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Article

First mitochondrial genome of *Amblyomma triste* Koch, 1844 (Acari: Ixodidae): Evidence for studying species within the *A. maculatum* group

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Abstract

The tick species that comprise the *Amblyomma maculatum* group (Acari: Ixodidae) are widely distributed in the Nearctic and Neotropical regions, ranging from the United States of America (USA) to Argentina. This group includes three species: *Amblyomma maculatum*, *Amblyomma tigrinum*, and *Amblyomma triste*, which parasitize a high number of vertebrates, including domestic mammals and humans, where they are the main vectors of *Rickettsia parkeri* s.s. The identification, distribution, and validity of the three species within the group have been controversial and continue to raise questions. Previous studies have mostly focused on the analysis of partial nuclear and mitochondrial genes, highlighting the need for new integrative analyses that contribute to clarifying their systematics and ecology. In this investigation, we obtained the first mitochondrial genome of *A. triste* (14,808 bp), which, upon comparison with the reported mitochondrial genomes of *A. maculatum*, exhibits a genetic distance of 4.2%, providing new evidence for the validity of the former species. The order, composition, and structure of the mitogenome of *A. triste* are consistent with the characteristics reported for most Metastriata including taxa within *Amblyomma*, that count with several mitochondrial genomes published in the last few years. The attributes of the mitogenomes, such as the absence of paralogs and a small genome, confirm the utility in studies involving complexes of cryptic species as it also serves as a robust tool for inferring phylogenies. Furthermore, we reported new molecular markers at the mitochondrial level which can be used in phylogenetic studies for other tick species, especially those with controversial or challenging taxonomy. We highlight the need of sequencing the mitochondrial genome of *A. tigrinum*, which is part of the *A. maculatum* group, as well as experimental crosses of populations from different places in the Americas to obtain additional evidence for species recognition.

Key words: Metastriata, Pathogens, Public Health, Systematics, Ticks

Introduction

Ticks (Acari: Ixodida) are obligate hematophagous ectoparasites capable of transmitting multiple pathogens such as bacteria, helminths, protozoa, and viruses (de la Fuente *et al.* 2008; Baneth 2014; Bezerra-Santos *et al.* 2022). Ixodida comprises three families: Argasidae (soft ticks), Ixodidae (hard

ticks), and Nuttalliellidae (Nava *et al.* 2017; Guglielmone *et al.* 2020; 2021; 2023). Currently, Ixodidae is the most diverse family with approximately 759 species and 15 genera, where *Amblyomma* is the third largest in terms of species number (136), surpassed by *Ixodes* and *Haemaphysalis* with 266 and 176 species, respectively (Guglielmone *et al.* 2023). *Amblyomma* species have received special attention due to their involvement in pathogen transmission (e.g., *Anaplasma*, *Ehrlichia*, *Hepatozoon*, *Rickettsia*) and by the presence of cryptic species (complexes or groups) in America, such as the *Amblyomma cajennense*, *Amblyomma maculatum*, and *Amblyomma ovale* (Dumler *et al.* 2001; Demoner *et al.* 2013; Rivera-Páez *et al.* 2017; Suwanbongkot *et al.* 2019), and the *Amblyomma testudinarium* and *Amblyomma marmoreum* complexes in Asia and Africa, respectively (Mohamed *et al.* 2022; Cotes-Perdomo *et al.* 2023b).

The *A. maculatum* group is widely distributed from the United States of America (USA), Argentina, Belize, Bolivia, Brazil, Chile, Colombia, Costa Rica, Ecuador, French Guiana, Guatemala, Honduras, Mexico, Nicaragua, Paraguay, Peru, Uruguay, and Venezuela (Mendoza-Uribe & Chávez-Chorocco 2004; Guzmán-Cornejo *et al.* 2006; Mertins *et al.* 2010; Abarca *et al.* 2012; Lado *et al.* 2018; Rivera-Páez *et al.* 2018; Ossa-López *et al.* 2022; Guglielmone *et al.* 2023). Currently, the phylogenetic position of the *A. maculatum* group within *Amblyomma* is unclear (Santodomingo *et al.* 2021; Cotes-Perdomo *et al.* 2023a). In addition, the three species of the *A. maculatum* group are morphologically and phylogenetically closely related, casting doubts on their identification and taxonomic recognition (Koch 1844; Mendoza-Uribe & Chávez-Chorocco 2004; Estrada-Peña *et al.* 2005; Guzmán-Cornejo *et al.* 2006; Mertins *et al.* 2010; Abarca *et al.* 2012; Paddock *et al.* 2015; Lado *et al.* 2018; Guglielmone *et al.* 2023). For example, some studies suggest the possibility that *A. maculatum* and *A. triste* are conspecific (Nava *et al.* 2017; Lado *et al.* 2018). Other species such as *A. tigrinum* always cluster into a well-defined monophyletic lineage (Lado *et al.* 2018), and differs from *A. maculatum* and *A. triste* in the adult morphology, and ecological preferences. However, the larvae and nymphs of these three species are extremely difficult to differentiate using morphological traits (Guglielmone *et al.* 2000; Mendoza-Uribe & Chávez-Chorocco 2004; Nava *et al.* 2007; 2017; Lado *et al.* 2018; Guglielmone *et al.* 2021; 2023).

For the *A. maculatum* group, Lado *et al.* (2018) reported four morphotypes with distinct geographical distribution: i) *A. triste* s.s., defined as morphotype I, including specimens from Argentina, southern Brazil, Paraguay, and Uruguay; ii) *A. maculatum* s.s., defined as morphotype II and including specimens from Colombia (Department of Santander), eastern United States of America (USA), Guatemala, Honduras, Mexico, Nicaragua, and Venezuela; iii) morphotype III, which presents a combination of morphological characters limiting the strict assignment to *A. maculatum* s.s. or to *A. triste* s.s.; this morphotype includes ticks from southern and southwestern USA (Arizona and Texas), and northern Mexico; and iv) morphotype IV, which also exhibits a combination of characters of *A. maculatum* s.s. and *A. triste* s.s., and is found in Chile, Ecuador, and Peru. Ossa-López *et al.* (2022) confirmed the wide distribution of *A. maculatum* s.s. (morphotype II) in Colombia and indicated that the sequences of the individual from the Department of Santander analyzed by Lado *et al.* (2018), consistently clustered with other samples from Colombia (from the departments of Caldas, Cundinamarca, and Tolima) instead of clustering with sequences from the USA.

Similarly, Ossa-López *et al.* (2022) confirmed the presence of *A. triste* s.s. (morphotype I) in Colombia using morphological and molecular data, therefore, two out of the three species within the *A. maculatum* group are found in the country, and *A. tigrinum* is currently excluded from the Colombian territory due to lack of verified records (Guglielmone *et al.* 2011; Guglielmone & Robbins 2018; Guglielmone *et al.* 2021; Ortíz-Giraldo *et al.* 2021; Guglielmone *et al.* 2023).

The medical and veterinary importance of *A. maculatum* is well-documented due to its role as the principal reservoir of *Hepatozoon americanum*, and together with *A. triste*, both tick species are

vectors of *Rickettsia parkeri* s.s. (Mathew *et al.* 1998; 1999; Paddock *et al.* 2004; Venzal *et al.* 2004; Paddock & Goddard 2015; Colombo *et al.* 2016; Romer *et al.* 2020). For that reason, more research on reproductive compatibility, distribution, taxonomy, and molecular affinities is needed to clarify the controversial specific taxonomy and to contribute to the understanding of their role in pathogen transmission (Mathew *et al.* 1998; 1999; Ewing *et al.* 2000; Paddock *et al.* 2004; Venzal *et al.* 2004; Colombo *et al.* 2016; Allerdice *et al.* 2020; Romer *et al.* 2020; Cuervo *et al.* 2021; Ossa-López *et al.* 2022; Guglielmone *et al.* 2023).

Mitochondrial genomes (mitogenomes) have proven to be important for the taxonomy, systematics, and population genetics of ticks, emerging as a valuable source of informative molecular markers (Wang *et al.* 2019; Uribe *et al.* 2020; Kneubehl *et al.* 2022; Cotes-Perdomo *et al.* 2023a). Mitochondrial genomes of 120 tick species have been used in phylogenetic analyses (Kelava *et al.* 2021), recovering controversial relationships at the family and genera levels. The absence of paralogous and small size make the mitogenomes a robust molecular marker for inferring phylogenetic hypotheses in studies of cryptic diversity (Zaharias *et al.* 2020; Cotes-Perdomo *et al.* 2023a; 2023b). In this study, sequencing techniques were combined to generate the first mitogenome of *A. triste*, aiming to evaluate its composition (content and organization) and establish its phylogenetic relationships and differentiation from other closely related species within the genus *Amblyomma*.

Material and methods

Sample collection and DNA extraction

In the year 2018, two male ticks from the *A. maculatum* group were found parasitizing a capybara (Hydrochoerus hydrochaeris, Rodentia, Caviidae) at the vereda Las Plumas, municipality of Arauca, Department of Arauca (6◦36′ 18′’N; 70◦31′ 51′’W), Colombia (Ossa-López *et al.* 2022). The collections were executed within the framework permit granted by the National Environmental Licensing Authority (ANLA) to the Universidad de Caldas as stipulated in resolution 02497 of December 31, 2018. The specimens were stored in cryogenic tubes with absolute alcohol and were morphologically identified following the keys of Kohls (1956); Estrada-Peña *et al.* (2005); Barros-Battesti *et al.* (2006); Nava *et al.* (2017); Lado *et al.* (2018) and Guglielmone *et al.* (2021; 2023). The specimens were confirmed as *A. triste*, primarily based on the presence of a thick and highly sclerotized spine and a thin and weakly sclerotized seta on the tibia of legs II-IV. In addition, the spiracular plates (peritrema) are almost oval, with short and wide dorsal projection, and longer spines on coxa IV (Ossa-López *et al.* 2022). The total DNA from each individual was extracted using the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's recommended protocols, with an extended incubation period (16 h) in lysis buffer at 56 °C.

Amplifying and sequencing

The molecular confirmation was made with partial sequences of three mitochondrial genes, *12S*, *16S* and *cox1* (Ossa-López *et al.* 2022). DNA from a single tick was used for subsequent molecular processes and the sequencing of the complete mitogenome. The fragments were obtained by conventional PCR and sanger sequencing (Macrogen Inc., South Korea) (Supplementary Material 1). The sequences were analyzed using Geneious® Prime 2022.1 software (Kearse *et al.* 2012).

Two strategies were implemented for the amplification and sequencing of the mitogenome. Initially, two fragments that complete ~10kb (70% of the mitogenome) were amplified by long-range PCR and pooled together at equimolar concentration to prepare a Nextera XT DNA library, which was sequenced using Nova Seq 6000 150 PE (150 × 2 bp; 10 Gb/sample) in Illumina platform.

Subsequently, primers were designed along the missing region using as a reference the mitogenome of *A. maculatum* (MW719251; available in the National Center for Biotechnology Information – NCBI database) for primer-walking sequencing by Sanger (Supplementary Material 2).

Assembly, annotation, and alignment

For each raw file from Illumina sequencing, the quality was checked using FastQC (Andrews 2010). The cleaning, filtering, and adapter trimming of each raw file was made across Trimmomatic (Bolger *et al.* 2014). The partial *cox1* sequence was used as a scaffold for reference-guided assembly using the "Map to Reference(s)" option in Geneious® Prime 2022.1. Subsequently, the protein-coding (PCGs; coding sequences - CDSs), transference RNAs (tRNAs), and the two ribosomal RNAs (rRNAs) genes were identified using MITOS web server (Bernt *et al.* 2013). The annotation was manually verified with the ORFs searcher in Geneious Prime® 2023.2.1. The genes obtained from the *Amblyomma aureolatum* transcriptome from SRA repository in GenBank (SRR4301110; Martins *et al.* 2017) assembled *de novo* by Uribe *et al.* (2020) were included in our analysis, beside the following mitogenomes available in the NCBI: *Amblyomma cajennense* s.s. (OP901701; OP901707), *A. maculatum* (MW719251), *Amblyomma mixtum* (OP901702; OP901703), *Amblyomma ovale* (MT554102; MT554103), *Amblyomma patinoi* (OP901704), *Amblyomma sculptum* (NC_032369; OP901706), *Amblyomma tonelliae* (OP901705), and *Dermacentor nitens* (NC_023349) used as outgroup.

Phylogenetic analyses

The genes were aligned and filtered separately using MAFFT (Katoh & Standley 2013) and BMGE (Criscuolo & Gribaldo 2010). The CDSs + rRNAs filtered genes were concatenated in a matrix at the nucleotide level (Matrix-NT), which was the subject of phylogenetic analyses by probabilistic methods such as Bayesian Inference (BI; Rannala & Yang 1996; Yang & Rannala 1997) and Maximum Likelihood (ML; Felsenstein 1981). BI analyses were performed using MrBayes v3.2.7a (Ronquist *et al.* 2012), two parallel runs, 2000000 generations, in which the initial 25% of sampled data were discarded as burn-in; and ML analyses was performed in IQ-TREE (Nguyen *et al.* 2015) and the robustness was assessed using 1000 bootstrap pseudoreplicates. ModelFinder (Kalyaanamoorthy *et al.* 2017) was used to select the best evolutionary model based on the Bayesian Information Criterion (BIC; Schwarz, 1978): GTR+I+G+F for BI and GTR+F+I+G4 for ML. FigTree v. 1.4.3 (Rambaut 2007) was used to visualize all the phylogenetic trees. Genetic distances were estimated using the *p-distance* method with the MEGA 11 program (Tamura *et al.* 2021) and corroborated with the identity percentage matrix in Geneious Prime® 2023.2.1.

Results

Content and organization of the mitochondrial genome *of* Amblyomma triste

The complete mitogenome of the male tick sequenced is available in GenBank as record OR497835. This molecule is completely circular, has a length of 14,808 bp, and it contains (i) 13 CDSs: *cox1-3*, *nad1-6*, *nad4L*, *atp6*, *atp8* and *cytb*; (ii) two rRNA genes: a small subunit ribosomal RNA gene (*rrn*S or *12S*), and a large subunit ribosomal RNA gene (*rrn*L or *16S*); (iii) 22 tRNAs; and (iv) two intergeneric spaces: between *12S* and tRNA-Ile, and between tRNA-Leu and tRNA-Cys, corresponding to the non-coding regions 1 and 2 (NCR1 and NCR2) respectively (Figure 1; Table 1).

In the organization of the mitogenome there are two overlaps in the same strand between: *atp6* and *atp8*, and between *nad4* and *nad4L*; and six overlaps in different strands between: *nad2* and

tRNA-Trp; tRNA-Trp and tRNA-Tyr; tRNA-Tyr and *cox1*; tRNA-Arg and tRNA-Asn; tRNA-Asn and tRNA-Ser; and between tRNA-Glu and *nad1*. All the tRNAs vary in length from 54 to 68 nucleotides (Figure 1; Table 1). Prediction of the tRNA secondary structure showed that 20 of the tRNAs have the standard cloverleaf structure, while tRNA-S1 (trnS1) and tRNA-C (trnC) were missing the D-arm or DHU arm (Figure 2).

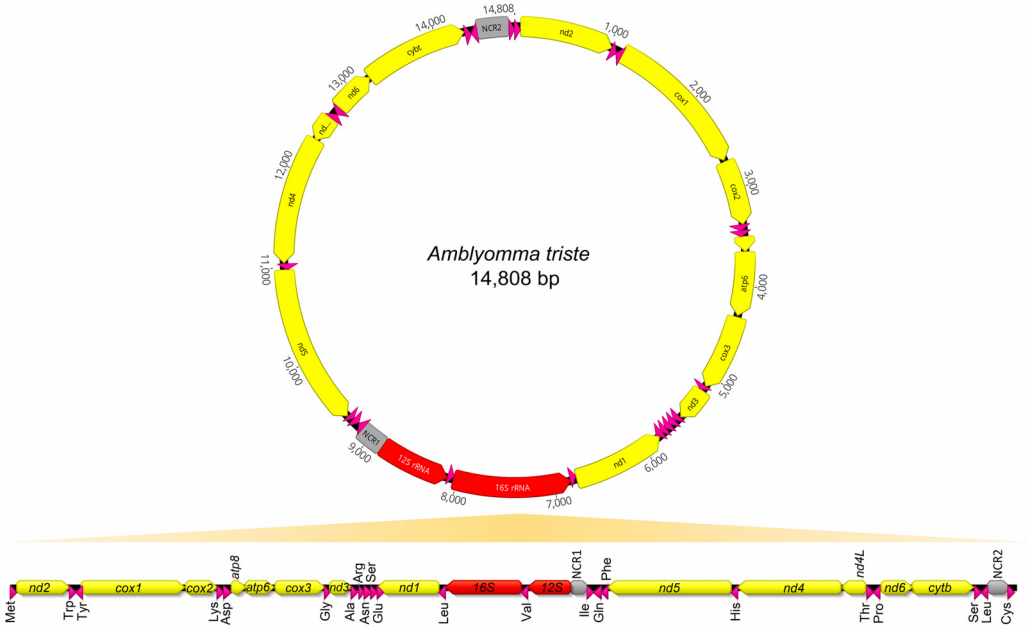

FIGURE 1. Circular and linear representation of the mitochondrial gene order of *Amblyomma triste*. Gene scaling is approximate, and the end of the arrow indicates the direction of the genes (the genes encoded in the H-strand or "+" and L-strand or "-"). All genes have standard nomenclature including the 22 tRNA genes, which are designated by a three-letter code for the corresponding amino acid. The CDS genes are in yellow, the rRNAs in red, the tRNAs in pinkish-purplish color, and the two potential control regions (CR), are non-coding in gray.

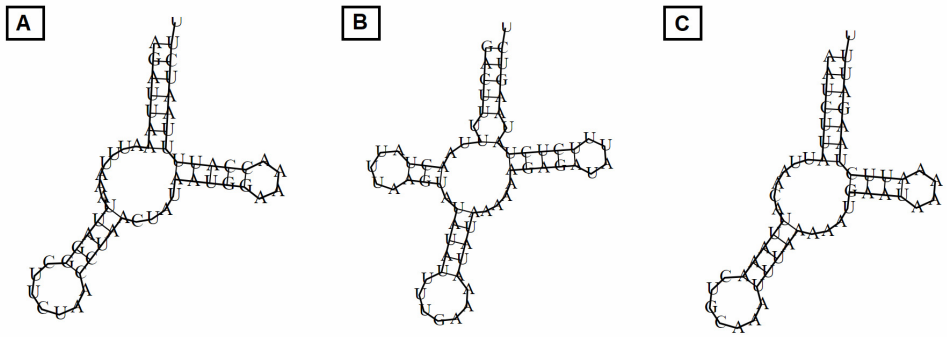

FIGURE 2. Predicted secondary structure of the mitochondrial tRNA genes of *Amblyomma triste* obtained using MITOS. A. trnS1(aga), lacks DHU arm; B. trnS2(tca), normal cloverleaf structure; C. trnC(tgc), lacks DHU arm.

TABLE 1. Mitochondrial genome organization of *Amblyomma triste.*

Genes/regions	Positions and nt sequence lengths(bp)	Strand	Initiation and stop codons
tRNA-Met (M)	1–64 (64)	H	
*nad*2	65–1030 (1194)	H	ATT/TAA
tRNA-Trp (W)	1030–1091 (62)	H	
tRNA-Tyr (Y)	1090–1151 (62)	L	
*cox*1	1144–2682 (1539)	H	ATT/TAA
*cox*2	2698–3370 (673)	H	ATG/T
tRNA-Lys (K)	3371–3435 (65)	H	
tRNA-Asp (D)	3436–3497 (62)	H	
*atp*8	3498–3653 (156)	H	ATT/TAA
*atp*6	3647–4309 (663)	H	ATG/TAA
*cox*3	4325–5102 (778)	H	ATG/T
tRNA-Gly (G)	5103–5162 (60)	H	
*nad*3	5163–5507 (345)	H	ATT/TAA
tRNA-Ala (A)	5516–5578 (63)	H	
tRNA-Arg (R)	5583–5640 (58)	H	
tRNA-Asn (N)	5640–5703 (64)	H	
tRNA-Ser^{AGA} (S1)	5702–5758 (57)	H	
tRNA-Glu (E)	5761–5823 (63)	H	
*nad*1	5803–6763 (961)	L	ATT/T
tRNA-Leu^{UUA} (L_2)	6764–6826 (63)	L	
*rrn*L	6836–8035 (1200)	L	
tRNA-Val (V)	8039–8100 (62)	L	
*rrn*S	8101–8788 (688)	L	
Non-coding region (NCR1)	8789–9111 (323)		
tRNA-Ile (I)	9112–9177 (66)	H	
tRNA-Gln (Q)	9180–9247 (68)	L	
tRNA-Phe (F)	9250–9308 (59)	L	
*nad*5	9313–10971 (1659)	L	ATT/TAA
tRNA-His (H)	10972–11035 (64)	L	
*nad*4	11040–12362 (1323)	L	ATG/TAA
*nad*4L	12356–12631 (276)	L	ATG/TAA
tRNA-Thr (T)	12638–12698 (61)	H	
tRNA-Pro (P)	12703–12764 (62)	L	
*nad*6	12771–13205 (435)	H	ATT/TAA
*cyt*b	13210–14286 (1077)	H	ATG/TAA
tRNA-Ser^{UCA} (S2)	14295–14358 (64)	H	
tRNA-Leu^{CUA} (L1)	14361–14419 (59)	L	
Non-coding region (NCR2)	14421–14750 (330)		
tRNA-Cys (C)	14753–14806 (54)	H	

Genetic distances and phylogenetic analyses

The concatenated matrix with CDSs indicates that *A. triste* has a 95.8% of identity with *A. maculatum* (MW719251), a record provided by the Oklahoma State University Tick Rearing Facility, USA (Brenner & Raghavan, 2021; Table 2). *A. ovale* has also a 80.6% of identity with *A. aureolatum*, and the species of the *A. cajennense* complex between 76.1 and 81.9% (Table 2).

TABLE 2. Percent identity matrix of concatenated protein-coding genes (CDS).

Species		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*A. triste* (This study)	-													
2	*A. maculatum* (MW71925)	95.8	-												
3	*A. aureolatum* (SRR4301110)	76.7	76.9	-											
4	*A. ovale* (MT554102)	77.6	77.2	80.6	-										
5	*A. ovale* (MT554103)	77.6	77.3	80.6	97.8	-									
6	*A. cajennense* s.s. (OP901701)	78.1	78	76.2	76.7	76.7	-								
7	*A. cajennense* s.s. (OP901707)	78	77.9	76.2	76.7	76.6	99.8	-							
8	*A. patinoi* (OP901704)	77.9	77.8	76.2	76.7	76.6	88.1	88.1	-						
9	*A. mixtum* (OP901702)	78.3	78	76.7	76.8	76.9	87.6	87.5	87.6	-					
10	*A. mixtum* (OP901703)	78.2	77.9	76.6	76.8	76.9	87.4	87.3	87.6	95.6	-				
11	*A. sculptum* (NC_032369)	78	77.6	76.2	76.4	76.3	85.7	85.6	85.3	85.5	85.7	-			
12	*A. sculptum* (OP901706)	78.1	77.6	76.1	76.4	76.3	85.6	85.6	85.3	85.5	85.7	99.4	-		
13	*A. tonelliae* (OP901705)	78.4	78	77	77.5	77.4	82	81.9	82.2	82.3	82.3	82	81.9	-	
14	*D. nitens* (NC_023349)	73.6	73.5	71.1	71.9	71.9	72.8	72.7	72.7	73.5	73.1	73.1	73.1	72.7	-

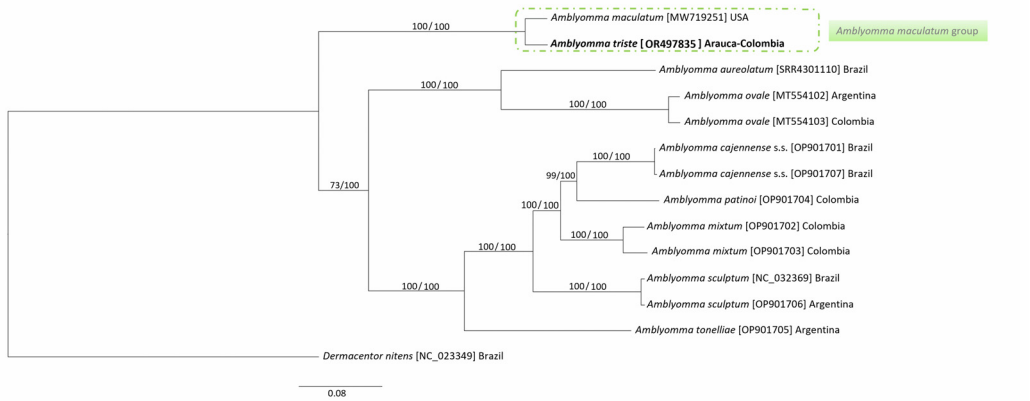

FIGURE 3. Phylogenetic tree based on the concatenation of 13 CDS and two rRNA genes. The tree was inferred with the best-fit substitution model and the topology shown corresponds to Bayesian Inference (BI: MrBayes v3.2.7a) and Maximum Likelihood (ML: IQ-TREE). Branch support indicated bootstrap percentages (ML/BI). Accession codes are in square brackets and mitogenome obtained in this study are shown in bold.

The interspecific genetic distances between *A. maculatum* and *A. triste* from each of the genes (CDSs and rRNA) are shown in Table 3. The *nd3* and *nd5* genes exhibited the greatest divergence, with 5.4% and 5% respectively. The phylogenetic relationships of 14 tick species based on the

concatenated Matrix-NT, using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses, are shown in Figure 3. The topologies of the trees using ML and BI were identical, recovering a highly supported (100%) clade constituted by the two species of the *A. maculatum* group (*A. maculatum* and *A. triste*). Another well supported (73/100% respectively for ML/BI) was constituted by taxa of the *A. ovale* group. Similarly, the taxa of the *A. cajennense* complex were recovered in a separated clade with a 100% of support in both ML/BI reconstructions (Figure 3).

TABLE 3. Pairwise distance (in percentage) of mitochondrial genes (CDS and rRNA) between *A. maculatum* and *A. triste*, according to the *p-distance* method.

	Genes	*A. maculatum* (MW71925)
A. triste (This study)	*atp6*	3.9
	atp 8	4.3
	cox 1	4.2
	cox 2	3.0
	cox 3	3.8
	cybt	3.7
	nd1	3.6
	nd2	3.7
	nd3	5.4
	nd4	4.1
	nd4L	3.3
	nd5	5.0
	nd6	3.4
	12S	2.1
	16S	2.9

Discussion

Content and organization of the mitochondrial genome of Amblyomma triste

The mitogenome of *A. triste* obtained in the present study is the first one recorded for this species, and it is like the mitogenomes reported for other arthropods including ticks (Montagna *et al.* 2012; Liu *et al.* 2013; Cameron *et al.* 2014; Brenner & Raghavan 2021).

In ticks, mitogenomes exhibit size ranges between 14 to 16 kb, circular organization, double-stranded DNA, with 37 genes: 13 CDSs, 22 tRNAs genes, and two rRNA genes, as well as two control or non-coding regions (NCRs); the arrangement of the genes of is similar to that found in the majority of species of Ixodidae ticks (Burger *et al.* 2013; Simon & Hadrys 2013; de Lima *et al.* 2017; Li & Liang 2018; Wang *et al.* 2019; Kelava *et al.* 2021; Cotes-Perdomo *et al.* 2023a).

In the *A. triste* mitogenome, the start codons (ATT and ATG) for the CDSs correspond to the typical start codons found in insects, which are the same as those commonly adopted by ticks (ATN) (Liu *et al.* 2013; Wang *et al.* 2019; Uribe *et al.* 2020; Brenner & Raghavan 2021). Regarding the termination codons (TAA and T), the CDSs correspond to the termination codons, which in ticks are mainly TAA and TAG, but sometimes "T" or "TA" may be converted into a complete termination codon by polyadenylation after translation (Montagna *et al.* 2012; Liu *et al.* 2013; de Lima *et al.* 2017; Uribe *et al.* 2020; Brenner & Raghavan 2021; Chavatte & Octavia 2021).

The mitochondrial rRNA genes (*12S* and *16S*) of *A. triste* exhibit a complex functional structure with a relatively slow evolution rate; this is particularly important as these genes have long been used as markers in tick population genetics, and phylogenetic and systematic studies (Araya-Anchetta *et al.* 2015; Wang *et al.* 2019).

Tick mitogenomes contain unique copy of the *12S* and *16S* rRNA genes, and due to gene rearrangement, the position of the rRNA genes shifts, whereas the gene order and the location in the N strand remain unchanged; therefore, the combined use of CDSs and rRNA genes is recommended for taxonomic studies, particularly when involving cryptic species and complexes (Burger *et al.* 2014; Mans *et al.* 2019; Wang *et al.* 2019).

The size and structure of the tRNA genes in this study (54 to 68 nucleotides) are consistent with the ranges reported for other tick species (50 to 90 bp), and most tRNA genes have a complete cloverleaf structure (Liu *et al.* 2013; Wang *et al.* 2019; Chavatte & Octavia 2021), except for trnS1 and trn-C, which lacks DHU arm or D-arm. This is a common feature in most animal species, including ticks (Liu *et al.* 2013; Cameron *et al.* 2014; Williams-Newkirk *et al.* 2015; Wang *et al.* 2019; Chavatte & Octavia 2021). Mitochondrial tRNA-C secondary structures are variable among tick species with some missing D-arm and/or T-arm and some having standard cloverleaf structure (Burger *et al.* 2012; Montagna *et al.* 2012; Williams-Newkirk *et al.* 2015).

Similarly, tick mitogenomes may contain two non-coding regions (NCRs), as in Metastriata, or one as in *Ixodes* (non-Australasian Prostriata), and Argasidae. In both cases, these regions have a conserved location within the mitogenome (Montagna *et al.* 2012; Kelava *et al.* 2021). Alternatively, they may be specific to the group, for example, Metastriata and Australasian *Ixodes* (Prostriata) have two non-coding regions, but with different locations within the mitogenome (Montagna *et al.* 2012; Liu *et al.* 2022). Changes in the non-coding regions of the mitochondrial DNA suggest potential differences in replication/transcription mechanisms, as NCRs contain regulatory elements (Burger *et al.* 2012; Liu *et al.* 2013; Wang *et al.* 2019; Chavatte & Octavia 2021).

Genetic distances and phylogenetic analyses

Mitogenomes can provide a deeper understanding of tick systematics, allowing the resolution of phylogenetic relationships that are problematic when using partial genes (Wang *et al.* 2019; Kelava *et al.* 2021; Mohamed *et al.* 2022). In this study, the interspecific distances of CDSs+rRNA genes between *A. triste* and *A. maculatum* were 4.2%, similar to the reported by Cotes-Perdomo *et al.* (2023b) when comparing the complete genomes of two species of the *A. marmoreum* complex (*Amblyomma nuttalli* and *Amblyomma sparsum*) with an interspecific distance of 4.4%. In comparison with other *Amblyomma* species, the intra-specific distances found were: *A. ovale* (2.2%); *A. cajennense* s.s. (0.2%); *A. mixtum* (4.4%); *A. sculptum* (0.6%), divergence percentage is consistent with the findings reported by Cotes-Perdomo *et al.* (2023a) for the *A. cajennense* complex. For other tick genera, Reynolds *et al.* (2022) reported intra-specific divergences ranging from 5.8% to 10.6% for *Dermacentor albipictus*, suggesting the existence of a species complex, and recommend further evaluation of other genes (*nad1*, *nad2*, *nad5*, *cox1*, and *atp8*) that may contribute to highlighting the genetic differences.

Traditionally, the *A. maculatum* group has been studied using divergences of gene fragments such as *12S*, *16S*, and *cox1*, which have been the most commonly used and reported genes for hard ticks (Beati & Keirans 2001; Marrelli *et al.* 2007; Beati *et al.* 2013; Paternina *et al.* 2016; Rivera-Páez *et al.* 2017; 2018; Lado *et al.* 2018). Our results demonstrate that these genes do not exhibit the highest interspecific divergences. For that reason, further research involving mitogenomes or other genes that display greater genetic distances should be conducted. This could be useful for differentiation of cryptic species such as *A. cajennense* complex, *A. marmoreum* complex and *A. ovale* complexes.

As reported by Ossa-López *et al.* (2022), the genetic distances of each of the evaluated genes (1.5–1.8% for *12S;* 2.3–7.1% for *16S,* and 4.9–7.0% for *cox1*) obtained between *A. triste* and *A. maculatum* from Colombia are larger than those reported by Lado *et al.* (2018), although they are lower than those found between *A. tigrinum* compared to all morphotypes that comprise the *A. maculatum* group (3.9–4.3%). The phylogenetic results also confirmed differences between *A. maculatum* from USA and Colombia based on genetic distances of each of the evaluated genes (1.2–1.8 for *12S*; 1.5–5.8% for *16S*, and 3–3.7% for *cox1*).

Although this study emphasizes the divergence percentages for the CDSs + rRNA genes, with *12S* and *16S* showing low percentages of 2.1% and 2.9% respectively, in comparison to the CDSs: *nd3*, *nd5*, *atp8*, and *cox1* stand out with interspecific divergences of 5.4%, 5.0%, 4.3%, and 4.2% respectively. Three of these genes (*nd5*, *atp8*, and *cox1*) align with the recommendations of Reynolds *et al.* (2022), and should be explored in phylogenetic studies, using the primers designed in our study for *A. maculatum* (MW719251), which can be implemented for other populations or species (Supplementary Material 2).

Finally, there are still several questions and gaps in the systematics and diversity of hard ticks. This study shows that the *A. maculatum* group comprises at least two species in Colombia. We highlight the need to establish both intra- and inter-specific divergences among the species within the *A. maculatum* group, new information on the mitogenomes of the different morphotypes reported by Lado *et al.* (2018), which originate from diverse geographic areas, is needed. Also, obtaining and evaluating the mitogenome of *A. tigrinum*, is crucial for elucidating both intra- and interspecific divergences within the species that comprise the *A. maculatum* group, and to carry out experimental crosses to obtain additional evidence for species recognition.

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