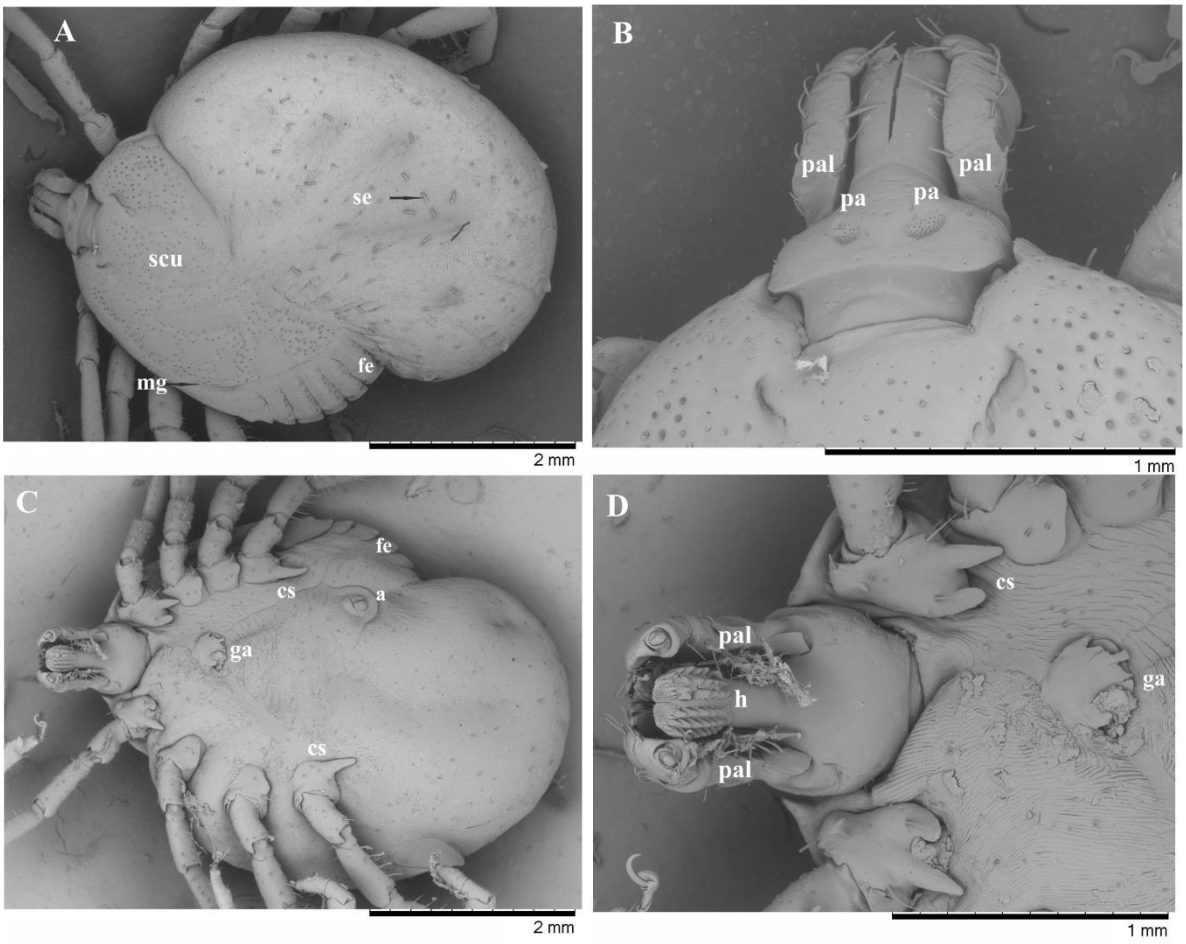

Fig. 2. Scanning Electron Microscopy (SEM) of *A. mixtum* gynandromorph. (A) dorsal view; (B) dorsal basis capitulum; (C) ventral view; (D) ventral basis capitulum. (a) anal aperture, (cs) coxal spur, (fe) festoons, (ga) genital aperture, (mg) marginal groove, (h) hypostome, (pal) palps, (pa) porose area, (scu) scutum, (se) setae.

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4.4. CAPÍTULO 4

Comparative analysis of germ cells and DNA of the genus *Amblyomma:* adding new data on *Amblyomma maculatum* and *Amblyomma ovale* species (Acari: Ixodidae).

Parasitol Res
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ORIGINAL PAPER

Comparative analysis of germ cells and DNA of the genus *Amblyomma*: adding new data on *Amblyomma maculatum* and *Amblyomma ovale* species (Acari: Ixodidae)

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Abstract Among tick species, members of the subfamily Amblyomminae have received special attention, since they serve as vectors for pathogens such as *Rickettsia* spp. and display cryptic species complexes that make their taxonomical classification challenging. *Amblyomma ovale*, *Amblyomma maculatum*, and other species of the genus *Amblyomma* have shown a long history of taxonomic controversies. Spermiotaxonomy has proved to be a valuable tool in the solution of systematic conflicts in Metazoa that can aid molecular and external morphological analyses in ticks and, overall, provide more robust analyses and results. With this in mind, this study included histological analyses of the reproductive system of the species *A. ovale* and *A. maculatum*, as well as the description of morphohistological characters of the male reproductive system of ticks of the genus *Amblyomma*, in order to evaluate these characters within the current clustering proposals. In addition, 16S rDNA and COI (mitochondrial) molecular markers were used to study the genetic relationships of the species. The results show that the tick male reproductive system and its germ cells contain useful candidate characters for taxonomical analyses of Ixodida.

Keywords *Cryptic species* · Spermiotaxonomy · Ticks · Histology · Molecular markers

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Introduction

Tick (Ixodidae) systematics has been discussed for decades, and periodically, the phylogeny of several groups is revisited through new analytical tools. Among the most known tick subfamilies, Amblyomminae has received special attention, since it harbors cryptic species that, until recently, were considered polymorphic by authors that followed the analyses of Hoogstraal and Aeschlimann (1982). Some important revisions have been made using molecular and biogeographical phylogeny techniques such as those developed by Black and Piesman (1994), Burger et al. (2012), Beati et al. (2013), and Nava et al. (2014), which confirmed the polyphyletic origin of the subfamily, its current distribution in the American continent, and the real taxonomic status of some species of medical and veterinarian importance, such as the *Amblyomma cajennense* sensu lato complex.

The *Amblyomma maculatum* group includes the following species: *A. maculatum* (Koch, 1844); *Amblyomma neumanni* (Ribaga, 1902); *Amblyomma parvitarsum* (Neumann, 1901); *Amblyomma tigrinum* (Koch, 1844); and *Amblyomma triste* (Koch, 1844) (Camicas et al. 1998). Camicas et al. (1998) clustered them within the *Amblyomma ovale* group that includes *A. ovale* and *Amblyomma aureolatum*, in the revisited version of the subgenus *Anastosiella*, originally proposed by Santos Dias (1963).

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The taxa *A. maculatum*, *A. triste*, and *A. tigrinum* were described as valid species by Koch (1844), based on adult specimens from the USA, Uruguay, and Brazil, respectively. However, Neumann (1899) considered that these three taxa belonged to a single species, namely *A. maculatum*, which was adopted by subsequent authors, until the study of Kohls (1956), who revalidated the three species based on morphological characters. Since then, although considered to be separate species, the identification of these tick species has been controversial, in particular the distinction between *A. maculatum* and *A. triste* (Estrada-Peña et al. 2005; Guglielmone et al. 2013; Lado 2015; Mendoza Uribe and Chavez Chorocco 2004; Mertins et al. 2010). Lado (2015), based on different mitochondrial and nuclear molecular markers, suggests that *A. triste* should be synonymized with *A. maculatum*.

On the other hand, *Amblyomma aureolatum* (Pallas, 1772) and *Amblyomma ovale* (Koch, 1844) have been described as the "*ovale*" complex by Aragão and da Fonseca (1961). These authors redescribed the male and female of both tick species, discussed the systematics, and convincingly supported the use of the name *A. aureolatum* and *A. ovale* (Guglielmone et al. 2003).

Against this backdrop, this study aimed to reanalyze the systematics and phylogenetic positions of representatives of Amblyominnae subfamily, including the species *A. ovale* and *A. maculatum* collected in Colombia, adding new data related to their reproductive system and germ cell morphology given the association of this species with epidemiological issues and public health in North and South America (Ferrari et al. 2012; Guglielmone et al. 2003; Lado et al. 2015; Nava et al. 2008). Furthermore, this study compiled the results obtained until now, based on the descriptions by Sampieri et al. (2014, 2016a, b), on the anatomy and morphology of the male tick reproductive system, mainly the genus *Amblyomma* with occurrence in Brazil and Colombia.

A list of useful characters in taxonomic analyses was obtained from the results generated in these studies adding the new data obtained here which were contrasted with the molecular data generated from the analysis of the mtDNA COI and 16S rDNA gene sequences, in order to confirm if the male reproductive system and its germ cells are a source of candidate characters for tick systematics.

Materials and methods

Study species and molecular evaluation

During the months of August 2014, January and August 2015, and May 2016, ticks of the species *A. ovale* and *A. maculatum* were directly collected from dogs (*Canis familiaris*) in the municipalities of Saldaña (3° 55′ 36″ N, 74° 58′ 33″ W) and Ibagué (04° 23′ 50″ N, 75° 8′ 12″ W) in the department of Tolima, Andean region of Colombia. The ticks were taxonomically identified based on their external morphology, using a light microscope (Leica M205C stereomicroscope), following Aragão and da Fonseca (1961), Jones et al. (1972), and Estrada-Peña et al. (2005). In addition, specimens from each species were prepared for scanning electron microscopy (SEM) (Hitachi Scanning Electron Microscope model TM3000), according to the techniques described by Corwin et al. (1979).

Following the morphological identification, a molecular assessment was performed for the species *A. ovale* and *A. maculatum*, as well as for the species analyzed in terms of their reproductive system by Sampieri et al. (2014, 2016a, b), being these: *A. aureolatum*, *A. triste*, and *Amblyomma sculptum* (Berlese, 1888) (Ixodidae, Amblyomminae). The species *Rhipicephalus sanguineus* s.l. (tropical linage) and *Ornithodoros rostratus* (Aragão, 1911) were used as outgroups I and II, respectively. In the same way, the reproductive system of *A. ovale* and *A. maculatum* (Colombia) were processed and analyzed by histological techniques as described in Sampieri et al. (2016a, b).

Specimens of each species, *A. aureolatum*, *A. triste*, *A. sculptum*, *A. ovale*, *A. maculatum*, and *Rhipicephalus sanguineus* s.l., were individually processed for the molecular analyses. For this purpose, DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocol, and tested by two PCR protocols, one targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene and the other the 16S rDNA gene. For the COI gene PCR, we used primers LCO1490 (F) 5-GGTCAACAAATCATAAAGATATTGG-3 and HCO2198 (R) 5-TAAACTTCAGGGTGACCAAAAAATCA-3 (Folmer et al. 1994). For the 16S rDNA gene, we used primers F 5′-CTGCTCAATGATTTTTTAAATTGCTGTGG-3′ and R 5′-CCGGTCTGAACTCAGATCAAGT-3′ (Norris et al. 1996). In addition to the *Amblyomma* species used for the morphological studies, the species *A. cajennense* s.s. (Brazil) and *A. mixtum* (Colombia) were also amplified, as representatives of the "*cajennense*" complex.

The PCR products were visualized on horizontal 1% agarose gels with 1X TBE pH 8.0 running buffer at 110 V/50 mA, stained with SYBR Safe® dye and photo-documented on a GelDoc-It®2310 Imager (UVP). The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen®), according to the manufacturer's instructions, and sent to Macrogen Inc. (South Korea) for DNA sequencing. The sequenced fragments were analyzed and edited using Geneious Trial v8.14 (Drummond et al. 2009) and Sequencher 4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA). In addition, the sequences were searched by MegaBlast against the public databases and deposited in Genbank and Barcode of Life Data Systems (BOLD). The sequences for each gene

were aligned using ClustalW (Thompson et al. 1997), included in the program MEGA version 7 (Tamura et al. 2013). Intraspecific nucleotide divergences were estimated with the program MEGA, using the Kimura 2 Parameter distance model (K2P; Kimura 1980). Species confirmation was carried out through a similarity analysis, based on maximum likelihood (ML), with the K2P model and 1000 bootstrap replications, using the program MEGA.

Morphohistology and matrix construction

For this study, we initially used the morphological characters of the male reproductive system obtained by Sampieri et al. (2014, 2016a, b). The characters were centered on three representative species of the genus *Amblyomma*: *A. aureolatum* (A), *A. triste* (B), and *A. sculptum* (C), and the species *Rhipicephalus sanguineus* s.l. (tropical lineage) and *Ornithodoros rostratus* were used as outgroup 1 (GE1) and outgroup 2 (GE2), respectively. In addition to the previously mentioned species, the reproductive system of *A. ovale* (D) and *A. maculatum* (E) were analyzed through histological techniques. Since these specimens were collected in situ while feeding on the blood of their natural hosts and their feeding was interrupted, it was not possible to describe the complete morphohistology of their reproductive system, given that the system's development is directly related to feeding.

Candidate characters with relevant variations were obtained from the observation of the morphology and ultramorphology of the male reproductive system of these species, and were coded based on the character state, such as apomorphic = 1 and plesiomorphic = 0. Then, a character matrix containing the taxa, characters, and character state for each species (0 or 1) was constructed. We determined the qualitative Jaccard index based on 10 morphological characters (Moreno 2001); furthermore, we used cluster analysis of the single linkage based on the Jaccard index in order to compare the segregation pattern of the species included in the study. The cluster analysis was performed using the software PAST 3.11 (Hammer et al. 2001).

This study was submitted and approved by the Ethical Committee in Animal Use (CEUA) from the Biological Sciences Institute of UNESP, Rio Claro, SP, Brazil, number 017/2012, Protocol 1422.

Results

Study species and molecular assessment

The morphological analyses showed that the ticks collected from dogs in Colombia were most similar to *A. maculatum*, as indicated by the scutal ornamentation, coxal spur patterns, and presence of tubercles at the postero-internal angle of all festoons, except for the middle one (Fig. 1a–c). Although, the *A. maculatum* specimens from Colombia typically were shown to have a pair of stout ventral spurs on the distal extremity of metatarsi II, III, and IV (Fig. 1c). The determination of *A. ovale* was carried out based on the scutal ornamentation patterns in the females, marginal grooves of the males (incomplete in *A. aureolatum* and complete in *A. ovale*), and differences in spurs of coxa I in both sexes.

The molecular analyses of the 403 bp 16S rDNA gene fragment and 480 bp COI gene fragment showed topologically similar phylogenetic trees (Fig. 2a, b) and allowed a clear determination of the study species, with the exception of *A. maculatum* and *A. triste*, which showed interspecific genetic distances too small to be considered different species, ranging between 0.0 and 2.1 for 16S and between 0.0 and 5.2 for COI, with the highest values found for *A. maculatum*

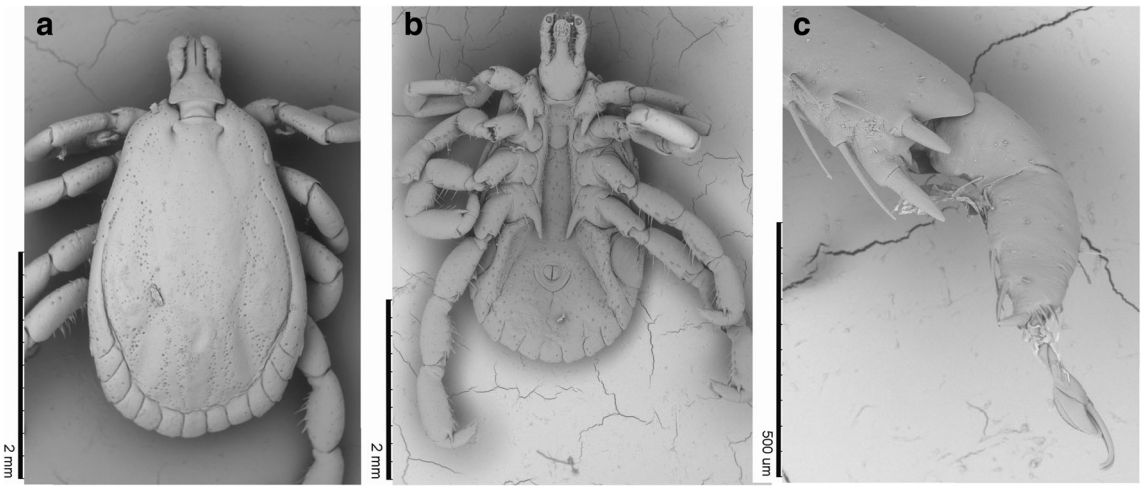

Fig. 1 *A. maculatum* specimens from Ibagué- Colombia. **a** Male dorsal view *A. maculatum.* **b** Male ventral view *A. maculatutum*. **c** Spines on metatarsus IV of female *A. maculatum*

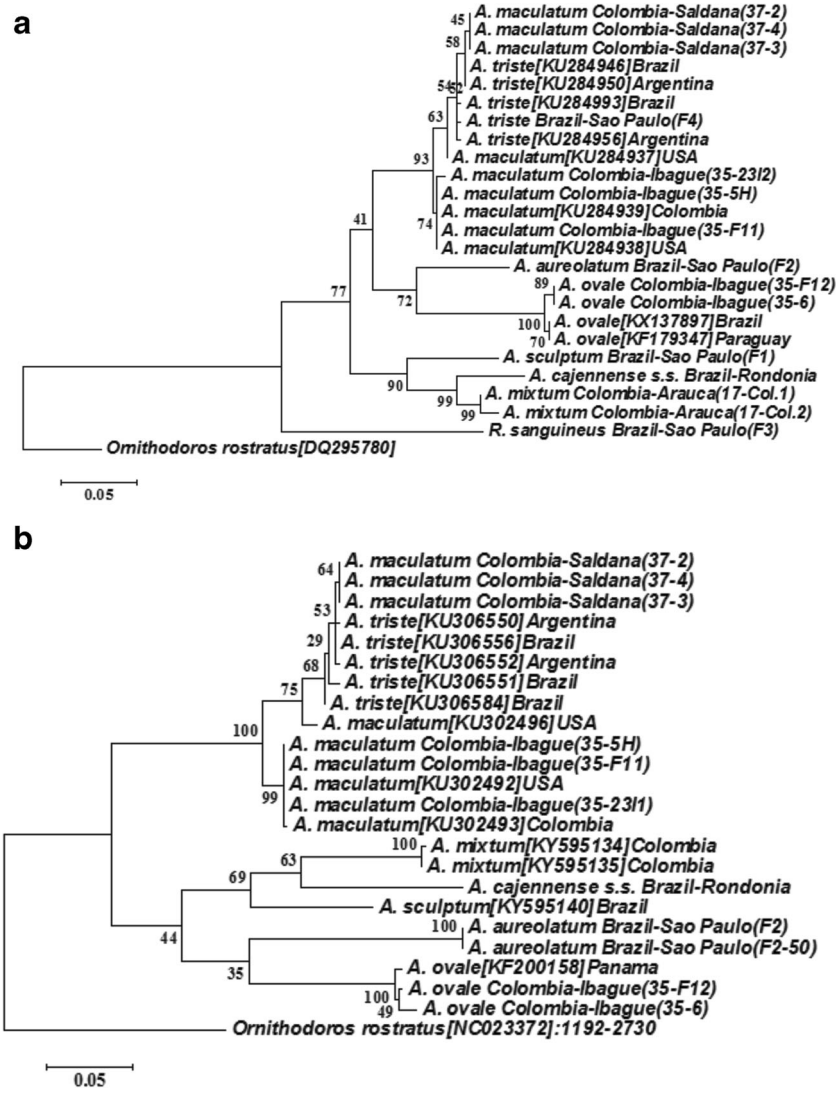

Fig. 2 **a** Maximum likelihood (ML) tree using the sequences of the mtDNA 16S rDNA gene. **b** Maximum likelihood (ML) tree using the sequences of the mitochondrial cytochrome c oxidase subunit I (COI) gene

(Tables 1 and 2). These interspecific genetic distances allowed us to cluster the species belonging to the *A. cajennense* s.l., *Amblyomma maculatum*, and *Amblyomma ovale* complexes (Fig. 2a, b). For the genus *Amblyomma*, interspecific genetic distances ranged from 6.1–23.8 for 16S and 16.2–26.9 for COI, excluding the differences found between *A. triste* and *A. maculatum*, which ranged between 0.3–2.1 and 0.2–5.2 for 16S and COI, respectively (Tables 1, 2).

Table 1 Kimura 2 parameter (K2P) distances (in percentage) for the mtDNA 16S rDNA gene

Tick species	*A. ovale*	*A. aureolatum*	*A. maculatum*	*A. triste*	*A. cajennense* s.s.	*A. mixtum*	*A. sculptum*	*R. sanguineus*	*O. rostratus*
A. ovale	0.0–0.9								
A. aureolatum	14.6–14.9	–							
A. maculatum	14.6–15.3	12.5–13.9	0.0–2.1						
A. triste	15.3–16.0	13.5–14.2	0.3–2.1	0.0–0.9					
A. cajennense s.s.	23.4–23.8	18.6	15.3	15.0–16.0	–				
A. mixtum	20.6–22.6	15.3–16.8	13.1–14.9	13.1–15.6	6.1–7.4	1.2			
A. sculptum	19.0–20.1	15.6	15.3	15.6–16.4	13.2	10.8–12.1	–		
R. sanguineus	28.7–29.1	24.5	22.5–23.7	23.3–23.7	26.7	26.2–27.4	24.5	–	
O. rostratus	34.0–35.0	35.9	27.4–27.8	27.8–29.1	38.4	35.4–37.3	32.7	36.9	–

Table 2 Kimura 2 parameter (K2P) distances (in percentage) for the mitochondrial cytochrome c oxidase subunit I (COI) gene

Tick species	*A. ovale*	*A. aureolatum*	*A. maculatum*	*A. triste*	*A. cajennense* s.s.	*A. mixtum*	*A. sculptum*	*O. rostratus*
A. ovale	0.8–1.7							
A. aureolatum	20.4–21.2	0.0						
A. maculatum	18.9–21.5	25.1–26.0	0.0–5.2					
A. triste	18.9–20.1	25.0–25.7	0.2–5.2	0.2–1.3				
A. cajennense s.s.	21.1–21.7	25.7	26.2–26.9	25.4–26.6	–			
A. mixtum	20.0–20.9	23.3–23.6	20.2–21.1	20.2–21.1	16.2–16.4	0.2		
A. sculptum	22.0–22.3	21.6	21.4–22.2	21.6–21.9	17.0	16.9–17.1	–	
O. rostratus	28.6–29.2	31.8	27.7–30.2	29.5–29.9	32.8	32.8	27.4	–

Morphohistology

The male reproductive system of *A. maculatum* and *A. ovale* studied here showed a basic morphology similar to other species of the genus and the family Ixodidae. For both species, it was not possible to observe if the testicles were connected to the distal region. However, both species showed germ cells organized in packets lined with a simple epithelium along the testicles up to a certain spermiogenesis developmental stage.

In *A. maculatum*, only two spermatid developmental stages were observed: (a) the initial developmental stage (sp I), where no cell limits are evident and the nucleus is round, occupying most of the cytoplasm (Fig. 3a, b) and (b) mature spermatids (spm) in the interior of the seminal vesicles that display clear cell limits, with the central region of the cytoplasm enclosing the membranous complex, a filiform, and extensive nuclear process; caudal region without constriction, with a round pole; and anterior region with a round rimless operculum (Fig. 3c).

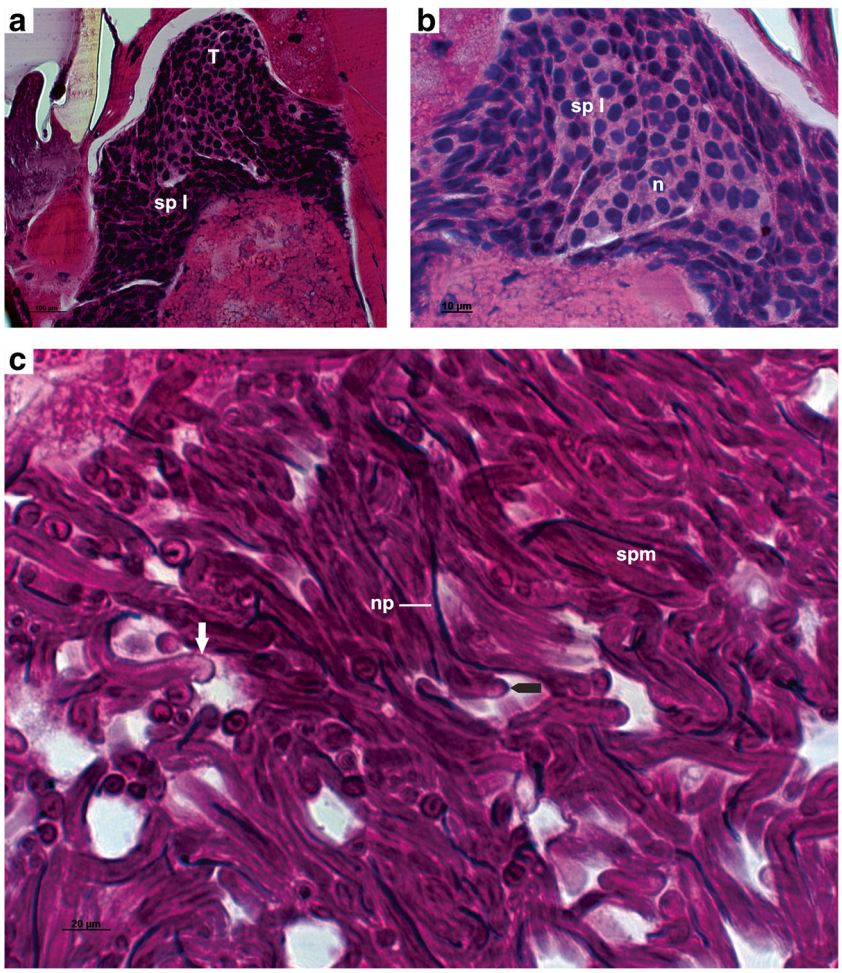

Fig. 3 Histological sections of the *Amblyomma maculatum* reproductive system (Ibagué - Colombia). **a**–**c** Testis housing spermatids I (sp I). Mature spermatids (spm) exhibiting a round operculum (arrow)

In *A. ovale*, several spermiogenesis stages were observed, for a total of six morphologically distinct stages. The first stage displayed an identical morphology to that described for *A. maculatum* (Fig. 4a, b). In the second stage (sp II), the cells begin a hypertrophy process with a cytoplasmic enlargement and no evident cell limits (Fig. 4c). The spermatid cell limits were observed only by the third development stage (sp III), indicating the beginning of formation of membranous cisternae. In this stage, the nucleus is enlarged and round, with one of two visible nucleoli, and the presence of cytoplasmic bridges between cells (Fig. 4d). The most marked morphological alterations can be observed at stage IV, in which cell limit enlargement is evident, the spherical cell shape becomes amoeboid, the nucleus becomes compact, and the cytoplasm is very heterogeneous (Fig. 4e, f). The next stage, sp V, is characterized by cell elongation and membrane cisternae fusion, as well as a marked condensation and elongation of the nucleus, which at this moment is referred to as the nuclear process; in addition, there is a total loss of spermatocyte organization (Fig. 4g).

The sixth and last stage (sp VI) of spermiogenesis exhibits a typical tick spermatic cell: elongated and filiform,

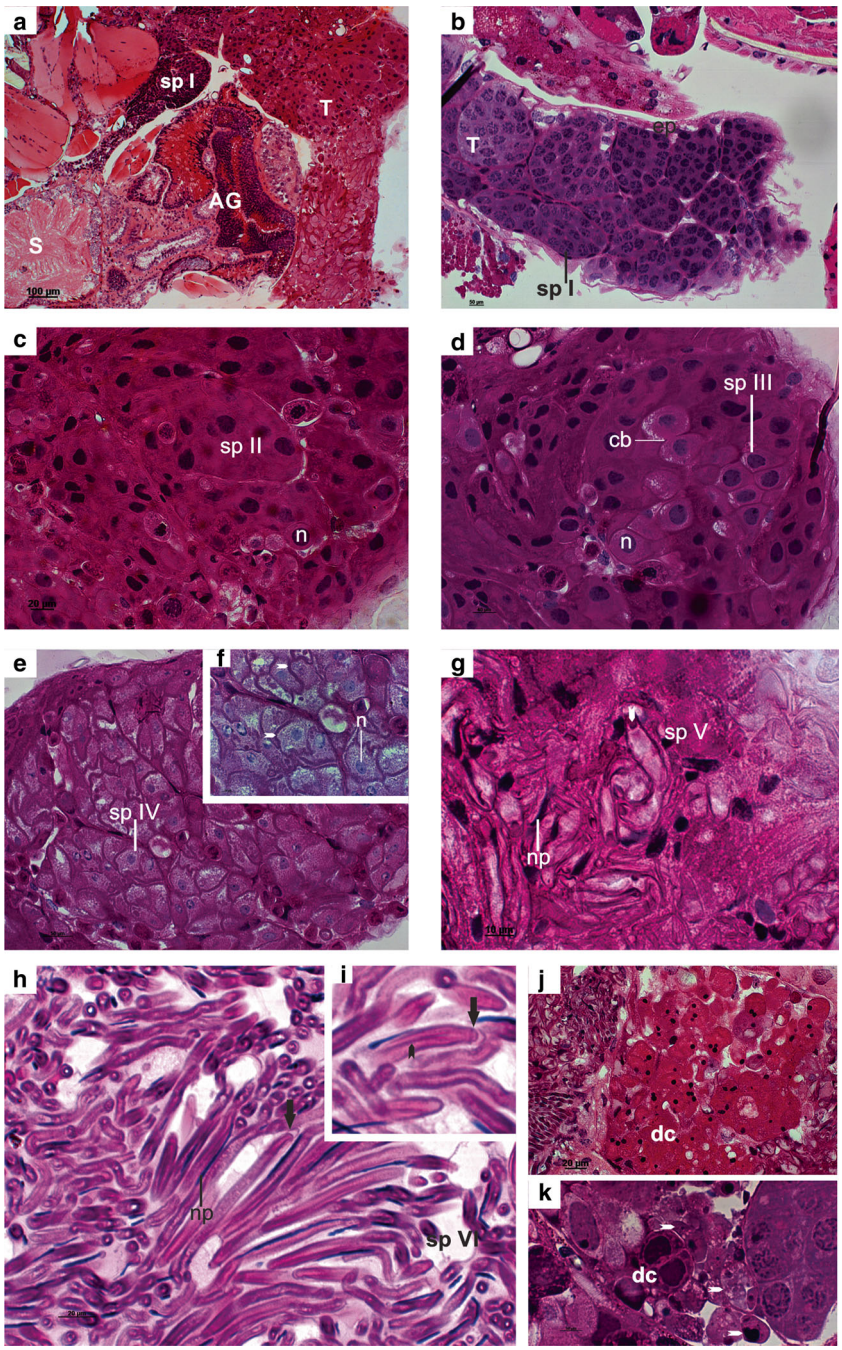

Fig. 4 Histological sections of the *Amblyomma ovale* reproductive system (Saldaña - Colombia). **a** Overview of the reproductive system, highlighting the accessory glad complex (AG) and testis (T) housing spermatocysts and germ cells in different stages. **b–d** Spermatocysts housing spermatids I (sp I), II (sp II), and III (sp III). **e**, **f** Spermatid IV (sp IV) going through hypertrophy and cisternae formation (arrowhead). **g** Spermatid V (sp V) characterized by the elongation process. **h**, **i** Spermatids VI (sp VI) matured, highlighting the nuclear process (np) and the operculum (arrow). **j**, **k** Spermatids undergoing degeneration process (DC). cb = cellular bridges; n = nucleus; S = singanglium

with a nuclear process highly stained by hematoxylin and positioned in the cellular cortex, with a membranous complex in the center of the cytoplasm that longitudinally crosses the spermatid. Furthermore, sp VI of *A. ovale* characteristically shows a cone-shaped operculum with a rim at its base, evident in histological sections, with a tail-like non-constricted posterior cell region and cone-shaped pole (Fig. 4h, i). In *A. ovale* and *A. maculatum*, many degenerating germ cells were observed, which displayed autophagy and varied apoptosis stages that included apoptotic bodies (Fig. 4j, k).

Character selection for matrix construction

The species *O. rostratus* (Argasidae), used as outgroup, has plesiomorphic characters, since it is known that representatives of the family Argasidae show basal characters and it is likely that the ancestor that gave rise to Ixodidae was similar to the members of this family. Therefore, the characters chosen and the stages used in this study were (1) testicle anatomy—single testicle (0), paired testicles with connection (1), and individualized paired testicles (1); (2) seminal vesicle—lateral disposition (0) and dorsal (1); (3) spermatogenesis—finalized at the immature stage (0) and finalized at the adult stage (1); (4) cytoplasmic bridges—absent (0) and present (1); (5) nucleus shape and chromatin condensation in the spermatid with cisternae formation—round with centralized condensed chromatin (0) and oval with peripheral condensed chromatin (1); (6) number of stages of membranous cisternae formation in spermatids—three (0) and two (1); (7) nuclear process formation—not evident (0) and evident (1); (8) operculum rim—present (0) and absent (1); (9) midline constriction of the tail-like in mature spermatid—presente (0) and ausente (1); and (10) nucleus shape in mature spermatid—filiform (0) and spiral (1).

The data gathered is included in Table 3, which displays the following results: (a) characters 1 and 2 are apomorphic for species A–E and GE1; (b) character 3 shows a synapomorphy between all taxa, except for B; (c) characters 4 and 6 cluster species A–D and GE1; and (d) characters 5, 8, 9, and 10 do not show any evident clustering and will be discussed below. The qualitative

Table 3 Morphological characters matrix used for evaluation of the male reproductive system of ticks

Taxons \ *Character*	1	2	3	4	5	6	7	8	9	10
A	1	1	0	1	1	1	1	0	0	1
B	1	1	1	1	1	1	1	1	0	1
C	1	1	0	1	1	1	1	0	1	0
D	1	1	0	1	0	1	1	0	1	0
E	1	1	0	--	--	--	1	1	1	0
GE1	1	1	0	1	1	1	0	1	1	1
GE2	0	0	0	0	0	0	0	0	0	0
	ABCDE	**ABCDE**	**ACDE**	**ABCD**	**ABC**	**ABCD**	**ABCDE**	**BE**	**CDE**	**AB**

GE *Grupo Externo*, ***A*** *A. aureolatum*, ***B*** *A. triste*, ***C*** *A. sculptum*, ***D*** *A. ovale*, ***E*** *A. maculatum*

Table 4 Jaccard's index of similarity—morphological characters used for evaluation of the male reproductive system of ticks

	A	B	C	D	E	GE1	GE2
A	1						
B	0.66	1					
C	0.66	0.43	1				
D	0.54	0.33	0.81	1			
E	0.40	0.40	0.75	0.75	1		
GE1	0.54	0.54	0.54	0.43	0.55	1	
GE2	0.17	0.05	0.17	0.25	0.16	0.11	1

GE *Grupo Externo*, ***A*** *A. aureolatum*, ***B*** *A. triste*, ***C*** *A. sculptum*, ***D*** *A. ovale*, ***E*** *A. maculatum*

Jaccard index showed that the analysis of the 10 morphological characters allowed to differentiate each of the species studied, since the similarity coefficient is always less than 1 (Table 4). The cluster analysis of the single linkage based on the Jaccard index, in order to compare the segregation pattern of the species included in the study, clustered the five species of the subfamily Amblyomminae, excluding the species *Rhipicephalus sanguineus* s.l. (tropical lineage) and *Ornithodoros rostratus*, used as outgroup 1 (GE1) and outgroup 2 (GE2), respectively (Fig. 5).

Discussion

The external morphology of *A. ovale* and *A. maculatum* corresponded to that proposed by Aragão and da Fonseca (1961), Estrada-Peña et al. (2005) and Jones et al. (1972). The 16S and COI genes analyses confirmed the current taxonomic proposals, supporting the separation of the subfamilies *Amblyominnae* and *Rhipicephalinae* and of the species complex within the genus *Amblyomma*. The 16S and COI genes indicate that *A. maculatum* and *A. triste* may in fact be a single species with a wide geographic distribution, as has been proposed by other authors (Estrada-Peña et al. 2005; Lado 2015). However, it is noteworthy that the intraspecific genetic distance of *A. maculatum* is the highest of all the species studied, which could indicate that the external morphological evaluation has its limitations, and many individuals that are characterized as *A. maculatum* can actually correspond to other species of the "*maculatum*" complex. This can only be confirmed through experimental crosses between species of the complex from different localities.

The taxonomical analysis performed in this study presents important information regarding the phylogeny of the ticks of the genus *Amblyomma*, which has been the center of constant controversies and revalidations in the past years. Despite the evident need for a greater sample size of taxa for the genus, as well as a broader character selection for cladogram construction, several homology hypotheses are informative and consistent with the well-established clusters. The novel aspect of this study is the implementation of morphological characters not used until now, in order to understand the relationships between tick species. As the studies by Sampieri et al. (2016a, b) have shown, spermiotaxonomy is a promising tool for separating Ixodida species, and their phylogeny can benefit from the information generated through this analysis and the understanding of tick morphology and ultrastructure of the reproductive system and its germ cells.

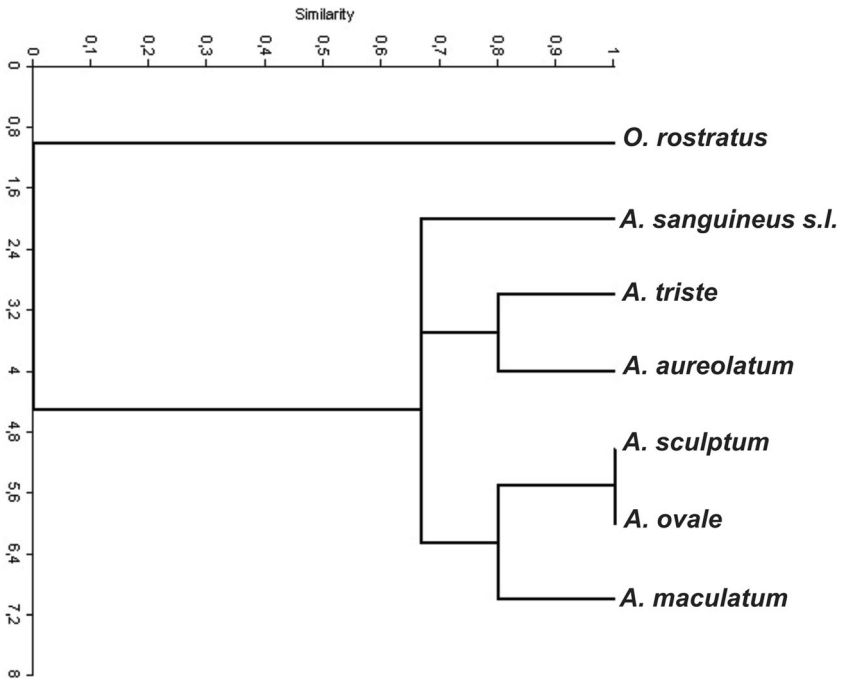

Fig. 5 Cluster analysis of the single linkage with Jaccard distance in order to compare the segregation pattern of the species included in the study. Characters (1, 2, 7–10). **A. sculptum* is different from *A. ovale* in character 5 (character not included)

This species separation is possible by analyzing characters 8, 9, and 10 (Table 3), which do not generate plausible clustering hypotheses, since in all three cases, the species *R. sanguineus* was clustered with *Amblyomma* species. For character 8, *A. sculptum*, *A. ovale*, and *A. aureolatum* were excluded from the cluster, while for character 9, *A. triste* and *A. aureolatum* were excluded, and for character 10, *A. sculptum*, *A. ovale*, and *A. maculatum* were not part of the cluster. In view of this, the three characters (8—presence of operculum rim, 9—midline constriction of the tail-like region, 10—nuclear process morphology) can be used together to analyze the ultramorphology of mature spermatids of each species and allow their separation, since these characters, along with operculum shape (unique to each species studied to date), can aid in confirming two or more closely related species. This information allows us to suggest that *A. maculatum* (Colombia) and *A. triste* (Brazil), belonging to the "*maculatum*" complex in the Americas, are representatives of different species, according to our morphohistological study (Similarity 0.4) (Table 4); however, these results must be complemented with studies at the ultrastructural level.

Characters 1 and 2 cluster taxa A–E and GE1, thereby clustering all of the species of the genus *Amblyomma* and *R. sanguineus* and suggesting that these are a synapomorphy of the family Ixodidae. In a similar manner, the homology proposed by characters 4 and 6 most likely clusters this family, since they only exclude taxon E (due to the lack of information on these characters for this taxon). In the case of character 3, the only species excluded from the cluster is *A. triste*, which is questionable whether this character is eligible for this type of analysis. An incorrect interpretation of the studies of Sampieri et al. (2016b) could have occurred regarding the presence of spermatocytes II in cell division in adults of *A. triste* (which is why we concluded that the first analysis of character 3 was not eligible for cladistic analysis). Character 5 suggests an improbable clustering and could have been observed from morphological differences generated from technical artifacts.

Character 7 suggests an interesting clustering, since taxa A–E are included, and it shows the formation of a nuclear process that could probably be a synapomorphy of the subfamily Amblyomminae. In this analysis, the characters observed from previously published studies and the data presented herein show the relevance of identifying novel characters for research in taxonomy and phylogeny, since family- and subfamily-level clustering is possible in these cases and, in the same way, the separation of cryptic species can be complemented with this information (Table 4) and cluster characters (1, 2, 7–10) (Fig. 5).

For a spermiotaxonomy robust analysis, a wide sampling and experimentation is required, but still can be a useful tool in solving problems of this nature when DNA analysis is ambiguous or not conclusive, as in the case of the comparison between *A. maculatum* and *A. triste*.

Although this is a preliminary study, the data obtained indicate that the morphology and ultrastructure of the male reproductive system in ticks can generate eligible characters for establishing homology hypotheses and cladistic studies. A robust phylogeny for Ixodida can be constructed through detailed studies of these characters, making use mainly of ultrastructural analyses and relating ticks with other Parasitiformes with widely studied reproductive systems and spermatozoids, thus contributing to an integrative taxonomy of this group.

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4.5. CAPÍTULO 5

Rickettsial infection in ticks (Acari: Ixodidae) of domestic animals in Colombia.

Rickettsial infection in ticks (Acari: Ixodidae) of domestic animals in Colombia

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Abstract

Currently, rickettsiosis is an emerging or re-emerging disease, with a worldwide distribution associated to transmission by vector arthropods. *Rickettsia* species belong to the spotted fever group (SFG) and are transmitted by hard-bodied ticks (Acari: Ixodidae) that act as vectors and reservoirs. In Colombia, little is known of the role of each species of the family Ixodidae in rickettsial circulation and transmission. We carried out molecular detection and characterization of *Rickettsia* species from 204 specimens of the family Ixodidae (7 species), collected from domestic hosts during active feeding. Sampling took place in 17 municipalities of 10 departments of Colombia, from August 2014 to May 2016. We performed PCR amplification of fragments of the citrate synthase "*glt*A" gene and outer membrane protein "*omp*A" gene. Of the 204 samples analyzed, 11.3% (23) were positive for rickettsial infection. We found three *Rickettsia* species belonging to the spotted fever group, constituting the first reports of *Rickettsia rickettsii* in various departments. Furthermore, we found the first presence of *Candidatus* Rickettsia andeanae in the Colombian territory, a

species with an unknown pathogenic role for man. These results raise awareness regarding the need to increase epidemiological control measures, as well as to consider new endemic regions in Colombia for Rocky Mountain Spotted Fever (RMSF).

Keywords: Spotted Fever, *Candidatus* Rickettsia andeanae, endemic region, *Rickettsia rickettsii*.

Introduction

The genus *Rickettsia* encompasses strict intracellular bacteria that are transmitted by arthropods and mainly infect endothelial cells (Walker, 1982; Oteo et al., 2014). The genus is divided into four large groups: the typhus group (TG), the spotted fever group (SFG), the transitional group (TRG) and the ancestral group (AG) (Londoño et al., 2017). Rodents and ticks are the main reservoirs of rickettsias; the latter become contaminated by ingestion of the bacteria during feeding on the infected host, or through transovarial or trans-stage transmission (Soares et al., 2012).

The first reported outbreak of tick-borne rickettsiosis in Colombia occurred between 1934 and 1936 in the municipality of Tobia, department of Cundinamarca; thus its name "Tobia spotted fever" for Colombia, which corresponded to Rocky Mountain Spotted Fever (RMSF), caused by *Rickettsia rickettsii*. This disease affected 20% of the population and led to the death of 62 out of 65 patients registered (Patiño, 1941; Patiño et al., 1937). Following a prolonged epidemiological silence, in 2003 and 2004, Hidalgo et al. (2007a) confirmed *R. rickettsii* as the causal agent of the death of two patients in the same region of Cundinamarca. Recently, three important SFG rickettsiosis outbreaks have occurred in Colombia, all caused by *R. rickettsii*: Necoclí - Antioquia (2006), with five patients deceased out of 14 registered cases (Acosta et al., 2006); Los Córdobas – Córdoba (2007), with 11 confirmed cases and the death of six patients (Hidalgo et al., 2007b), and Turbo – Antioquia (2008), with four patients deceased out of 15 reported cases (Pacheco et al., 2008).

Faccini-Martinez et al. (2015) isolated *R. rickettsii* and molecularly detected *Rickettsia amblyommii* from *Amblyomma patinoi* in the department of Cundinamarca (Faccini-Martinez et al., 2016). Londoño et al. (2014) registered specimens of *Amblyomma*

ovale in the departments of Antioquia and Córdoba, infested by *Rickettsia* sp. strain Atlantic rainforest, which has shown to be pathogenic to humans. Gómez-Quintero et al. (2017) registered a probable case of rickettsial infection by a member of the SFG in a patient of the same region of the Colombian Orinoquía where Rivera et al. (2016) found the presence of *Amblyomma mixtum*, a proven vector of *R. rickettsii*.

In this context and considering the medical importance of the different species of the genus *Rickettsia*, the aim of this study was to detect and molecularly characterize *Rickettsia* species associated with hard-bodied ticks, collected from domestic hosts in various departments of the Colombian territory.

Materials and Methods

From August 2014 to May 2016, hard-bodied ticks (Acari: Ixodidae) were directly collected from domestic hosts during active feeding. Overall, 204 tick specimens were obtained, belonging to seven species of the family Ixodidae (Table 1). The specimens corresponded to 197 adults and 7 nymphs, collected from the following domestic hosts: cattle (*Bos taurus*), domestic dogs (*Canis lupus familiaris*), horses (*Equus caballus*), donkeys (*Equus asinus*), and one domestic pig (*Sus scrofa*), in addition to two tick samples collected from the vegetation, in 17 municipalities of 10 departments of Colombia. Voucher tick specimens were deposited at the following Brazilian tick collection: "Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva" (University of São Paulo, São Paulo, Brazil).

For the molecular detection and analysis of *Rickettsia* species, ticks were individually submitted to DNA extraction, using the DNeasy Blood and Tissue kit (Qiagen, Chatsworth, California), following the manufacturer´s protocol. Extracted DNA samples were tested by PCR, using primers CS-78 and CS-323, targeting a ~401-bp fragment of the citrate synthase gene (*glt*A) for presumably all *Rickettsia* species (Labruna et al., 2004). This gene fragment is conserved among species of the spotted fever group (SFG), but is variable among species not belonging to the SFG. To avoid false negatives, the positive and negative samples for *glt*A were further tested by another PCR protocol, using primers Rr190.70p and Rr190.602n, targeting a ~530-bp fragment of the 190-kDa outer membrane protein gene (*omp*A), present only in SFG *Rickettsia* species (Regnery et al., 1991). In each set of reactions, negative

control tubes containing water and a positive control tube containing DNA of *Rickettsia parkeri* strain NOD were included.

PCR products were purified with the QIAquick PCR purification kit (Qiagen) and sent to the Universidad de los Andes (Bogotá-Colombia) for DNA sequencing. The *glt*A and *omp*A gene sequences were analyzed using Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) to determine the closest similarities with other *Rickettsia* species. Species confirmation was carried out through a similarity analysis among gene *omp*A, based on Maximum Likelihood (ML), with K2P substitution model and 1,000 bootstrap replications, using the program MEGA version 7 (Tamura et al., 2013).

Table 1. Hard-bodied tick species and specimens analyzed in this study.

Tick species	Number of specimens/Stage	Municipality
A. dissimile	1 Nymph	Yopal.
A. maculatum	19 Adults	Ibagué; Saldaña; Norcasia.
A. mixtum	51 Adults/2 Nymphs	Arauca; Neira; Nunchía; Yopal; S. J. de Arama.
A. ovale	10 Adults	Ibagué; Saldaña.
D. nitens	33 Adults	Leticia; Arauca; San Jacinto; Nunchía; Yopal; Ibagué.
R. microplus	34 Adults/2 Nymphs	Leticia; Arauca; Fortul; Saravena; San Jacinto; S. J. de Arama; Ibagué; Restrepo.
R. sanguineus s. l.	49 Adults/3 Nymphs	Leticia; Medellín; Arauca; Saravena; Dorada; Yopal; Puerto Salgar; Ibagué; Saldaña; Cali; Restrepo.
	Total : 204	

Results

We found 23 tick specimens positive for rickettsial infection (11.3%), from a total of 204 tick specimens comprising seven species tested by PCR for rickettsial infection (Table 2). Three rickettsial agents were detected (Fig. 1): *R. rickettsii* in *A. mixtum* (11 infected/53 tested; 20.7% infection rate); *Candidatus* Rickettsia andeanae in *Rhipicephalus sanguineus* sensu lato (2/52; 3.8%), *A. ovale* (4/10; 40%) and *A. maculatum* (3/19; 15.8%); and *Rickettsia* sp. strain Colombianensi in *A. mixtum* (2/53; 3.8%) and *Amblyomma dissimile* (1/1; 100%). The three species belong to the spotted fever group (Table 2).

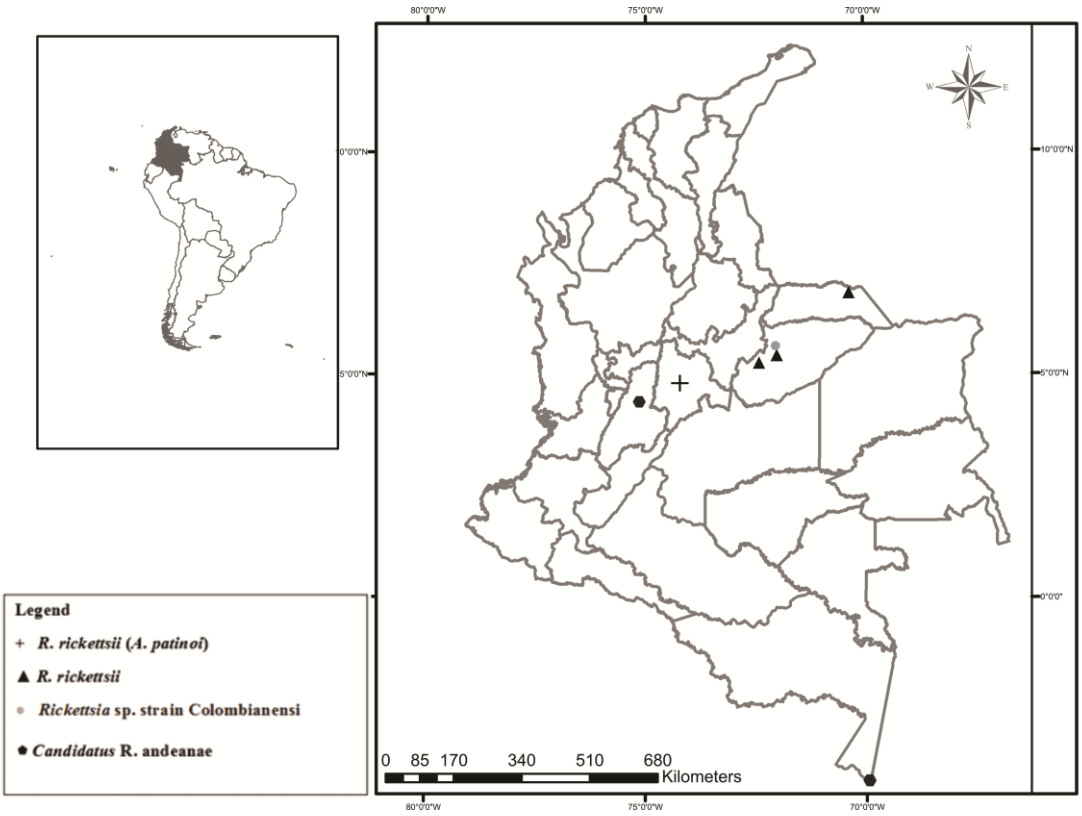

Fig.1. New registers of *Rickettsia* species. **(+)** First reported outbreak of a tick-borne rickettsiosis in Colombia (Tobia-Cundinamarca) - *Rickettsia rickettsii*.

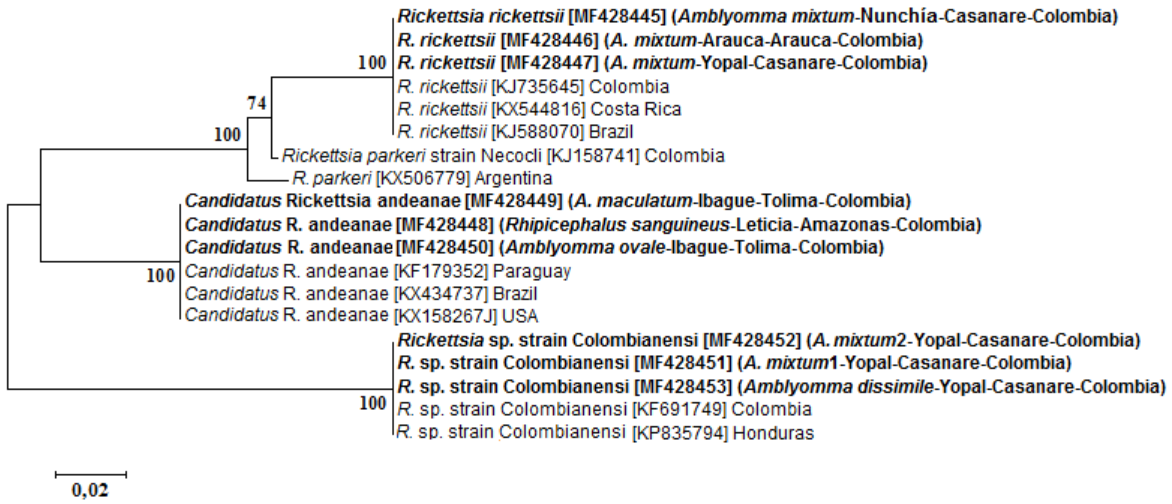

Fig.2. Maximum Likelihood (ML) tree using sequences of the 190-kDa outer membrane protein gene (*omp*A) present only in SFG *Rickettsia* species. *Rickettsia* species present in hard-bodied ticks (Acari: Ixodidae) from various regions of Colombia.

Table 2. Results of molecular identification of rickettsiae - infected ticks from domestic animals in Colombia.

Department	Municipality	Host	Tick species (number of specimens/stage)	No. infected/No. tested (%)	Closest GenBank identity (gene: accession number)
Amazonas	**Leticia**	*Bos taurus*	*R. microplus* (14/Adults)	0/14 (0)	
		B. taurus - Equus caballus	*D. nitens* (4/Adults)	0/4 (0)	
		Canis lupus familiaris	*R. sanguineus* s. l. (6/Adults)	2/6 (33.3)	100% *Candidatus* R. andeanae [*omp*A: KX158267]
Antioquia	**Medellín**	*C. lupus familiaris*	*R. sanguineus* s. l. (2/Adults)	0/2 (0)	
Arauca	**Arauca**	*E. caballus - B. Taurus - Sus scrofa*	*D. nitens* (15/Adults)	0/15 (0)	
		E. caballus - B. taurus-	*A. mixtum* (23/Adults)	6/23 (26.1)	100% *Rickettsia rickettsii* [*omp*A: KJ735645]
		E. caballus - C. lupus familiaris	*R. sanguineus* s. l. (5/Adults)	0/5 (0)	
		B. taurus	*R microplus* (2/Adults)	0/2 (0)	
	Fortul	*B. taurus*	*R. microplus* (2/Adults)	0/2 (0)	
	Saravena	*B. Taurus - C. lupus familiaris*	*R. microplus* (3/Adults; 2 Nymps)	0/5 (0)	
		C. lupus familiaris	*R. sanguineus* s. l. (2/Adults)	0/2 (0)	
Bolívar	**San Jacinto**	*E. caballus- Equus asinus*	*D. nitens* (4/Adults)	0/4 (0)	
		E. asinus	*R. microplus* (1/Adults)	0/1 (0)	
Caldas	**Dorada**	*C. lupus familiaris*	*R. sanguineus* s. l. (2/Adults)	0/2 (0)	
	Neira	*B. taurus*	*A. mixtum* (5/Adults)	0/5 (0)	
	Norcasia	*C. lupus familiaris*	*A. maculatum* (2/Adults)	0/2 (0)	
Casanare	**Nunchía**	*E. caballus - B. taurus -* Vegetation	*A. mixtum* (12/Adults)	3/12 (25)	100% *Rickettsia rickettsii* [*omp*A: KJ735645]
		E. caballus	*D. nitens* (2/Adults)	0/2 (0)	
	Yopal	*C. lupus familiaris - E. caballus*	*R. sanguineus* s. l. (6/Adults)	0/6 (0)	
		E. caballus	*D. nitens* (4/Adults)	0/4 (0)	
		E. caballus - E. asinus	*A. mixtum* (10/Adults)	2/10 (20)	100% *Rickettsia rickettsii* [*omp*A: KJ735645]
		Vegetation	*A. mixtum* (2/Nymps)	2/2 (100)	*Rickettsia* sp. strain Colombianensi 100% [*omp*A: KF691749]
		Vegetation	*A. dissimile* (1/Nymps)	1/1 (100)	*Rickettsia* sp. strain Colombianensi 100% [*omp*A: KF691749]
Cundinamarca	**Puerto Salgar**	*C. lupus familiaris*	*R. sanguineus* s. l. (2/Adults)	0/2 (0)	
Meta	**S. J. de Arama**	*B. taurus*	*A. mixtum* (1/Adults)	0/1 (0)	
		B. taurus	*R. microplus* (2/Adults)	0/2 (0)	
Tolima	**Ibagué**	*B. taurus*	*R. microplus* (7/Adults)	0/7 (0)	
		B. taurus- C. lupus familiaris	*R. sanguineus* s. l. (12/Adults)	0/12 (0)	
		C. lupus familiaris	*A. maculatum* (3/Adults)	3/3 (100)	100% *Candidatus* R. andeanae [*omp*A: KX158267]

		C. lupus familiaris	*A. ovale* (8/Adults)	4/8 (50)	100% *Candidatus* R. andeanae [*omp*A: KX158267]
		E. caballus - *E. asinus*	*D. nitens* (4/Adults)	0/4 (0)	
	Saldaña	*C. lupus familiaris*	*R. sanguineus* s. l. (8/Adults)	0/8 (0)	
		C. lupus familiaris	*A. maculatum* (14/Adults)	0/14 (0)	
		C. lupus familiaris	*A. ovale* (2/Adults)	0/2 (0)	
Valle del Cauca	**Cali**	*C. lupus familiaris*	*R. sanguineus* s. l. (2/Adults; 3 Nymps)	0/5 (0)	
	Restrepo	*C. lupus familiaris*	*R. sanguineus* s. l. (2/Adults)	0/2 (0)	
		B. taurus	*R. microplus* (3/Adults)	0/3 (0)	
			Total: 204 (196 Adults; 8 Nymphs)	23/204 (11.3)	

The partial gene sequences for *glt*A and *omp*A of *R. rickettsii*, *Candidatus* Rickettsia andeanae and *Rickettsia* sp. strain Colombianensi were both 100% identical to the corresponding sequences available for each species in Genbank (Table 2, Fig. 2). GenBank nucleotide sequence accession numbers for the partial sequences generated in the present study are [MF428454-MF428463] for the *glt*A gene, and [MF428445-MF428453] for the *omp*A gene.

Discussion

This study demonstrated the presence of three *Rickettsia* species of the SFG, based on PCR amplification of *glt*A and *omp*A gene fragments, in six municipalities of four departments of Colombia (Fig. 1): *R. rickettsii*, in Arauca (Arauca), Yopal and Nunchía (Casanare); *Candidatus* R. andeanae, in Leticia (Amazonas) and Ibagué (Tolima); and *Rickettsia* sp. strain Colombianensi, in Yopal, (Casanare).

In Colombia, *R. rickettsii* has been the only species of the SFG identified in human cases as well as in *A. patinoi* ticks (Patiño et al., 1937; Faccini-Martinez et al., 2015). Furthermore, the central (department of Cundinamarca) and northwestern (departments of Cordoba and Antioquia) regions of Colombia have constituted the two known endemic regions for rickettsiosis (Acosta et al. 2006; Hidalgo et al. 2007a, 2011). In this study, we provide the first reported presence of *R. rickettsii*, the etiological agent of the most severe spotted fever in the world, in three municipalities of three departments. In the municipalities of Arauca (Arauca), Yopal and Nunchía (Casanare), we found the presence of *R. rickettsii* in *A. mixtum*, the second species of the *Amblyomma cajennense* complex recently registered for

Colombia (Rivera et al., 2016) and a proven vector for *R. rickettsii*. Serological studies in human and animals conducted in the Colombian Orinoquía (departments of Arauca, Casanare, Guaviare, Meta, and Vichada) showed a high seroprevalence against SFGR (Miranda et al., 2011; Riveros-Pinilla et al., 2015) and, recently, Gómez-Quintero et al. (2017) reported a probable case of infection by a *Rickettsia* species of the SFG. Overall, we propose that the molecular confirmation of *R. rickettsii* in three municipalities of the Orinoquía provide clear evidence that this region must be considered the third endemic region for rickettsiosis in Colombia.

We present the first register for Colombia of *Candidatus* R. andeanae, in *R. sanguineus* s. l. in Leticia (Amazonas), as well as in *A. ovale* and *A. maculatum* in Ibagué (Tolima). This *Rickettsia* species has been registered in Brazil, Peru, Argentina, USA, Chile and Paraguay, and its role as a human pathogen is unknown (Ferrari et al., 2013; Witter et al., 2016). However, the study of this species is relevant, since Paddock et al. (2015) reported that a high prevalence of *Candidatus* Rickettsia andeanae leads presumably to the exclusion of *R. parkeri* in adult *A. maculatum* in Kansas and Oklahoma (USA), an event that could be occurring in the populations of *A. maculatum* analyzed herein, where all ticks studied were adults.

Rickettsia sp. strain Colombianensi was typed by Miranda et al. 2012, in *A. dissimile* and *Rhipicephalus microplus* on iguanas in Montería (Colombia); however, there has been no evidence suggesting that this *Rickettsia* species is transmissible to humans (Miranda and Mattar, 2014). Here, we report the first register of *Rickettsia* sp. strain Colombianensi for the species *A. mixtum*, as well as the first report of infection for *A. dissimile* in the department of Casanare. These findings raise the need for studies addressing the possible pathogenic potential of this *Rickettsia* species.

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5. Considerações Finais e Conclusões

Atualmente muitos problemas na taxonomia de carrapatos tem origem em grande parte devido a um número cada vez mais comprovado de espécies crípticas ou pseudocrípticas, que atuam como vetores de um grande número de rickettsias.

No presente estudo que levou em consideração a estreita interação entre vetor e patógeno, levantou-se dados referentes à identificação de espécies de carrapatos duros e sua contaminação por bactérias do gênero *Rickettsia*, bem como a sua distribuição geográfica e as relações carrapato/hospedeiros/patógenos. Ressalte-se que na Colômbia os estudos que visam contribuir para o melhor entendimento das questões taxonômicas, assim como das relações entre carrapatos e patógenos são ainda muitos escassos e fragmentados.

Os dados obtidos no presente estudo resultaram do uso de diferentes ferramentas, tais como histologia, de ultramorfologia (MEV) e da aplicação de marcadores moleculares, o que permitiu comprovar a presença de oito espécies de carrapatos duros, considerados os principais vetores e reservatórios de bactérias do gênero das rickettsias, patógenos responsáveis pela transmissão das febres manchadas (GFM), consideradas de grande importância médica e veterinária.

Foi aqui comprovado pela primeira vez a presença da espécie *Amblyomma mixtum* para a Colômbia. O complexo *A. cajennense* s.l. que é composto por seis espécies, tem distribuição desde a Argentina até os EUA. *Amblyomma cajennense* s.l. especificamente é considerado o principal vetor de *R. rickettsi* para humanos nas Américas, sendo três de suas espécies vetoras de *R. rickettsi* (*A. mixtum*, *A. patinoi* e *A. sculptum*), as duas primeiras ocorrendo também na Colômbia. Assim, o estudo da espécie *A. mixtum* torna-se de grande importância na epidemiologia das rickettsioses devido ao deslocamento e a colonização desta espécie de carrapato, também devido às mudanças climáticas e à intervenção antrópica.

Outro resultado interessante que foi obtido no presente estudo foi a variabilidade morfológica e molecular encontrada entre os carrapatos duros das populações amostradas, caso do ginandromorfo de *A. mixtum*, inclusive caracterizado como o primeiro registro para a espécie. Além deste intrigante registro, encontrou-se variações morfológicas entre as populações de *R. sanguineus* do departamento do Casanare e as demais populações

da Colômbia, principalmente nas fêmeas; os resultados moleculares sugeriram mistura de populações, demonstrada pelas sequências do ITS2, ou ainda, a inclusão de todas as populações na "linhagem tropical" através do gene 16S.

Dados semelhantes foram observados na população de *D. nitens* oriunda da cidade de Leticia, cujas variações morfológicas não foram tão marcantes, porém, os genes 16S e COI apresentaram uma distância genética maior do que a esperada entre os indivíduos pertencentes a uma mesma espécie. Esses dados enfatizaram a necessidade de se dar continuidade aos estudos destas duas espécies na Colômbia, fazendo inclusive uma abordagem de cruzamentos entre indivíduos de populações distintas, com o intuito de se determinar se tais variações seriam apenas plasticidade intraespecífica, ou se de fato seriam um complexo de espécies, como foi o caso de outros representantes de Ixodidae já registrados.

No mesmo contexto, fez-se apontamentos também quanto as controvérsias diante do complexo *A. maculatum*. Foram encontrados alguns exemplares na Colômbia que pareceram estar mais relacionados com *A. maculatum* de outros países da América, enquanto outros à espécie *A. triste*. As distâncias genéticas foram significativas para considera-los como indivíduos de uma mesma espécie, mas em outros casos estas diferenças foram muito pequenas para se considerar que *A. maculatum* seria uma espécie diferente de *A. triste*. As análises histológicas das células germinativas dos machos mostraram, no entanto, que existem sim diferenças marcantes quando são comparadas as espécies de *A. triste* do Brasil e *A. maculatum* da Colômbia, sugerindo tratarem-se de espécies distintas.

O registro da ocorrência de três espécies de rickettsias, entre elas a *R. rickettsi*, veio contribuir com o conhecimento da circulação e transmissão das rickettsioses na Colômbia. Ficou aqui demonstrada que as duas regiões endêmicas, previamente relatadas para rickettsioses na Colômbia (região central "Cundinamarca" e região noroeste "Cordoboa e Antioquia"), (ACOSTA et al., 2006; HIDALGO et al., (2007a, 2007b); PACHECO et al., 2008; PATIÑO et al., 1937), devem agregar-se a região da Orinoquía, por sua grande prevalência de vetores do gênero *Amblyomma*, incluindo a ocorrência de *A. mixtum*. Nestas regiões, comprovou-se a presença de *R. rickettsi*, infectando *A. mixtum*, e ainda nesta fez-se necessário um estudo mais detalhado, sobre a patogenicidade do vetor.

Da mesma forma, pode-se concluir a necessidade de se continuar as investigações com foco em outras duas espécies de rickettsias registradas no presente trabalho: *Candidatus* Rickettsia andeanae (primeiro registro para a Colômbia) e *Rickettsia* sp. strain Colombianensi, ambas ainda sem comprovação de patogenicidade para humanos. Nos EUA já houve registros que mostraram *Candidatus* Rickettsia andeanae e *Rickettsia parkeri*, infectando adultos de *A. maculatum* simultaneamente, e ainda relatos de que a presença da última seria inibida pela presença da primeira.

Desta forma os resultados aqui apresentados, vieram alertar na Colômbia a necessidade do apoio governamental em pesquisas que tenham como objetivo fornecer informações que auxiliem na busca de medidas epidemiológicas nos estados colombianos amostrados, salientando-se que a região da Orinoquía, já pode ser considerada a terceira região endêmica para rickettsioses. Além disso deve-se levar em consideração a necessidade de um acompanhamento da ocorrência de *A. mixtum* nesta e em outras regiões da Colômbia, devido ao grande fluxo comercial de bovinos, não só entre os estados deste país, mas também entre diferentes países, como no caso da Venezuela.

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