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Original article

Phylogenetic relationships of the *Amblyomma cajennense* complex (Acari: Ixodidae) at mitogenomic resolution

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ABSTRACT

The genus *Amblyomma* is the third most diverse in the number of species within the Ixodidae, with practically half of its species distributed in the Americas, though there are also species occurring in Africa, Asia, and Australia. Within the genus, there are several species complexes with veterinary and public health importance. The *Amblyomma cajennense* complex, in the Americas, is represented by six species with a wide distribution, from Texas to northern Argentina. We combined two sequencing techniques to generate complete mitogenomes of species belonging to the *Amblyomma cajennense* complex: genome skimming and long-range PCRs sequencing methods. Thus, we generated seven new mitochondrial genomes for all species of the *Amblyomma cajennense* complex, except for *Amblyomma interandinum*. Genetic distances between the mitogenomes corroborate the clear differentiation between the five species of the *Amblyomma cajennense* complex. The phylogenetic relationships of these species had previously been evaluated by combining partial nuclear and mitochondrial genes and here these relationships are corroborated with a more robust framework of data, which demonstrates that the conjunction of mitochondrial and nuclear partial genes can resolve close relationships when entire genes or genomes are unavailable. The gene order, structure, composition, and length are stable across these mitogenomes, and they share the general characteristics of Metastriata. Future studies should increase the number of available mitogenomes for this genus, especially for those species from the Indo-Pacific region and Africa, by means of a better understanding of their relationships and evolutionary process.

1. Introduction

Ticks are included in the order Ixodida, characterized by their hematophagous habits, parasitizing all groups of vertebrates except for fish (Sonenshine and Roe, 2014). Ticks are parasites with veterinary and public health relevance because of the deleterious physical effects that tick parasitism provokes in hosts and due to their role as vectors of multiple pathogens (bacteria, protozoa, and viruses) that affect animals and humans, causing diseases such as rickettsiosis, anaplasmosis, babesiosis, Lyme disease, relapsing fever, Crimean Congo hemorrhagic fever, and tularemia, among others, which places them as one of the animal groups with the greatest economic and health importance on the planet (Jongejan and Uilenberg, 2004; Magnarelli, 2009).

Systematically, the order Ixodida comprises three extant families, Argasidae, Nuttalliellidae, and Ixodidae, and this last family is by far the most diverse with 15 genera and approximately 760 species (Guglielmone et al., 2015 and updates; Guglielmone et al., 2021; Apanaskevich et al., 2022). Within the Ixodidae, the genus *Amblyomma* is the third most diversified in species (after *Ixodes* and *Haemaphysalis*), with 136 species, and practically half of these species (67 spp.) are found in the

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Americas, including the Caribbean, but there are also *Amblyomma* representatives in Africa (sub-Saharan), Asia, and Australia (Guglielmone et al., 2021). Climate change has caused alteration in the distribution of several species, putting human communities at risk of health problems, mostly in margined populations (Gray et al., 2009; Monzón et al., 2016). Thus, *Amblyomma* is a priority clade to develop evolutionary frameworks that allow inferring the potential historical biotic and abiotic factors that prompted the diversification of these animals (Binetruy et al., 2020; Kelava et al., 2021).

The use of molecular techniques has been shown to be instrumental to determine evolutionary relationships within Neotropical *Amblyomma* species groups (Labruna et al., 2009a; Beati et al., 2013; Lado et al., 2016, 2018; Tarragona et al., 2022). This is the case of the *Amblyomma cajennense* complex, which comprises six species: *Amblyomma cajennense* s.s., *Amblyomma sculptum, Amblyomma mixtum, Amblyomma patinoi, Amblyomma tonelliae*, and *Amblyomma interandinum* with a wide distribution in the Americas, from Texas to northern Argentina, including the Caribbean Islands (Nava et al., 2014). Combined works have decoded the circumscription of these species (Labruna et al., 2011; Mastropaolo et al., 2011; Estrada-Peña et al., 2014; Martins et al., 2016) and have inferred their phylogenetic relationships using partial molecular markers (Beati et al., 2013; Tarragona et al., 2022). Beati et al. (2013) also inferred that the diversification of the *Amblyomma cajennense* complex started from the Miocene to the Pleistocene and was the result of climatological and geological phenomena in the Americas.

The *Amblyomma cajennense* complex contains some of the most important tick species from a veterinary and public health perspective (Scoles and Ueti, 2013; Bermúdez et al., 2016). The ticks of the complex are important pests to domestic animals and humans in the Neotropical region (Glugliemore et al., 2021). It is also one of the main vectors of *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever in some areas of the Americas, which has been recognized for its particular aggressiveness to humans and domestic fauna (Parola et al., 2013; Labruna et al., 2014; Nava et al., 2017; Guglielmone et al., 2021; Nogueira et al., 2022).

The mitochondrial genomes (mitogenomes) have been widely used to reconstruct evolutionary histories at different hierarchical levels and across diverse clades (Ding et al., 2019; Harasewych et al., 2019; Hassanin et al., 2021; Irisarri et al., 2020; Uribe et al., 2022). The absence of paralogy, a high degree of recombination, and the absence of missing data across the taxa, make this circular molecule a powerful tool to infer robust phylogenies (Zaharias et al., 2020). Thus, in this work, we have combined two sequencing techniques to generate complete mitogenomes within the *Amblyomma cajennense* complex with the aim to: *i.* to reconstruct the phylogenetic relationships of the main lineages within the *Amblyomma cajennense* complex with a novel and robust dataset; *ii.* establish mitogenomic composition patterns across species; *iv*, develop general primers to amplify complete mitogenomes across all *A. cajennense* complex species.

2. Methodology

2.1. Sample collection and DNA extraction

A total of seven adult specimens belonging to five species from the *Amblyomma cajennense* complex were analyzed for this study (Table 1). Ticks were morphologically determined following Jones et al. (1972) and Nava et al. (2014). These samples were fixed and stored in 96% ethanol at −20 °C. The DNA was extracted from half of each specimen using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following the provider's recommendations, with an extended lysis period of 16 h at 56 °C.

2.2. Amplifying and sequencing

Partial sequences were obtained by conventional PCR and sanger sequencing of cytochrome *c* oxidase 1 (*cox1*) and large subunit of ribosomal RNA (*16S*) partial mitochondrial genes using universal primers (Folmer et al., 1994; Palumbi et al., 1991, respectively). The following PCR conditions were used: initial denaturation at 95 °C for 5 min (min), followed by 40 cycles of 94 °C for 30 s (s), annealing at 47 °C (for the *cox1*) and 50 °C (for *16S*) for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 3 min. With the partial mitochondrial genes were made the molecular confirmation of the species.

Two methods of massive sequencing were used to obtain the mitochondrial genome sequences of each sample. First, a TruSeq Nano DNA Kit was used to prepare a library for genome DNA extracted from *A. patinoi*. The library was sequenced using Nova Seq 6000 150 PE (150 × 2 bp; 10 Gb/sample) across Illumina platform. This method is known as genome skimming (GS). The remaining six mitogenomes were sequenced across long-range PCR. To this, primers that allowed the amplification of the entire mitogenome in four overlapped fragments were designed (see Table 2). The design of these primers requested the alignment of all mitogenomes available to *Amblyomma* in order to choose the conservative regions across the mitogenome structures. Long-range PCR reactions contained 0.2 μl of Takara LA Taq DNA

Table 1
Analyzed samples for this study. ♂: male, ♀: female. Also are indicated the size of each mitogenome with its mean coverage values.

Code	Species	Sex	Host/Method	Locality	Date	No. NCBI	Mean coverage	Std Dev
T01	*A. mixtum*	♂	dragging	Santa Marta, Colombia	jun-16	OP901702	82.7	91.8
T02	*A. mixtum*	♀	Cow	Santa Marta, Colombia	jun-16	OP901703	167.8	108.3
A.c	*A. cajennense*	–	–	Mato Grosso, Brazil	–	OP901707	801.4	1203.8
T19	*A. cajenense* s.s.	♀	dragging	Mato Grosso, Brazil	–	OP901701	19,509.9	32,504.2
A.s	*A. sculptum*	–	dragging	Formosa, Argentina	–	OP901706	12,335.8	12,527.5
T18	*A. tonelliae*	♀	dragging	Santiago del Estero, Argentina	–	OP901705	3040.6	2831.9
SA20	*A. patinoi*	♀	Horse	Villeta, Colombia	jul-12	OP901704	1376	437.2

Table 2
Primers employed to amplify the mitogenomes within the *Amblyomma cajennense* complex.

Primer name	Estimated Amplicon size	Primer sequence 5′–3′	Position
Caj1F	~ 2237 bp	TTTAAGCRATGGTCTCTTAAACCAA	tRNALys
Caj2R		TTCTAAATTCAGGCCGAAACTGAA	tRNAArg
Caj2F	~ 3471 bp	TTCAGTTTCGGCCTGAATTTAGAA	tRNAArg
Caj3R		CAACCGGCTATCDCATTTACTGGAC	Control region 1
Caj3F	~ 4236 bp	GTCCAGTAAATGHGATAGCCGGTTG	Control region 1
Caj4R		CCATAATTTACATCTCGTTGAATATG	Cytb
Caj1R	~ 4802 bp	KKTTGGTTTAAGAGACCATYGCTT	tRNALys
Caj4F		CATATTCAACGAGATGTAAATTATGG	Cytb

Polymerase (5 units/μl), 2.5 μl of LA Buffer II 10x (Mg2+plus), 3 μl of dDNTPs (2.5 mM each), 0.2 μl of BSA, 0.5 μl of each primer (10 mM), 0.8 μl (20–100 nm) of the template DNA and water (dH_2O) to complete 25 μl. The reaction began with an initial denaturation at 95 °C for 5 min; 40 cycles with 98 °C for 10 s (extra-denaturalization), annealing at 50 °C for 30 s, and an extension at 68 °C for 60 s for kb of the expected product; and finishing with a final extension at 68 °C during 10 min.

The products were purified by precipitation with ethanol, and the overlapped fragments for a given mitogenome were combined in equimolar ratios. Following, these pooled fragments were trimmed in fragments of 250 pb in average using a Covaris Me220. Illumina libraries were constructed using the NEBNext Ultra II DNA Library Prep Kit for each sample and sequenced on the Ilumina MiSeq platform.

2.3. Assembly, annotation and alignment

The quality of each paired raw fastqc files generated was checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The trimming of each raw file was made across Trimmomatic (Bolger et al., 2014) if it was necessary. The partial sequences of *cox1* for each species were used as a template with the "Map to Reference" option in the software Geneious® Prime 2022.1 (Kearse et al., 2012) to assemble and annotate the mitogenomes and obtain the circular sequences. Consequently, we used the MITOS web server (Bernt et al., 2013) for the identification of protein codifying genes (CDS), transference RNAs (tRNA), and the two rRNAs. Furthermore, the annotation was corroborated with the ORFs searcher in Geneious® Prime and alignments gene by gene with other tick mitogenomes obtained from GenBank for a thorough correction of the start and end codons.

2.4. Phylogenetic reconstruction

Alignments of the mitochondrial genomes generated in this study, along with those obtained from GenBank, both of the genus *Amblyomma* and *Dermacentor* (as outgroups) were used for the phylogenetic analyses (Table A.1). Homologous CDS genes at the amino acid level and nucleotide level were aligned based on the amino acid codification to conserve the reading frames using Translator X *server* (Abascal et al., 2010). This server has incorporated programs to process the protein-coding genes, first performing the alignments (Katoh et al., 2005) and then filtering the alignments by quality (Castresana, 2000). The rRNA genes (*12S* and *16S*) were also aligned and filtered separately using MAFFTS (Katoh et al., 2019) and GBLOCK (Castresana, 2000) servers, respectively. With that, two concatenated matrices were created, the first with CDSs + rRNAs at the nucleotide level (Matrix NT), and the second, with CDSs at the amino acid level (Matrix AA). Both matrices were phylogenetically analyzed by probabilistic methods such as Bayesian Inference (BI; Rannala and Yang, 1996; Yang and Rannala, 1997) and Maximum Likelihood (ML; Felsenstein, 1981).

For the ML analysis, two strategies for the best fit substitution model selection were implemented using ModelFinder (Kalyaanamoorthy et al., 2017). One strategy was to select the best fit homogeneous model (HoM) matrix using the command -m TEXTONLYNEW, and the second was to choose the best mixture model (MM) using -mset matrix+C20, matrix+C30, matrix+C40, matrix+C50, matrix+C60 command. In both cases, the model was selected under the Bayesian Information Criteria (BIC) (Schwarz, 1978). For the BI analysis, the site-heterogeneous CAT-GTR model (HeM: Lartillot and Philippe, 2004) was used discarding constant sites ("-dc" option). Two independent MCMC chains were run, sampling every cycle for $>$ 20,000 cycles each. Majority-rule consensus trees were obtained after discarding the first 10% cycles as burn-in. Convergence was checked a posteriori using the tools implemented in PhyloBayes (max- diff $\langle$ 0.1, maximum discrepancy $<$ 0.1, and effective sample size $\rangle$ 100).

Despite the fact that the mitogenome for *Amblyomma interandinum* is not available, a concatenated matrix was created with the partial mitochondrial genes available for this species (JX987857: *12S*; KT820358: *16S*; KT820363: *cox1*; KF787627: *cox2*) in order to confirm its phylogenetic position. These analyses were possible thanks to RxML (Stamatakis, 2006) under the best fit nucleotide substitution models for each partition (GTR+I + G), which were selected by Partition Finder (Lanfear et al., 2012) and the BIC (Schwarz, 1978).

2.5. Characterization and general comparisons of mitogenomes

A subset matrix was created including the newly generated mt genomes of the *Amblyomma cajennense* complex plus the two previously available *A. sculptum* mitogenomes, with *A. americanum* as an outgroup. This matrix was used to calculate the Relative Synonymous Codon Usage (RSCU) and the amino acid frequencies, as well as the corrected pairwise distances in MEGA 11 (Tamura et al., 2021) under the Kimura 2-parameter model (Kimura, 1980).

3. Results

All seven new mitogenomes are completely circular molecules, their composition and length are very similar and range between 14,731–14,815 bp. Each of them contains 13 protein codifying genes, 2 rRNA genes, and 22 tRNA with the same arrangement. The two biggest intergeneric spaces appear between *12S* and *tRNA-Ile*, and between *tRNA-Leu*-and *tRNA-Cys*; whereas there are two overlaps in the same strand between: *atp6–atp8*, and *nad4–nad4L*; and three overlaps in different strands between: *tRNA-Glu–nad1, tRNA-Trp–tRNA-Tyr*, and *tRNA-Tyr–cox1*. All the tRNAs present a secondary clover-like structure. tRNAs nucleotide content is 11.1% of G, 16% of C, 29.2% of A, and 43.7% of T. See the general mitochondrial genome scheme of the *Amblyomma cajennense* complex in Fig. 1.

The codon usage is biased towards codons that have in their third positions an A or a T, except for CCG (Arg) and AGG (Ser 2) (Table A.2). The interspecific genetic distances within the *Amblyomma cajennense* complex show values between 14.02% and 23.01%, being the highest values observed with *A. tonelliae* in relation to the rest of the complex; the genetic distance across specimens included in this study are shown in Table 3, where the higher genetic divergence was between the two specimens of *A. mixtum* included (4.74%).

The phylogenetic hypotheses were calculated by BI and ML methods employing two matrices. The first matrix was built with the CDSs + rARNs at the nucleotides level (NT) and the second one, with CDSs at the amino acid level (AA). The resulting topologies are compatible and show minor differences in the statistical support values. The relationships among the *Amblyomma cajennense* species complex are the same in all the topologies as seen in Fig. 2, including those constructed with partial genes, Fig. 3. *A. tonelliae* appears branching first as sister of the remaining species of the complex. *A. cajennense* s.s. appears as a sister group of *A. patinoi*, being this two the sister group of *A. mixtum*, and these three related species were recovered as lineage of *A. sculptum*. These five species of the *Amblyomma cajennense* complex were recovered the sister clade of *A. americanum* in all topologies.

The principal disparities through the topologies are given by changes in the position of species like *A. maculatum, A. geoemydae*, and *A. ovale*. In the topologies calculated based on NT matrices (as observed in Fig. 2), *A. maculatum* appears as the sister group to the *Amblyomma cajennense* complex + *A. americanum*, while in the AA analysis it appears as the sister of the group conformed by the rest of species except for *A. triguttatum* or as the sister of that last one (see all topologies in Figs. A.1-5). While in the NT topologies, *A. tholloni* and *A. fimbriatum* appear as the sister group of *A. marmoreum* + *A. hebraeum*, and *A. testudinarium* + *A. javanese* respectively, but in the AA trees they are grouped and next to *A. marmoreum* + *A. hebraeum*, being *A. ovale* the sister of the last four.

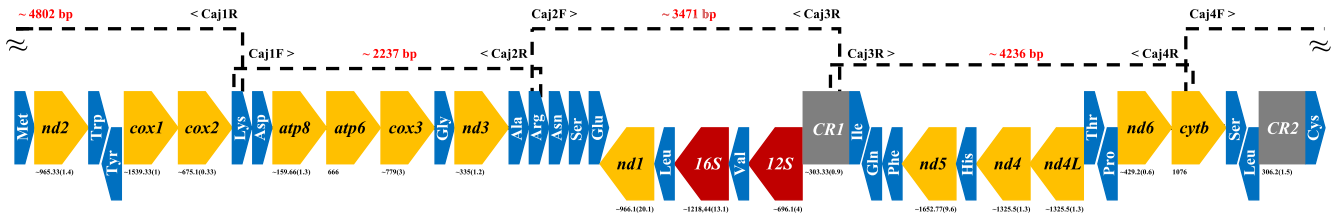

Fig. 1. Lineal representation of the common mitochondrial gene order of the *Amblyomma cajennense* complex [and Metastriata, except *A.* (*Africaniella*) *transversale*]. The protein-coding genes are in yellow, the rRNAs in red, the tRNAs in blue, and the two potential control regions (CR) in gray. The genes encoded in the major and minor strands are in the top and bottom positions, respectively. Flashing boxes circumscribe the amplified regions using their respective primers (as "caj"). The mean size of the amplified regions across the *Amblyomma cajennense* complex is in red. Numbers below CDS, rRNA, and CR genes indicate the mean size and standard deviation [~(SD)] of the sequenced species for the complex.

Table 3
Genetic distances within the *Amblyomma cajennense* complex.

Species		1	2	3	4	5	6	7	8	9
1	*A. americanum* (NC027609)									
2	*A. tonelliae* (T18)	0,2524								
3	*A. patinoi* (SA20)	0,256	0,2227							
4	*A. cajennense* (A.c.)	0,2565	0,2276	0,1407						
5	*A. cajennense* (T19)	0,256	0,2267	0,1402	**0,0038**					
6	*A. sculptum* (A.s.)	0,2521	0,2285	0,1787	0,1815	0,1804				
7	*A. sculptum* (NC020333)	0,2534	0,2301	0,1807	0,1829	0,1823	**0,0144**			
8	*A. sculptum* (NC032369)	0,2521	0,2287	0,1778	0,1816	0,1809	**0,0085**	**0,0087**		
9	*A. mixtum* (T01)	0,2525	0,2241	0,1429	0,1478	0,1471	0,1765	0,1789	0,1762	
10	*A. mixtum* (T02)	0,254	0,224	0,1438	0,1502	0,1498	0,1756	0,1779	0,1753	**0,0474**

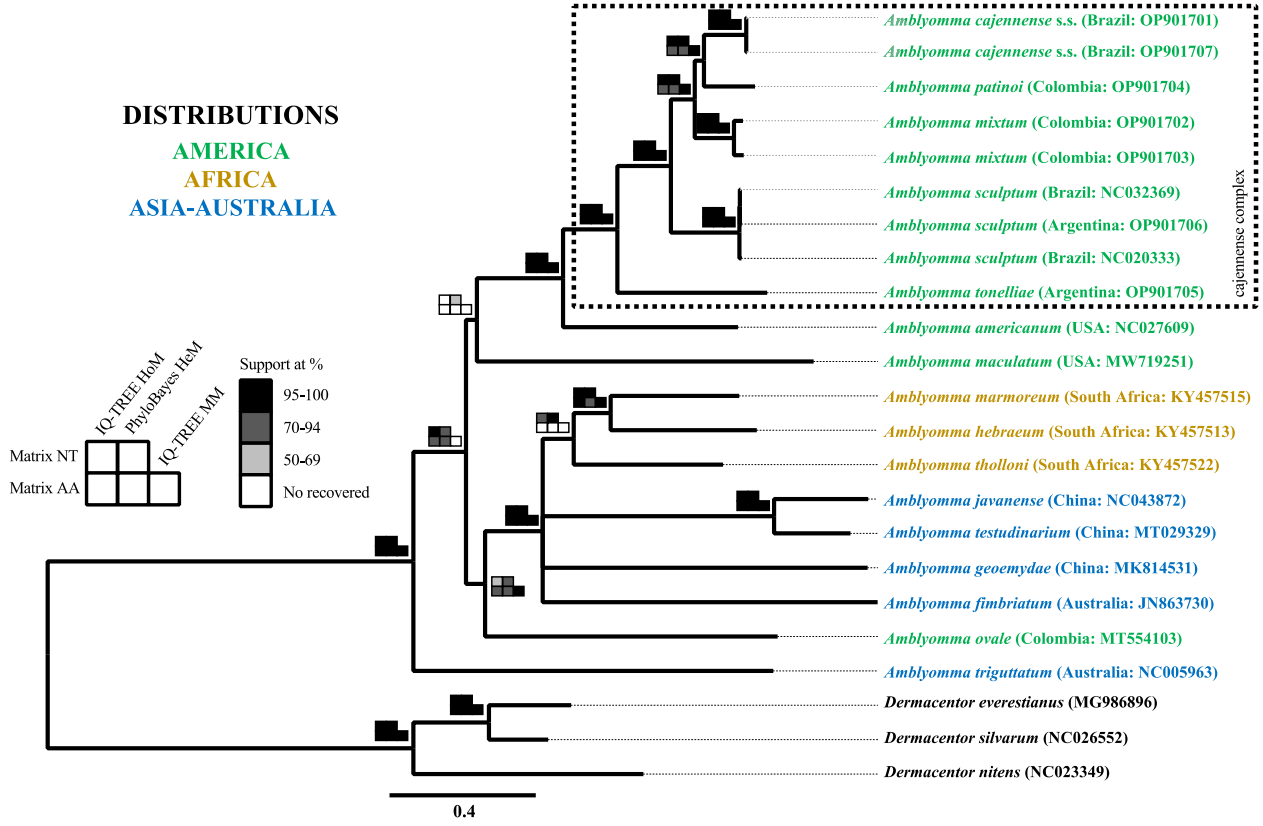

Fig. 2. Phylogenetic relationships of the *Amblyomma cajennense* complex in an *Amblyomma* context under the two matrixes (NT, AA), two probabilistic inferences (ML: IQ-TREE; BI: PhyloBayes), and their respectively evolutionary model (HoM, HeM, and MM). The reconstructed BI tree based on Matrix-NT and HeM is shown. Scale bar 0.4 substitutions/site. The weight of statistical support of each node is indicate by the color palette in the respective topology. The distribution of the species is indicated in color: American distribution, green; African distribution, yellow; Asia and Australian distribution, blue. The GenBank accession numbers of each specimen analyzed are included next to the country.

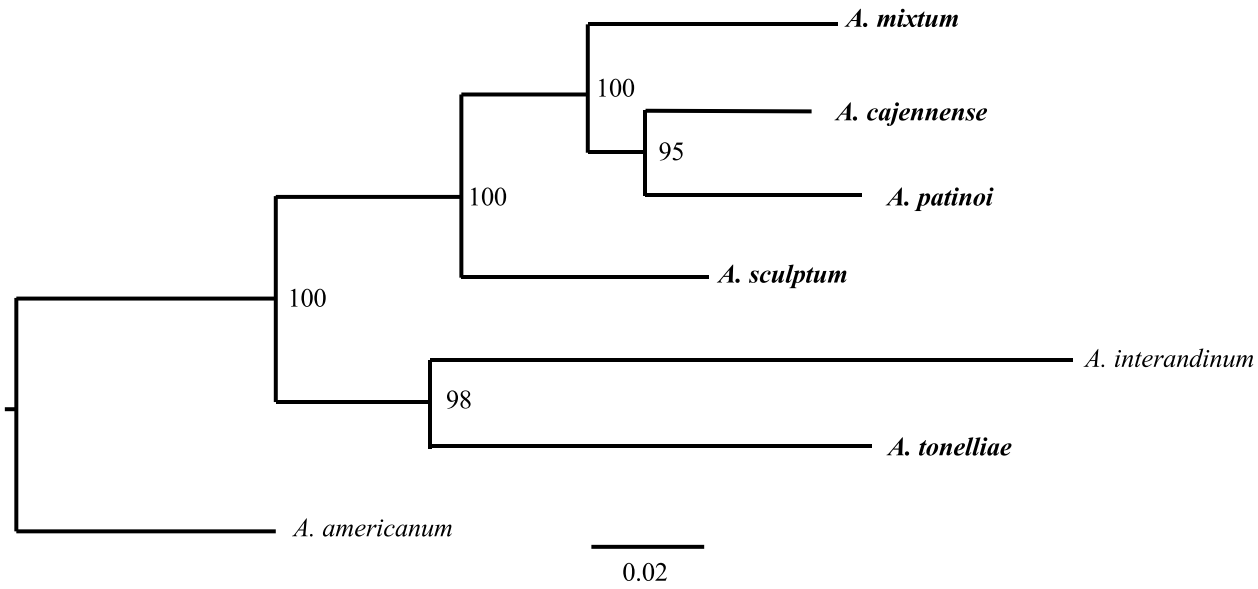

Fig. 3. Phylogenetic relationships of the *Amblyomma cajennense* complex using the partial *12S, 16S, cox1*, and *cox2* mitochondrial genes using the best fit evolutionary model under ML inference. *Amblyomma americanum* (NC027609) was used as outgroup. Node support indicated bootstrap percentages. Scale bar 0.02 substitutions/site. Names in bold indicate the sequences were generated in this study. Accession numbers for *Amblyomma interandinum* partial genes are as follows: *12S*: JX987857, *16S*: KT820358, *cox1*: KT820363, *cox2*: KF787627.

4. Discussion

Although the sequencing of the mitochondrial genomes has been increasing across animal clades, in part due to each time lower prices of novel sequencing techniques, in ticks, most of the investigations have aimed to increase the catalog in order to describe genomic composition, new gene rearrangements, and to establish the phylogenetic position of punctual species (Black IV and Roehrdanz, 1998; Shao and Barker, 2005; Burger et al., 20,012; 2013; 2014; Guo et al., 2016; Liu et al., 2018; Chang et al., 2019; Uribe et al., 2020; Chavatte and Octavia, 2021; Chandra et al., 2022; Lu et al., 2022). There is just one study aiming to concentrate the mitogenomic sampling on a particular clade and to answer concrete hypotheses (Burger et al., 2014). In this study, we have focused the mitogenomic sequencing effort on the *Amblyomma cajennense* complex to all species (except *A. interandinum*, from the Inter-Andean valley of Peru). This new mitogenomic information is a priority in order to better understand the evolutionary basis of this wide-distributed clade complex with a high economic and public health impact on the Americas.

4.1. Phylogenetic relationships

The poor resolution in the five topologies obtained in this study is mostly present in the phylogenetic relationships of the African and Indo-Pacific species with corresponding low statistical support values (Figs. A.1-5), which reveals the need to increment the molecular information about the *Amblyomma* genus in general, and in particular, from the species of those regions of the world.

Even though relationships within *Amblyomma* spp. are not clear, inside the *Amblyomma cajennense* complex we recovered the same internal relationships in all the topologies with strong support. The relationships observed correspond to those previously obtained by Beati et al. (2013) and Santodomingo et al. (2021). This result demonstrates that the conjunction of mitochondrial and nuclear partial genes (Beati et al., 2013; Santodomingo et al., 2021) can resolve close relationships when entire genes or genomes are unavailable, as also shown in the case of species belonging to the subgenus *Boophilus* (see Estrada-Peña et al., 2012; Burger et al., 2014; Labruna et al., 2009b). However, mitogenomes offer a better understanding of tick taxonomy and could solve deeper relationships than partial genes (Kelava et al., 2021; Mohamed et al., 2022) and, like this study, confirm with a robust mitogenomic dataset the previous hypothesis for the phylogenetic relationships of the complex. Likewise, here, we have decided to confirm the deep phylogenetic position of *Amblyomma interandinum* (within the *Amblyomma cajennense* complex, as the sister lineage of *A. tonelliae*) using a mitogenomic dataset, given its low statistical support in the previous hypotheses (Santodomingo et al., 2021).

4.2. Sequence pattern composition

The content of the new *Amblyomma* mitogenomes sequenced in this study corresponds with that of most animals, especially arthropods (Boore et al., 2005; Castellana et al., 2011). Their AT and GC contents are equal to the mt genome of *A. sculptum* (de Lima et al., 2017), and very similar to the rest of the Ixodidae (Chang et al., 2019; Irisarri et al., 2020; Liu et al., 2020; Uribe et al., 2020). The arrangement of the genes is the same found in the rest of *Amblyomma* and Metastriata (Fig. 1), except for *Amblyomma* (*Africaniella*) *transversale* where five gene inversions and translocations are present, however, this species must be classified outside *Amblyomma* given its phylogenetic position as well as other differences (Kelava et al., 2021).

The AT content in the Metastriata subfamily is about 75 – 80% (Liu et al., 2018; Wang et al., 2019, 2020) in accordance with the positive bias towards those codons finishing in A or T in the CDSs. It is common in mitochondrial genes of metazoans that the codon usage bias is correlated with neighboring nitrogenous bases since there is a positive selection for those codons whose translation efficiency is higher, which means that the rate of mutation from one base to another depends on the adjacent ones (Jia and Higgs, 2008). Usually, GC-rich regions show higher rates of evolution (Su et al., 2011), which can imply [according to de Lima et al. (2017)] a very conserved genetic structure in these genomes.

Genetic distances between the nine mitogenomes, now available for the *Amblyomma cajennense* complex, corroborate the clear differentiation between the five species. The pairwise identity of different species varies from 77 to 86%. It is necessary to increase the quantity of mitogenomic information across populations that allow for establishing a divergences range within the same species. Also, to assess the positive

selection and the role of mitochondrial adaptation in the diversification of the complex and its potential impact on the distribution of these species during times of climate change.

4.3. Genome skimming and conserved mitogenomic region for primer design

All the mitogenomes obtained in this study resulted from a combination of genome skimming and long-range PCRs sequencing methods. Genome skimming provides an efficient approach, especially when the quality of tissue samples is limited. WGS remains to be expensive, but for some kinds of data such as mitogenomes, it results in a good cost-benefit ratio (Zhang et al., 2019). Although the amount of copies of mtDNA is variable depending on many factors, most of them are not understood yet (Moraes, 2001; Cole, 2016), it is possible to find between 1000 and 5000 copies of the mitochondrial genome in most animal cells (Bogenhagen and Clayton, 1974; Sogl et al., 2000; Salminen et al., 2017), which is 10 to 100 more copies than in the nuclear DNA per cell, providing a higher read depth which allows the assembly and isolation of the mitogenome (Al-Nakeeb et al., 2017). A total of 83,597,882 reads were generated under the genome skimming (GS) through the Illumina platform for the gDNA of *A. patinoi*. From the total of these reads, 134,632 correspond to its mitogenome, generating a mean coverage of 1376 (Std Dev: 437.2) deep.

The remaining six mitogenomes were amplified by Long-range PCRs, which provide better cost-benefit when the aim of the project is to sequence mitogenomes of several samples and the quality of the DNA is high. Four conservative regions across the mitogenomes of *Amblyomma* available in NCBI were detected by alignment (see Fig. 1). In these regions, we designed crossed primers in order to amplify the mitogenomes in four overlapped amplicons. Primer regions correspond to $tRNA^{Lys}$, $tRNA^{Arg}$, the intergenic space between 12S rRNA and $tRNA^{Ile}$, and close to the first 200 bp of the *cytb* gene, with all four pairs having the same annealing temperature (see Fig. 1 and Table 2). This Long-range PCR strategy demonstrated that these designed primers may amplify the mitogenome for all species of the *Amblyomma cajennense* complex, or at least for those that were included here. Lastly, this Long-range PCR strategy also could provide a powerful tool (besides nuclear markers) to evaluate the circumscriptions of the potential cryptic species behind the current *Amblyomma mixtum* concept.

5. Conclusions

We present here the first mitochondrial genomes for *A. cajennense* s. s., *A. mixtum, A. tonelliae*, and *A. patinoi*; and a new one for *A. sculptum*, providing a more robust phylogenetic hypothesis that supports the previous relationships inferred for the *A. cajennense* species complex. All these genomes share the general characteristics of the mitogenomes of the Metastriata subfamily. Also, it is important to highlight that the evolutionary hypothesis reached previously with partial genes (both mitochondrial and nuclear) is corroborated here by the mitogenomic dataset, which points to the establishment of this hypothesis. It is necessary to increase the number of available mitogenomes for this genus, especially for those species from the Indo-Pacific region and Africa, by means of a better understanding of their relationships and evolution process.

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Authors' contributions

ACP and JEU contributed to the lab work, data curation, formal analyses, and writing the first draft; LRC and SN made substantial contributions in the design of the study and discussion of the results; FRP and JCV contributed with the collection of samples, taxonomical analyses and the final draft. All authors contributed and approved the final version of the manuscript.

Data availability

Mitogenomes are available in the nucleotide database of NCBI with OP901701-7 access numbers.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ttbdis.2023.102125.

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