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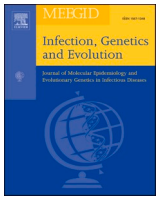

Phylogenetic description of *Trypanosoma cruzi* isolates from *Dipetalogaster maxima*: Occurrence of TcI, TcIV, and TcIV-USA

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ABSTRACT

Trypanosoma cruzi is the parasite responsible for Chagas disease. The parasite has been classified into six taxonomic assemblages: TcI-TcVI and TcBat (aka Discrete Typing Units or Near-Clades). No studies have focused on describing the genetic diversity of *T. cruzi* in the northwestern region of Mexico. Within the Baja California peninsula lives *Dipetalogaster maxima,* the largest vector species for CD. The study aimed to describe the genetic diversity of *T. cruzi* within *D. maxima*. A total of three Discrete Typing Units (DTUs) were found (TcI, TcIV, and TcIV-USA). TcI was the predominant DTU found (~75% of samples), in concordance with studies from the southern USA, one sample was described as TcIV while the other ~20% pertained to TcIV-USA, which has recently been proposed to have enough genetic divergence from TcIV, to merit its own DTU. Potential phenotype differences between TcIV and TcIV-USA should be assessed in future studies.

1. Introduction

Trypanosoma cruzi is the parasite responsible for Chagas disease (CD). A parasite that is naturally transmitted by insect vectors of the subfamily Triatominae. Other than a couple of studies citing the presence of *T. cruzi* outside of the American continent, this parasite appears to have a natural habitat in the Americas. As is true for most parasitic diseases (apart from malaria), no current vaccine is available to prevent the infection. Due to the enormous health impact of CD, it is considered one of the leading causes of disability-adjusted life years in the Latin American and Caribbean regions (Hotez et al., 2008).

Mexico is a country with a population of almost 130 million. Although the presence of vectors and the parasite has been confirmed for most of the Mexican territory, certain epidemiological aspects of CD remain unknown for some areas of the country. For example, the infection rate in vertebrate hosts and vectors, host feeding preferences of vectors, and genetic diversity of *T. cruzi* remain unrevealed for particular sections of the country. The Baja California Peninsula (BCP) is located in the northwestern corner of Mexico. From its northern border with the USA to its southern tip in the Cabo region, it covers more than 2000 km in length. Despite its dimension, it is a predominantly understudied region concerning CD.

Within the southernmost region of the BCP resides the endemic and largest Triatomine species described thus far: *Dipetalogaster maxima* (Ryckman and Ryckman, 1967). Few studies have focused on traits of the species in its natural habitat. In the field, *D. maxima* are easily collected due to its aggressive feeding behavior and whose feeding hosts are hypothesized to be quite diverse (Ryckman, 1986). A trait which in turn has been hypothesized to play an essential role on this vector's apparent *T. cruzi* low infection rate (Flores-López et al., 2021).

The genetic diversity of *T. cruzi* circulating in the BCP has not been previously reported. A description that is epidemiologically important due to the multiple lines of evidence that have shown there is a correlation between the genetic background of *T. cruzi* and the diverse pathological outcomes of CD (Andrade, 1974; de Lana et al., 1998; Postan et al., 1987; Revollo et al., 1998; Telleria et al., 2010). To date, there has been a significant genetic diversity described within *T. cruzi*, where the consensus points to a total of seven genetic lineages, termed Discrete Typing Units (DTUs) (Zingales et al., 2012). The DTUs described throughout the American continent are termed TcI-TcVI, whereas a more unusual DTU was described in bats and thus termed TcBat (Pinto et al., 2012). More recently, an additional lineage has been reported to be divergent enough from other previously described DTUs, which would potentially lead to a new DTU, and has been temporally referred to as TcIV-USA, for its previous categorization as TcIV within samples described from the southern USA (Flores-López et al., 2022).

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Table 1
Geographic localities of *Trypanosoma* spp. infected samples in *Dipetalogaster maxima*.

Sample ID	Geographic coordinates	Site code	Date of collection	Insect developmental stage
BAJA-16-7-N2-07	N 23°24.175′ W 110°12.307′	6	03/11/2016	Nymphal stage 2
BAJA-16-7-N3-03	N 23°24.175′ W 110°12.307′	6	03/11/2016	Nymphal stage 3
BAJA-16-7-N3-05	N 23°24.175′ W 110°12.307′	6	03/11/2016	Nymphal stage 3
BAJA-16-7-A-01	N 23°24.175′ W 110°12.307′	6	03/11/2016	Adult
BAJA-16-7-A-02	N 23°24.175′ W 110°12.307′	6	03/11/2016	Adult
BAJA-17-11-N3-03	N 23°51.641′ W 110°09.971′	2	22/7/2017	Nymphal stage 3
BAJA-17-13-N3-13	N 23°31.675′ W 109°46.862′	5	24/7/2017	Nymphal stage 3
BAJA-17-13-N3-12	N 23°31.675′ W 109°46.862′	5	24/7/2017	Nymphal stage 3
BAJA-17-11-N3-01	N 23°32.953′ W 109°48.652′	3	24/7/2017	Nymphal stage 3
BAJA-17-15-A-02	N 23°32.079′ W 109°46.876′	4	24/7/2017	Adult
BAJA-18-10-N3-03	N 23°59.501′ W 110°09.096′	1	19/6/2018	Nymphal stage 3
BAJA-18-10-N3-05	N 23°59.501′ W 110°09.096′	1	19/6/2018	Nymphal stage 3
BAJA-18-10-A-01	N 23°59.501′ W 110°09.096′	1	19/6/2018	Adult
BAJA-18-10-A-02	N 23°59.501′ W 110°09.096′	1	19/6/2018	Adult
BAJA-18-15-N1-02	N 23°32.079′ W 109°46.876′	4	19/6/2018	Nymphal stage 1
BAJA-18-15-N3-05	N 23°32.079′ W 109°46.876′	4	19/6/2018	Nymphal stage 3
BAJA-20-02-01	N 23°32′06” W 109°46′54”	4	27/07/2020	Undetermined

Collection dates are specified in the following order: day/month/year.

The objective of this study was the phylogenetic description of the DTUs currently circulating *D. maxima* within the BCP.

2. Methodology

2.1. Fieldwork and identification

The collection of *D. maxima* specimens was done by using the same individual collectors as baits. The method consisted of placing each collector on top of a granite outcrop, collecting all triatomine specimens that would approach the collector and placing each specimen in an individual vial filled with 95% ethanol. Specimens approached the collectors from a radius of approximately five meters. After 30 min of collecting specimens from a single location, collectors would move to another location that was at least five meters away. If no triatomines appeared within the first 15 min of initial collection, the collector would move to another granite outcrop at least seven meters from the initial position and start the collecting process. All fieldwork was done during a one-to-three-week period during the following years: 2016–2018 and 2020, between 10:00–16:00 h (Table 1). On average, a total of three to four collectors would work on a given field collecting season. The localities of the sampled specimens can be found in (Flores-López et al., 2021), while the localities of specimens that were infected with *T. cruzi* and whose sequence was used in this study can be found in Fig. 1 and Table 1. All specimens collected were identified as *D. maxima* using a dichotomous morphological identification key (Lent and Wygodzinsky, 1979).

2.2. Diagnosis of T. cruzi

DNA was extracted from the digestive tracts of every *D. maxima* specimen collected and used to extract DNA. A Purelink Genomic DNA extraction kit was used for the DNA extraction (Invitrogen, USA). Diagnosis for the presence of *T. cruzi* was made with the MM TcCLB.507467.90, as described previously (Rivas-García et al., 2020) (Supplementary Fig. 1). Every PCR had approximately 50 ng of DNA per PCR reaction. DNA from a culture-derived *T. cruzi* sample (strain CA-1-05) was used as a positive control in the PCR diagnosis amplification (kindly provided by Dr. Carlos Machado at the University of Maryland). From which a total of one microliter of DNA at a concentration of 20 nanograms per microliter was used in each PCR reaction.

2.3. Molecular marker PCR amplification and sequencing

A total of five molecular markers (MM) were used for the phylogenetic analyses. Four of these MM were nuclear, while one marker was mitochondrial. The nuclear MM were gene segments of the Dihydrofolate reductase-thymidylate synthase (Machado and Ayala, 2001), Trypanothione reductase (Machado and Ayala, 2001), RNA binding protein 19 (Yeo et al., 2011) and TcCLB.508213.20 genes (previously referred to as Tc00.1047053506529.310) (Flores-López and Machado, 2011). The mitochondrial gene segment used was the cytochrome oxidase subunit 2-NADH dehydrogenase subunit 1 (COII-NDI) (Machado and Ayala, 2001). All five MM have been previously shown to be helpful for the phylogenetic reconstruction of *T. cruzi* DTUs (Machado and Ayala, 2001; Shender et al., 2016). The primers and PCR conditions for each amplification are described in the original references.

All PCR amplicons were bidirectionally sequenced at MacroGen Inc. by the Sanger method in a 3130xl Genetic Analyzer (Applied Biosystems). All chromatograms were manually checked and edited in Geneious 2022.2.1. All sequences were deposited in GenBank (Accession Nos. OQ672324- OQ672330; OQ594717-OQ594728; OQ818001-OQ818028).

2.4. Phylogenetic analyses

All the sequences generated in this study were aligned with reference sequences published in GenBank that represent all DTUs (Supplementary Table 1). Alignments were performed with MUSCLE (Edgar, 2004) in Seaview (Gouy et al., 2010) and manually checked. The most appropriate DNA substitution model for each MM alignment was calculated with the Aikike Information Criterion in jModeltest2 (Darriba et al., 2012). Consensus phylogenetic trees were reconstructed in MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), using the most appropriate DNA substitution model obtained from jModelTest. Each molecular marker's homologous gene segments from *T. cruzi marinkellei* were used as outgroups in each phylogenetic analysis and in the concatenated analysis for all five molecular markers. The Markov Chain Monte Carlo calculation performed in MrBayes sampled thirty million generation runs. All runs had a standard deviation lower than 0.01 when the generation runs had finished. The consensus trees were compiled after discarding the first 25% of saved trees and visualized in FigTree (Rambaut and Drummond, 2008).

3. Results

Overall, 16 *D. maxima* individuals were found to be infected with *Trypanosoma cruzi*. To uncover the evolutionary history of *T. cruzi*, five molecular markers (MM) were sequenced and used for individual phylogenetic reconstructions. Of the 16 infected samples, only four were able to have all five MM amplified. In contrast, most samples only had four or fewer MM amplified (Table 2). A pattern not uncommon when amplifying *T. cruzi* DNA from field-collected specimens (Flores-López et al., 2022). The phylogenetic reconstruction was generated by

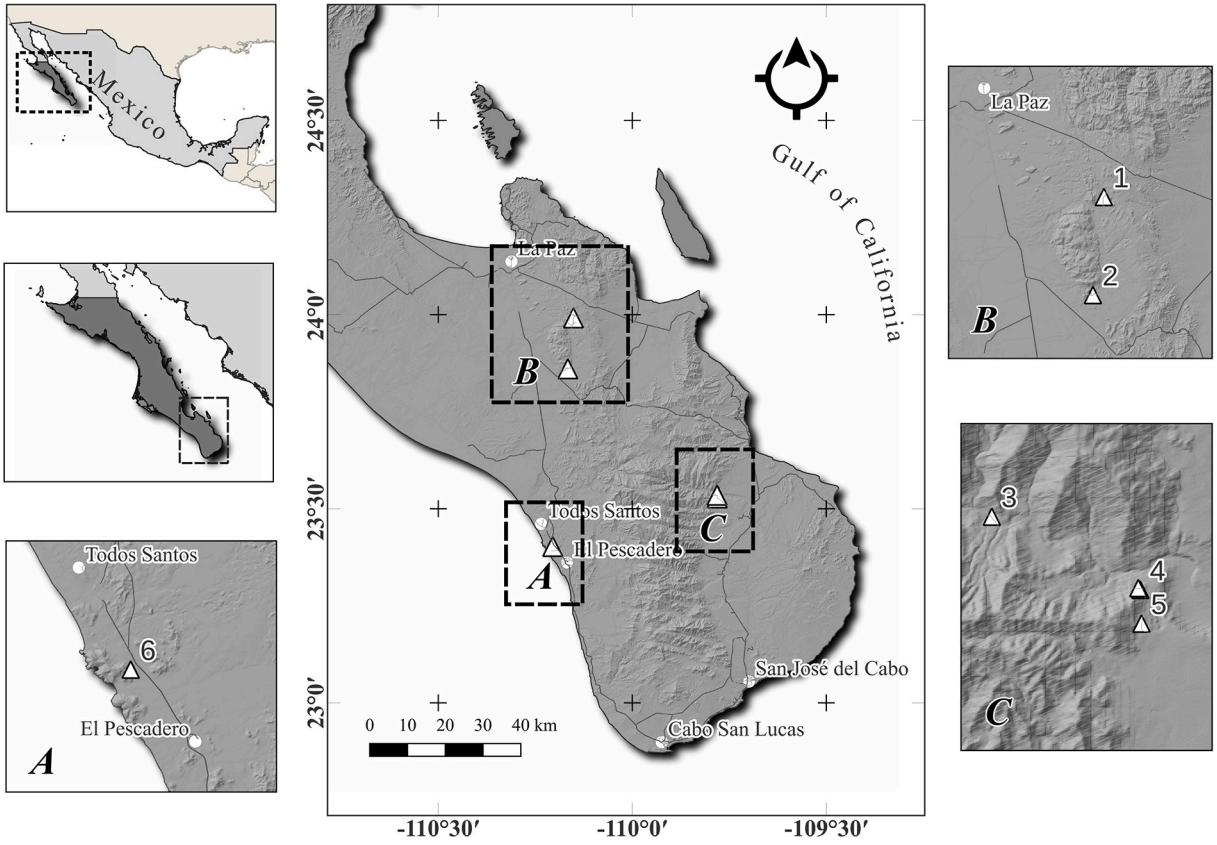

Fig. 1. Map of sites with positive *T. cruzi* samples of *D. maxima*. Numbers within subfigures A-C represent the "site codes" from Table 1. Geographic coordinates can be found in Table 1.

Table 2
DTU allocation for *T. cruzi*-positive samples.

Sample ID	COII-NDI	DHFR-TS	TRY2	TcCLB.508213.20	Rb19
BAJA-16-7-N2-07	TcI	–	TcI	TcI	TcI
BAJA-16-7-N3-03	TcI	TcI	TcI	TcI	TcI
BAJA-16-7-N3-05	TcI	–	–	–	–
BAJA-16-7-A-01	TcI	–	TcI	–	TcIV-USA
BAJA-16-7-A-02	TcI	TcI	TcI	TcI	TcI
BAJA-17-11-N3-03	–	–	–	TcIV-USA	
BAJA-17-13-N3-13	TcI	TcI	TcI	–	–
BAJA-17-13-N3-12	TcI	TcI	TcI	TcI	–
BAJA-17-11-N3-01	–	TcIV-USA	–	TcIV-USA	–
BAJA-17-15-A-02	TcI	TcI	TcI	TcI	TcI
BAJA-18-10-N3-03	–	–	–	TcIV-USA	TcIV-USA
BAJA-18-10-N3-05	–	–	–	TcI	–
BAJA-18-10-A-01	TcI	TcI	TcI	TcI	TcI
BAJA-18-10-A-02	TcIV	–	TcIV-USA	–	–
BAJA-18-15-N1-02	TcI	–	–	TcI	TcI
BAJA-20-02-01	TcI	–	–	–	–

COII-NDI: cytochrome oxidase subunit 2-NADH dehydrogenase subunit 1. DHFR-TS: Dihydrofolate reductase-thymidylate synthase. TRY2: Trypanothione reductase. Rb19: RNA binding protein 19. Cells with (–) represent MMs that could not be amplified.

individual gene trees as well as with a concatenated data set of all positive samples (Figs. 2 & 3).

Once all reference *T. cruzi* strains were included in the individual alignments, 1085 bp of aligned DNA sequence was used for the mitochondrial gene segment of the COII-NDI. Of the four nuclear MM, the DHFR-TS MM gene segment consisted of 1145 bp of aligned sequence, the Trypanothione reductase (TRY2) MM alignment consisted of 1160 bp, the TcCLB.508213.20 locus, a total of 600 bp of aligned DNA sequence was obtained, while the gene segment used for the RNA binding protein 19 (Rb19) consisted of 323 bp of aligned sequence. GenBank accession codes for every sequence used in the phylogenetic analyses can be found in supplementary Table 1.

The amount of polymorphic DNA present among the five MM allowed for a proper phylogenetic resolution in three out of the five MM used (COII-NDI, DHFR-TS, TRY2, and TcCLB.508213.20). The MM of the RNA binding protein did not result in a fully resolved tree (Fig. 2), while the TcCLB.508213.20 also did not produce a fully resolved tree. The basal node for *T. cruzi* DTUs in the RNA binding protein MM (Rb19) resulted in a polytomy (Fig. 2). Other than these two MM, the other three MM had fully resolved phylogenetic trees with good statistical support

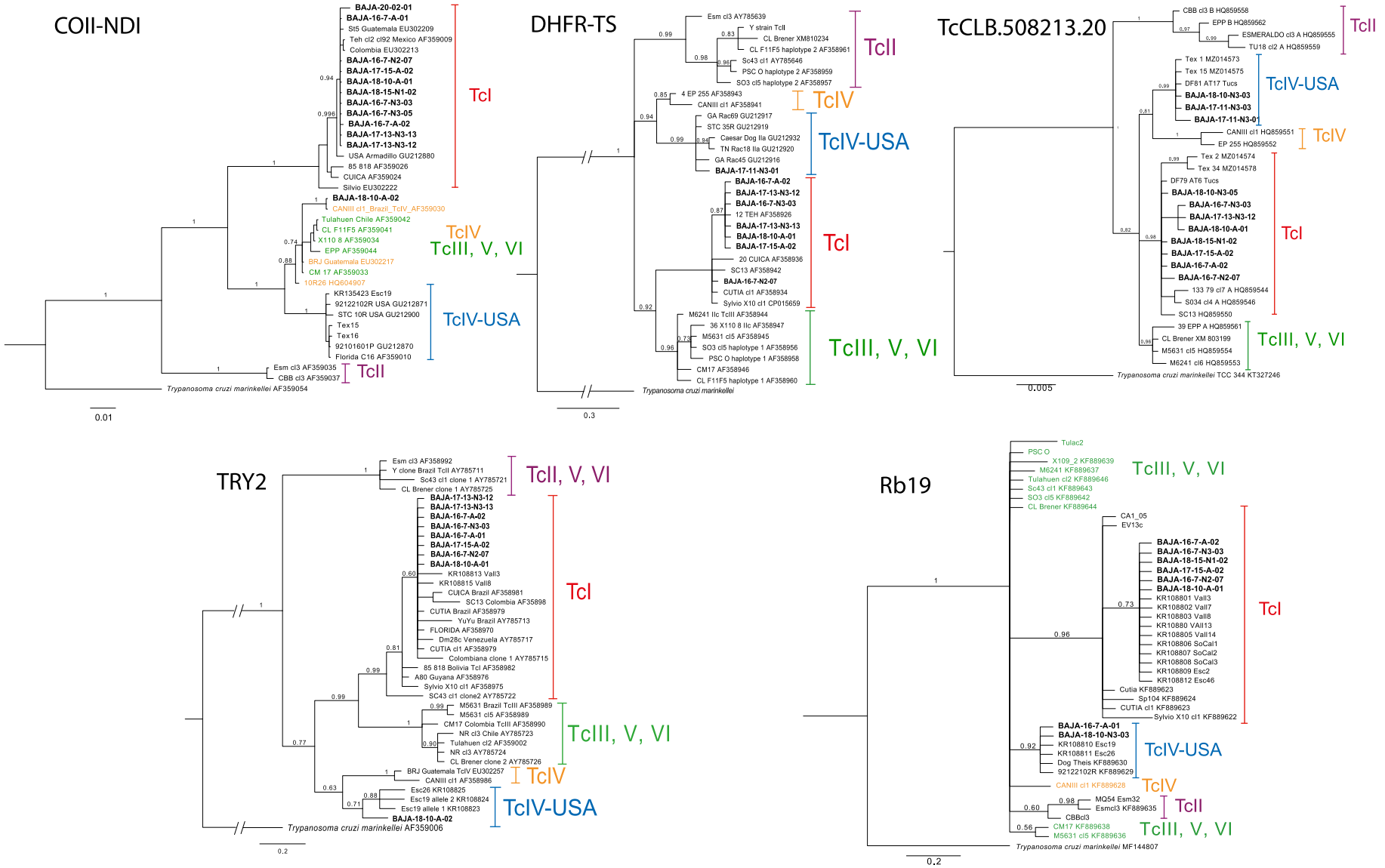

Fig. 2. Phylogenetic trees for each molecular marker used. Numbers above each internal node represent posterior probabilities from the consensus trees generated in MrBayes. COII-NDI: cytochrome oxidase subunit 2-NADH dehydrogenase subunit 1, reconstructed from a total of 1085 bp of aligned DNA sequence; DHFR-TS: Dihydrofolate reductase-thymidylate synthase, reconstructed from a total of 1145 bp of aligned DNA sequence; TcCLB.508213.20 consisted of a total of 600 bp of aligned DNA sequence; TRY2: Trypanothione reductase, reconstructed from a total of 1160 bp of aligned DNA sequence; Rb19: RNA binding protein 19, reconstructed from a total of 323 bp of aligned DNA sequence. Samples isolated in this study are shown in bold.

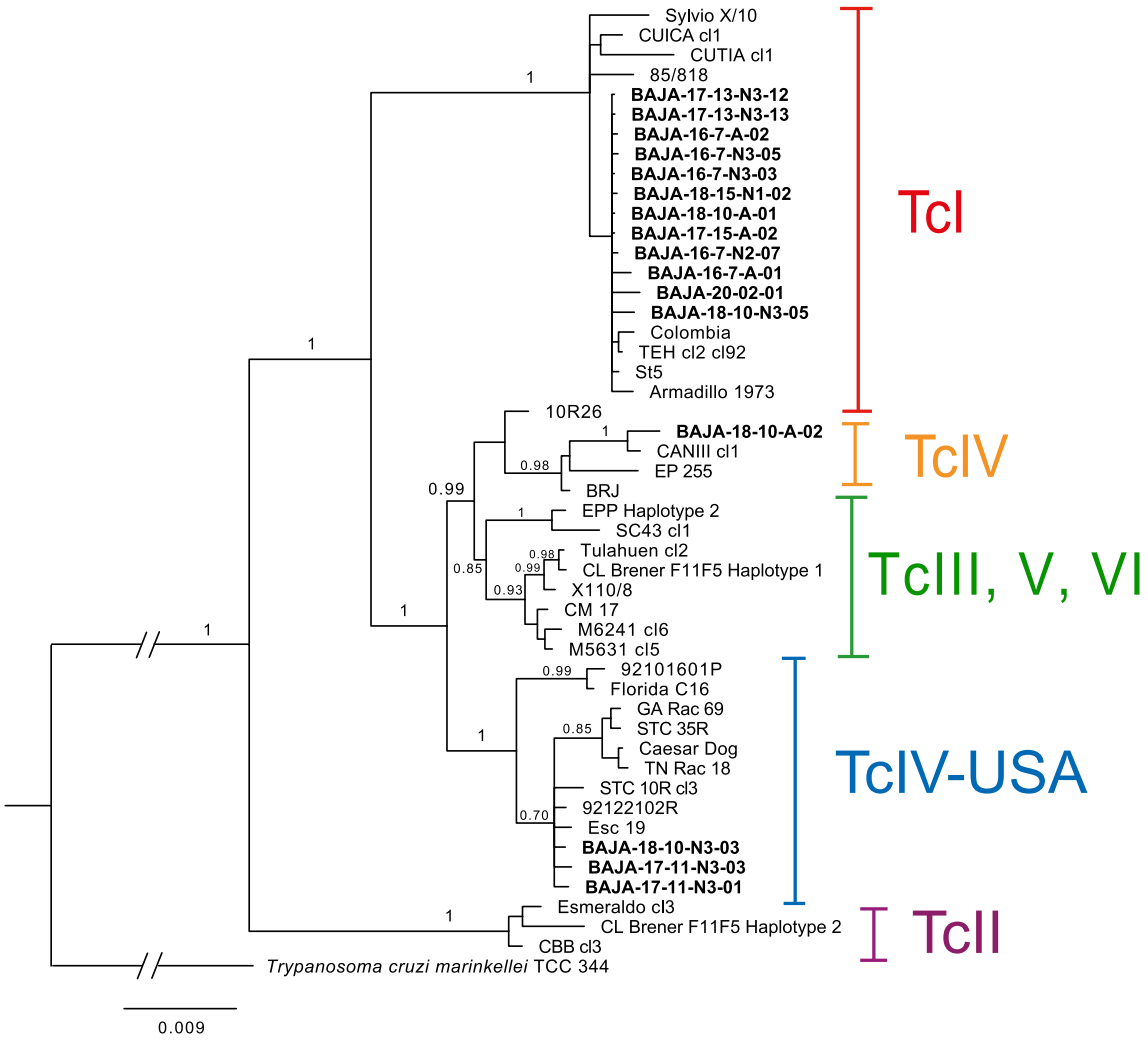

Fig. 3. Phylogenetic tree from the concatenated data set of all five MM. Numbers above each internal node represent posterior probabilities from the consensus trees generated in MrBayes. The concatenated data set consisted of COII-NDI, DHFR-TS, TRY2, TcCLB.508213.20, and Rb19 gene segments. GenBank accession codes for sequences used in analyses can be found in supplementary Table 1.

in each monophyletic clade representing the major DTUs of *T. cruzi*.

A concatenated data set was constructed from the individual DNA sequences from each MM. The phylogenetic tree generated from this data set confirmed the results obtained from the individual gene trees. It resolved two conflicting phylogenetic signals uncovered in two samples (Fig. 3). Specifically, samples BAJA-16-7-A-01 and BAJA-18-10-A-02 had conflicting phylogenetic histories between two MM (Table 2). The mitochondrial MM clustered BAJA 18–10-A-02 with CANIII cl1, which has been classified as DTU TcIV. At the same time, the TRY2 MM grouped the same sample with TcIV-USA (Fig. 2), and BAJA-16-7-A-01 clustered as TcI in the COII-NDI and TRY2 MM's, while the Rb19 MM clustered the sample with TcIV-USA (Fig. 2). However, both inconsistencies were resolved in the consensus tree generated from the concatenated data set. From this, it can be observed that sample BAJA-18-10-A-02 appears to be more closely related to reference strains of TcIV, while BAJA-16-7-A-01 appears to cluster within TcI (Fig. 3).

Thus, of 16 samples, 14 samples did not show any inconsistent phylogenetic backgrounds between molecular markers (Table 2). The Bayesian analysis of the mitochondrial locus uncovered two distinct *T. cruzi* DTUs. Eleven samples clustered with DTU TcI, while only one clustered with a sample historically classified as TcIV. Concerning the samples classified as TcI, they all cluster with previous TcI samples recovered from Central America, Mexico, and the USA, showing almost no variation within the samples isolated in this study (Fig. 2).

The nuclear segment for the Dihydrofolate reductase-thymidylate synthase gene revealed two distinct DTUs. One sample (BAJA 17–11-N3–01) grouped with high statistical support to the newly proposed clade TcIV-USA, while the rest clustered with TcI. A very similar outcome was observed for the other four nuclear markers. The phylogenetic tree for the gene TcCLB.508213.20 clustered three samples as TcIV-USA, while the other eight samples grouped with DTU TcI (Fig. 2). In contrast, the phylogenetic tree for Rb19 assembled two samples as TcIV-USA and the other six as DTU TcI. Most infections pertained to TcI, whereas a few samples were infected with TcIV-USA.

Overall, the combination of MM allowed a description of the DTUs present in *D. maxima* (Figs. 3 & 4) since the combination of data avoided any potential biases in the phylogenetic interpretation of a single MM (Fig. 2), which resulted in the identification of three distinct DTUs. TcI was the predominant DTU described, with 12 samples of the 16 pertaining to this DTU. Only one sample was described as TcIV, while three

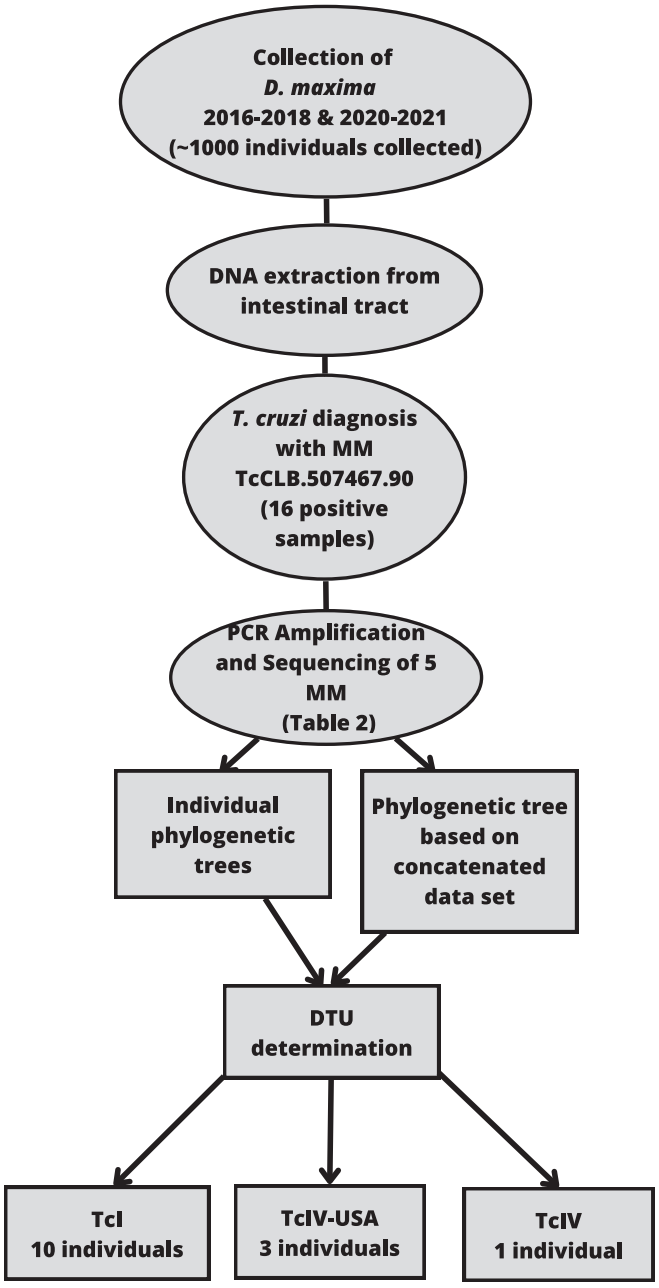

Fig. 4. Flowchart for the description of *T. cruzi* DTUs found within *D. maxima*.

were TcIV-USA (Fig. 3 and Table 2).

4. Discussion

Although two samples appeared to have conflicting phylogenetic signals when analyzing individual gene trees (Fig. 2), the discordance might be the result of several reasons: 1) a potential hybrid ancestry for these samples (BAJA-16-7-A-01 and BAJA 18–10-A-02), resulting from a recombination event between distinct genetic lineages; 2) a mixed infection of distinct DTUs; 3) a mixed infection of distinct kinetoplastid species (e.g., a mixed *T. cruzi* and *Leishmania* sp. infection) (Viettri et al., 2018); 4) a lack of sufficient phylogenetic informative sites from particular MM (TcCLB.508213.20 and Rb19).

Our results are consistent with other studies describing the phylogenetic diversity of *T. cruzi* in North America, where there is a predominance of TcI (Bosseno et al., 2002; Flores-López et al., 2022; Shender et al., 2016). Overall, the diversity found within TcI was not significant. Including sequences from Southern California in the phylogenetic analysis, it can be observed that the TcI genetic lineages described here appear to be indistinguishable from those described from Southern California by Shender and colleagues in 2016 (Shender et al., 2016) (Fig. 2). The genetic diversity of TcI between the BCP and California is low, which reflects a close genetic relationship between the TcI genetic lineages circulating in this area. However, using MM with a finer resolution (e.g., microsatellites) would be needed to provide a clearer picture of the phylogeographic nature of TcI in this area. Since studies of this nature have shown that using finer MM can uncover subdivisions within a *T. cruzi* DTU (e.g., the subdivision of TcI into distinct clades, which appear to be circulating in different hosts within South America) (Zumaya-Estrada et al., 2012).

The presence and overall prevalence of TcI and TcIV-related DTUs appear to correspond to studies from central and southern Mexico that have reported the use of multiple MM to describe the phylogenetic diversity of *T. cruzi* DTUs circulating in Mexico (Bosseno et al., 2002, 2009; Dorn et al., 2017). Although studies that have used other MMs have reported the presence of other DTUs more commonly described from South America (Ramos-Ligonio et al., 2012). Similar results have been reported from distinct southern USA states (Curtis-Robles et al., 2018; Garcia et al., 2017). Given the small amount of *T. cruzi* samples found within the large number of *D. maxima* individuals that were diagnosed for this study, it should be noted that the presence of additional DTUs currently circulating in the BCP cannot be ruled out. Additional DTUs circulating with a lower prevalence among the population (either vector or vertebrate hosts) are unlikely to be found until a much larger amount of *T. cruzi* positive specimens are studied.

Regarding the abundance of distinct *T. cruzi* DTUs in the study region, TcI is the most common DTU described from studies in North America. Thus, the predominance of TcI in samples of *D. maxima* which occupies the southernmost region of the BCP, has the potential to be helpful information for the public health sector, specifically with information regarding the most likely pathologies that could be observed in this region.

Regarding the genetic diversity found among TcI samples described in this study, the diversity found within Baja California appears to be identical to California strains. With the available information, the lack of genetic differences might suggest an absence of isolation of *T. cruzi* strains in the area studied. However, more refined markers (like microsatellites or MM with a more significant amount of polymorphisms) would be needed to address any potential genetic structure among these populations.

The classification of *T. cruzi* into DTUs has varied through the years (Barnabé et al., 2000; ISRJ, 1999; Zingales et al., 2012). As additional information (e.g., unsampled areas, hosts, vectors, and finer phylogenetic analyses) is described, a clearer picture will emerge regarding this complex parasite's phylogenetic diversity, and with this, modifications to its classification system should be expected. The taxonomic classification of TcIV has previously been subdivided into two distinct divergent clades, where one of the genetic lineages has been termed TcIV-USA or TcIV-US (Lewis et al., 2011; Messenger et al., 2012; Roellig et al., 2013; Shender et al., 2016; Yeo et al., 2011), which has recently been shown to have the same level of genetic divergence as that found when comparing distinct DTUs (Flores-López et al., 2022).

5. Conclusions

This study reveals that the genetic diversity of *T. cruzi* circulating in *D. maxima* in Baja California is similar to the lineages described in *Triatoma protracta* in California (Shender et al., 2016). The circulating

DTUs and the abundance of each DTU present are the same. The description of TcIV-USA in *D. maxima* broadens the distribution of this clade from the southern USA to Mexico. The presence of this clade in additional regions of Mexico is likely since studies of this nature are lacking from many regions of Mexico. The report of TcIV-USA in the southern tip of the BCP confirms the importance of this kind of study. Future studies should focus on filling the knowledge gaps regarding unexplored areas, vectors, and hosts. Additionally, improving the quality and quantity of genetic polymorphisms used to correctly identify DTUs present in a particular region, vector, or host will improve the description of the genetic diversity of *T. cruzi*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2023.105465.

Author contributions

Conceptualization: CAFL
Field work: CAFL, AGC
Data curation, validation: CAFL, EAEF
Formal analyses: CAFL, EAEF
Writing: CAFL

Data statement

All sequences have been deposited in GenBank (Accession Nos. OQ672324- OQ672330; OQ594717-OQ594728; OQ818001-OQ818028).

Declaration of Competing Interest

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

Data availability

Genomic sequences have been submitted to GenBank and thus are freely available

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