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Multilocus genetic analysis of *Trypanosoma cruzi* supports non-domestic intrusion into domestic transmission in an endemic region of Colombia

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ABSTRACT

Trypanosoma cruzi, the causative agent of Chagas disease, is primarily transmitted to humans by hematophagous bugs of the Triatominae subfamily. In the Colombian Caribbean region, particularly on Margarita Island, *T. cruzi* transmission is highly endemic and associated with vectors such as *Triatoma maculata* and *Rhodnius pallescens*. Additionally, *T. cruzi*-infected *Didelphis marsupialis* are commonly found in close proximity to human dwellings. Given the complex transmission dynamics involving various domestic and non-domestic hosts, this study aimed to analyze 145 *T. cruzi* clones from twelve strains isolated from *T. maculata, R. pallescens*, and *D. marsupialis* using spliced leader intergenic region (SL-IR) sequences and nine polymorphic microsatellite loci. The results indicate the presence of a single polymorphic *T. cruzi* population, suggesting sustained local transmission dynamics between triatomines adapted to *A. butyracea* forests and peridomestic areas inhabited by synanthropic mammal reservoir such as *D. marsupialis*. Notably, this population appears to lack substructure, highlighting the importance of adopting an alternative eco-health approach to complement traditional chemical vector control methods for more effective and sustainable interruption of transmission.

1. Introduction

The causative agent of Chagas disease, *Trypanosoma cruzi*, is a genetically diverse parasite classified into six Discrete Typing Units (DTUs), namely TcI – TcVI, along with a genotype associated with bats known as TcBat (Marcili et al., 2009; Ramírez et al., 2017, 2014). Among these, TcI is the most prevalent Discrete Typing Unit (DTU) in the northern Amazon basin. It is further divided into two major genotypes associated with domestic (TcI_{Dom}) and sylvatic ($TcI_{Sylvatic}$) transmission cycles (Cura et al., 2010; Ramírez et al., 2012). These genotypes are observed in Colombia and Venezuela and have not yet been described in other countries in the Amazon

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basin (Cura et al., 2010; Ramírez et al., 2012). The TcI in Colombia is subdivided into TcIa, TcIb, TcIc, TcId, and TcIe, each with distinct transmission dynamics (Hernandez et al., 2016; Ramírez et al., 2013; Ramírez et al., 2012). TcIa and TcIc are primarily linked to sylvatic cycles, found predominantly in wild environments (corresponding to $TcI_{Sylvatic)}$. TcIb and TcId are associated with domestic cycles, indicating a higher prevalence in settings with human-vector contact (corresponding to $TcI_{Dom)}$. TcIe is found in both domestic and sylvatic cycles but is more prevalent in sylvatic reservoirs. The emergence of the TcI_{Dom} genotype in Colombia signifies an increase in a specific TcI subdivision linked to domestic transmission, potentially impacting disease dynamics, host range, and human infection risk (Beatty et al., 2024; Hernandez et al., 2016; Ramírez et al., 2013; Ramírez et al., 2012). TcI_{Dom} genotype is considered emerging, with a wide distribution in the domestic cycle facilitated by insects capable of colonizing human dwellings under favorable conditions (Forattini and Barata, 1974). Studies investigating nuclear and mitochondrial genes in areas where domestic *R. prolixus* occurs have revealed the coexistence of sympatric TcI_{Dom} and $TcI_{Sylvatic}$ genotypes, often associated with oral Chagas transmission in various regions of the country (Beatty et al., 2024; Hernandez et al., 2016; Ramírez et al., 2013; Ramírez et al., 2012).

Rhodnius prolixus and *Triatoma dimidiata* are the main domestic species known to transmit *T. cruzi* in Colombian, where the prevalence of Chagas disease varies from 1.4% to 36.9% (Olivera et al., 2019). Moreover, in areas where these vectors are not present, non-domestic adventitious household-invading triatomines, such as *R. pallescens*, *Eratyrus cuspidatus*, *Triatoma venosa, P. geniculatus,* and *T. maculata*, play a relevant epidemiological role (Cantillo-Barraza et al., 2015; Cantillo-Barraza et al., 2014; Tovar Acero et al., 2017).

The Bolivar department in Colombia is a key region for the transmission of Chagas disease, as evidenced by studies conducted on Margarita Island, located within this department along the Caribbean coast. These studies have focused on understanding the diversity of triatomine insects and their infection rates in various environments on the island (Cantillo-Barraza et al., 2015; Cantillo-Barraza et al., 2010; Olivera et al., 2019). In Bolívar department, the prevalence was reported as 0.2% in children under 15 years old (Olivera et al., 2019). In Margarita Island, northern Colombia, the active *T. cruzi* transmission with absence of domestic triatomines has been well documented, with an infection prevalence of 1.7% (CI = 0.68–2.15%) (Cantillo-Barraza et al., 2015; 2014, 2010). However, peridomestic *T. maculata* and non-domestic *R. pallescens* (associated with *Attalea butyracea* palms) frequently invade dwellings in the region, with *T. cruzi* infection rates of 71.6% and 88.5%, respectively (Cantillo-Barraza et al., 2015; Cantillo-Barraza et al., 2014, 2010). Additionally, frequent reports of *T. cruzi*-infected *Didelphis marsupialis* around households suggest a potential infection risk (Cantillo-Barraza et al., 2015), which adds complexity to the *T. cruzi* peridomestic transmission cycle in this region due to the interactions between its populations, *T. maculata*, and domestic dogs(Cantillo-Barraza et al., 2015).

The research conducted in some endemic localities of Caribbean region, focusing on non-domestic vectors, suggests no definitive genetic structure within TcI populations from diverse hosts, indoors or outdoors (León et al., 2015). This challenges the assumption of uniform spread of TcI_{Dom} and $TcI_{Sylvatic}$ across Colombia's endemic landscape (Gomez-Palacio et al., 2016; León et al., 2015), highlighting the need for further investigation into TcI diversity and distribution relative to different vectors and host environments (Gomez-Palacio et al., 2016; León et al., 2019; León et al., 2015).

Despite the considerable efforts invested in developing Chagas disease prevention strategies at the local level, there's a significant gap in our understanding of *T. cruzi* transmission dynamics on Margarita Island. This is particularly notable given that the island lacks known colonies of domestic triatomines yet sustains endemic human infections. Furthermore, there is no evidence thus far to support other non-vectorial transmission routes, such as oral transmission or congenital transmission, in this area. To address this knowledge gap, we conducted a comprehensive multilocus genotype analysis. Our aim was to characterize and compare multiple clones of *T. cruzi* isolates obtained from various sources, including non-domestic *R. pallescens*, peridomestic *T. maculata*, and synanthropic *D. marsupialis*. This analysis is crucial for gaining insights into the transmission patterns and potential reservoirs of *T. cruzi* on Margarita Island, ultimately informing more targeted and effective disease control strategies.

Table 1
Geographical distribution, host, and *Trypanosoma cruzi* clones analyzed in this work.

Locality	Geographic coordinate	Biological source (ND = non-domestic /P = peridomestic)	Isolate ID code	Clones analyzed by SL-IR	Clones analyzed by microsatellites
Cicuco	9°15′10 "N 74°28′25" W	*R. pallescens* (ND)	P6	6	6
Talaigua Nuevo	9°18′15.61"N;	*D. marsupialis* (P)	D1	4	15
	74°33′52.29"W	*R. pallescens* (ND)	P2	2	10
		R. pallescens (ND)	P3	0	12
		T. maculata (P)	M1	2	11
		T. maculata (P)	M2	1	10
Mompós / Loma	9°13′33.93"N;	*D. marsupialis* (P)	D2	3	9
	74°27′49.70"W	*D. marsupialis* (P)	D3	0	10
Mompós /	9°14′59.82"N;	*R. pallescens* (ND)	P1	0	5
Tierrafirme	74°28′39.26"W	*D. marsupialis* (P)	D5	3	8
Margarita	9°09′15" N 74°16′26 "W	*R. pallescens* (ND)	P4	3	8
San Fernando	9°12′04"N 74°18′39" W	*R. pallescens* (ND)	P5	3	10
Total			12	27	114

2. Materials and methods

2.1. Study area and biological samples

Margarita Island is situated within Colombia's Bolívar department, nestled within the Magdalena River basin (Fig. S1). Sampling efforts focused on triatomine vectors and mammals across diverse localities, encompassing Cicuco, Talaigua Nuevo, Mompós - La Loma, Mompós - Tierrafirme, San Fernando, and Margarita itself (Table 1). Spanning about 40 km in linear distance, the study area boasts an average altitude of 30 m. Climate-wise, it experiences an annual temperature of 28 °C and receives an average rainfall of 1660 mm, as previously documented (Cantillo-Barraza et al., 2015; Cantillo-Barraza et al., 2010).

Non-domestic *R. pallescens* specimens were captured in palm trees of *A. butyracea* situated in proximity (<20 m) to domestic dwellings, following the methodology described by (Romaña et al., 1999). *T. maculata* specimens were collected by community members from peridomestic structures, predominantly found in pigeon and chicken coops with roofs constructed from palm leaves. These specimens were then brought to our community meetings for further analysis. Synanthropic mammals were captured using Tomahawk® live traps (Tomahawk Live Traps, Tomahawk, WI) and Sherman (H.B. Sherman Traps, Tallahasee, FL), baited with a mixture of peanut, banana, oat, and fish. Sets of 15 traps were strategically placed at 20 m intervals in peridomestic areas. Upon capture, mammals were anesthetized intramuscularly using a mixture of ketamine chloralhydrate (10%) and xylazine (2%). Blood samples were collected via cardiac puncture for subsequent analysis. Following sample collection, all captured mammals were safely returned to their natural habitat at the capture site. The capture and processing of mammals adhered to ethical standards and were conducted in accordance with the guidelines established by the University of Antioquia Ethics Committee (License 08–012-185). Additionally, permission from the national environmental authority was secured for the collection of biological specimens for research purposes (0524-27-05-2014). Measures were taken to ensure the welfare and well-being of the captured animals throughout the sampling process, including appropriate anesthesia and prompt release back into the wild. All captured animals were marked with an ear biopsy to facilitate recapture identification, as done in previous studies (Cantillo-Barraza et al., 2020). However, no recaptures were observed during the study periods.

2.2. Trypanosoma cruzi isolates and clones

A total of twelve *T. cruzi* isolates were obtained, consisting of four from *D. marsupialis*, six from *R. pallescens*, and two from *T. maculata* (Table 1). Isolates from mammalian hosts were collected by inoculating 200 µl of blood into NNN medium supplemented with RPMI and 10% fetal calf serum. Parasites from insects were obtained from fecal material of positive triatomines, which was then inoculated subcutaneously into BALB/C mice. Once flagellate forms were observed in the blood of mice, parasites were transferred to NNN medium. All isolates were incubated at 28 °C until reaching a concentration of 2.5×10^6 cells per ml, suspended in 10% glycerol, and stored at −80 °C until further analysis, including cloning (Cantillo-Barraza et al., 2014).

The cloning process involved an individual cell sorting protocol adapted from (Evans et al., 2015). In brief, the cell suspension was sorted into 96-well microplates (Thermo Scientific, Massachusetts, United States) containing RPMI medium using a BD FACSAria III Cell Sorter (BD Biosciences, New Jersey, United States). The sorter utilized a laser excitation of 480 nm, a cell strainer of 70 µm, and a drop envelope of 0.5, enabling precise placement of a single cell in each microplate well with high accuracy and efficiency. The microplates were then incubated at 28 °C, and cell growth was monitored microscopically after two weeks. Between 5 and 15 clones were analyzed from each *T. cruzi* isolate (Table 1).

2.3. DNA extraction and molecular identification of isolates

Genomic DNA extraction was performed on a 200 µl epimastigote pellet using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for cultured cells. The total DNA was eluted in 100 µl elution buffer, and its concentration was measured at 260 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, United States). Subsequently, the DNA samples were stored at −20 °C until further molecular processes.

A conventional PCR targeting satellite DNA was conducted using primers and PCR conditions as previously described by (Moser et al., 1989) to confirm the *T. cruzi* species of each isolate. The PCR conditions are detailed in Table S1. Subsequently, *T. cruzi* Discrete Typing Unit (DTU) was determined using a multiplex PCR targeting the intergenic spacer from a mini-exon (SL-IR) according to the protocol outlined by (Souto et al., 1996) (Table S1). PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and visualized under UV light for detection.

2.4. Genotyping of T. cruzi clones by SL-IR and microsatellites sequencing

2.4.1. SL-IR sequencing and analysis

Direct sequencing of the SL-IR region was performed on one to four randomly selected clones from each isolate, totaling 27 clones (Macrogen, Seoul, South Korea). Sequences were aligned using CLUSTALW in BioEdit v.7.2 (Hall, 1999). SL-IR-based TcI was determined by BLAST searches and compared to reference strains (Cura et al., 2010; Falla et al., 2009; Herrera et al., 2019; Herrera et al., 2009, 2007, 2013). Maximum likelihood (ML) tree was conducted using MEGA v.11 (Tamura et al., 2021). The best substitution model was calculated using an information-theoretic criterion (Bayesian Information Criterion, BIC) implemented in jModelTest (Posada, 2008). To assessing inter-clone diversity, SL-IR haplotypes were defined using DnaSP v.6 (Rozas et al., 2017), and median-

joining haplotype network (Bandelt et al., 1999) was constructed by incorporating *T. cruzi* sequences reported for Colombia (Table S1) using Network v.10 (https://www.fluxus-engineering.com). To minimize star-shaped haplotypes, a preprocessing step involved contraction within a radius of one mutational step (Forster et al., 2001).

2.4.2. Multilocus microsatellite analysis

A total of 114 clones, ranging from five to fifteen random clones per isolate, were subjected to microsatellite multilocus analysis (MMA). This analysis targeted nine microsatellite loci distributed across seven *T. cruzi* chromosomes. Conventional PCR was employed, with the 5′ primer labeled with a fluorescent dye, following protocols established by (Llewellyn et al., 2011; Llewellyn et al., 2009). Details of the PCR conditions are provided in Table S1. DNA fragment size detection was carried out in an automated DNA sequencer ABI 3130 (ThermoFisher), whereas allele classification was performed with GeneMapper v.3.7 software (ThermoFisher). Each allele class was determined through a histogram of size-frequency, binning to the closest, most abundant base pair size.

For genetic differentiation analyses based on MMA, we delineated three hierarchical levels: (i) Infrapopulation: This level comprises all clones derived from each isolate. (ii) Populations: These are sets of clones categorized based on ecotope, distinguishing between peridomestic and sylvatic environments. (iii) Metapopulation: This level encompasses clones derived from specific hosts, including *T. maculata*, *R. pallescens*, or *D. marsupialis*.

Henceforward, each clone was referred to a Multilocus-genotype (MLG) characterized by the alleles of each of 9 microsatellite loci. Hierarchically structured at multiple levels analyses were assessed using sample size corrected allelic richness (A_r) calculated in FSTAT v.2.9.4 (Goudet, 2003). The number of private alleles per locus (PA) and mean pair-wise shared allele distance (D_{AS}) were calculated in FSTAT v.2.9.4 (Goudet, 2003). Inbreeding coefficient (G_{IS}), a measure of the distribution of heterozygosity within populations, was estimated in each group of unique MLGs from each population in GenoDive v.3.06 (Meirmans, 2020). Test for population-specific departures from Hardy Weinberg Equilibrium (HWE) expectation and linkage disequilibrium (LD) between microsatellite loci pairs were calculated in ARLEQUIN v.3.11 (Excoffier et al., 2005). The extent of population subdivision between isolates from different ecotopes, host, and locality was evaluated using a pairwise genetic distance estimator (R_{ST}) in ARLEQUIN v.3.11 (Excoffier et al., 2005), and statistical significance assessed via 10,000 random permutations of alleles between populations. Similarly, within-population subdivision was examined in ARLEQUIN v.3.5.1.2 (Excoffier et al., 2005) using a hierarchal AMOVA. Population-level heterozygosity indices were also calculated in ARLEQUIN v.3.11 (Excoffier et al., 2005), and associated significance levels for p values were derived after sequential Bonferroni correction (Bonferroni, 1936). Individual clustering levels were defined via a neighbor-joining tree based on pair-wise distances D_{AS} (1- the proportion of shared alleles at loci / n) between multilocus genotypes MLGs.

3. Results

3.1. Margarita Island *T. cruzi* clones are TcI_{Dom}

Twenty-seven clones were genotyped using SL-IR sequence analysis, confirming them as belonging to the TcI_{Dom} type, with an identity of ≥96.7% to the Colombian *T. cruzi* CGC strain (AM259467) (Table S2). Thirteen haplotypes, labeled A through M (Hd =

Table 2
Haplotype, biological source, and frequencies of the defined haplotypes in SL-intergenic region sequence of *T. cruzi*. *D. m* = *D. marsupialis*; *T. m* = *T. maculata*; *R. p* = *R. pallescens*. Sample codes included in the Maximum Likelihood (ML) tree (Fig. 1a) are in bold.

Haplotype	Haplotype sequence						*D.m*	*T.m*	*R.p*	Freq. (%)	Sample code (clone number)
A	T	T	C	C	G	T	1	–	–	1 (3.7)	**DiPer11Mo** (1)
B	.	.	.	.	.	C	6	–	2	8 (29.6)	DiPer3Ta (3) **DiPer17Mo** (2) RpSil58Ci (1) RpSil59Ci (1)
C	G	.	.	.	.	C	1	1	2	4 (14.8)	DiPer11Mo (1) **DiPer21Mo** (1) RpSil42Ta (1) **TmPer32Ta** (1)
D	.	.	.	.	C	C	1	–	3	4 (14.8)	DiPer11Mo (1) **RpSil50Ma** (3)
E	G	.	.	T	.	C	1	–	–	1 (3.7)	**DiPer3Ta** (1)
F	G	A	.	.	C	C	–	1	–	1 (3.7)	**TmPer31Ta** (1)
G	G	.	.	.	.	.	–	1	1	2 (7.4)	RpSil51Ma (1) **TmPer32Ta** (1)
H	G	.	.	.	C	C	–	–	1	1 (3.7)	**RpSil42Ta** (1)
I	G	A	.	A	C	C	–	–	1	1 (3.7)	RpSil60Ci (1)
J	.	A	.	.	.	C	–	–	1	1 (3.7)	**RpSil62Ci** (1)
K	.	A	.	T	C	C	–	–	1	1 (3.7)	**RpSil42Ta** (1)
L	G	A	A	T	C	C	–	–	1	1 (3.7)	**RpSil52Ma** (1)
M	.	.	.	T	C	.	–	–	1	1 (3.7)	**RpSil51Ma** (1)
Total							10	3	14	27 (100)	

0.883 ± 0.001), were identified, with frequencies ranging from 3.7 to 29.6 (Table 2). Representative haplotype sequences from Margarita Island were grouped into the TcI_{Dom} (TcIa) clade (Fig. 1a). The haplotype network, incorporating 46 Colombian isolates of the TcI_{Dom} type, revealed that all haplotypes found in Margarita Island were unique to this area (Fig. 1b). They formed a distinct cluster, separated by more than four mutational steps from other haplotypes previously reported in humans, *R. prolixus*, *R. pallescens*, and *T. venosa* from Colombia (Herrera et al., 2009; Ramírez et al., 2012) (Fig. 1b).

3.2. Genetic diversity and clonal composition

Of the 114 clones genotyped using microsatellites, we identified a total of 75 unique MLGs. Among these, 44 (58.7%) were exclusively found in peridomicile habitats, while 31 (41.3%) were specific to sylvatic environments (Table 3). Notably, two MLGs were observed among isolates from *D. marsupialis* (D1) and *R. pallescens* (P1 and P2), captured in peridomestic and sylvatic ecotopes, respectively (Fig. 2). In terms of infrapopulation MLG diversity, calculated as the ratio of MLGs to the total number of clones at each isolate (MLG/N), we observed a range from 1 in isolates D2, D3, and P4 to 0.33 in D1 and M2 (Table 3). At the metapopulation level (host level), the mean MLG diversity (d) was calculated as the ratio of total MLGs to the total number of clones at each host. We found d = 0.74 (i.e., 31 MLGs / 42 clones) for *D. marsupialis*, d = 0.62 (i.e., 13 MLGs / 21 clones) for *T. maculata*, and d = 0.61 (i.e., 31 MLGs / 51 clones) for *R. pallescens* (Table 3).

The mean allelic richness (Ar) estimated across all clones was 2.9 (±0.06), with no significant differences ($p < 0.05$) observed among infra-, meta-, and population levels (Table 3). The overall pairwise shared allele distance (D_{AS}) across the entire sample was 0.62 (±0.05). Infrapopulation mean pairwise shared allele distances (D_{AS}) between clones varied from 0.38 (±0.03) in D5 to 0.97 (±0.06) in M1 (Table 3). The highest pairwise shared allele distance at the metapopulation level was observed in isolates from *T. maculata* (0.86 ± 0.06), followed by *R. pallescens* (0.62 ± 0.05) and *D. marsupialis* (0.56 ± 0.04) (Table 3).

A total of 19 private alleles were identified across the entire dataset, with an overall average of 1.28 (±0.32) private alleles per locus (Table 3). Infrapopulation (isolate-level) private alleles ranged from 0 (observed in D1, M1, M2, P1, and P2) to 6 in P3, which exhibited the highest mean private alleles per locus at 0.67 (±0.29) (Table 3). Additionally, private alleles at the metapopulation level (host-level) were more prevalent in *R. pallescens* (12 alleles) compared to *D. marsupialis* (seven alleles), with no private alleles detected in *T. maculata* (Table 3). In terms of inbreeding and Linkage Desequilibrium within *T. cruzi* subpopulations, no statistically significant values ($p > 0.05$) were found for the G_{IS} coefficient across samples. This suggests a minimal degree of inbreeding within these populations (Table 3). The linkage disequilibrium (LD) analysis between all pairs of loci revealed no significant deviations ($p > 0.05$) after applying the Bonferroni correction. Hence, it can be inferred that the nine loci segregate independently, allowing their combined use in

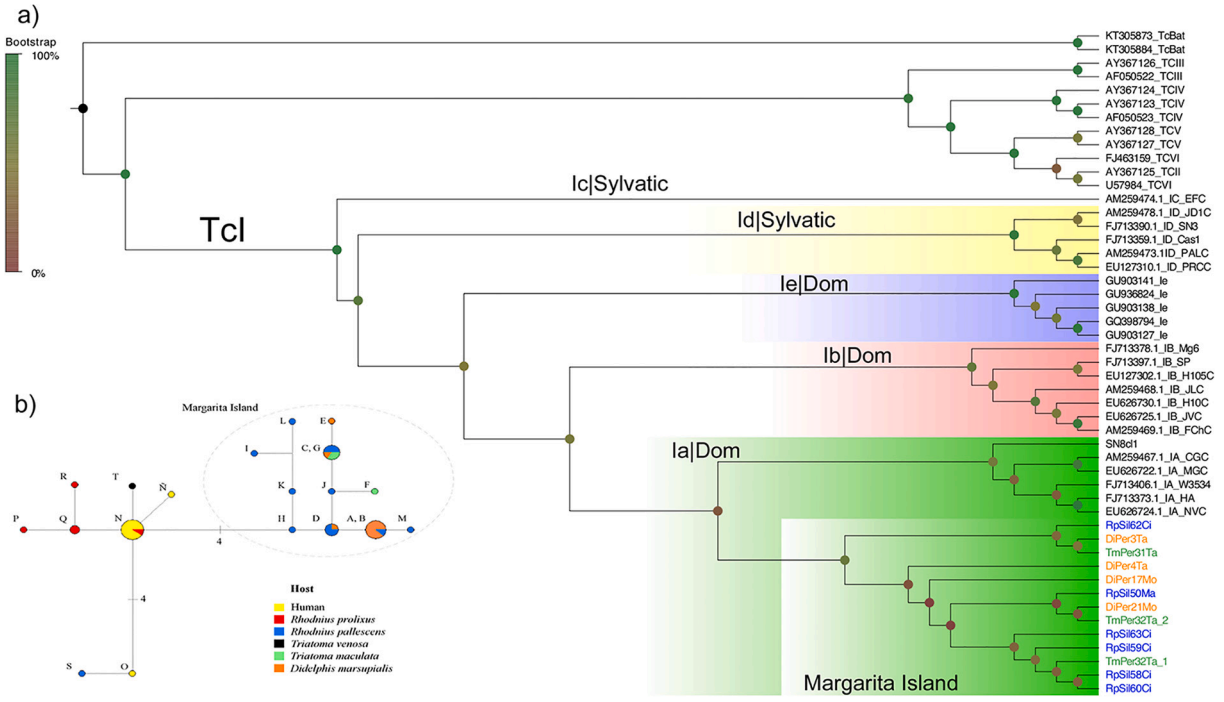

Fig. 1. (a) Maximum likelihood (ML) tree generated using the SL-intergenic region of *T. cruzi* clones from Margarita Island analyzed in this study, along with reference strains for TcI reported in America. Tips are colored as indicated in the inset. Nodes are represented by shapes and sizes denoting bootstrap support based on 1000 replicates, as indicated. (b) Median-Joining network constructed with SL-intergenic region haplotypes detected in Margarita Island (A-M) and reference *T. cruzi* strains belonging to TcI_{Dom} reported in Colombia. The size of each node is proportional to the frequency of the haplotype. The number of mutational steps ≥4 is shown.

Table 3
Population genetic parameters across *Trypanosoma cruzi* I sampled in Margarita Island, based on MLG analysis. Notation: MLG/N = Multilocus Genotypes/number clones in the infrapopulation; d = mean MLG diversity; A_r = Allelic richness as a mean over loci ± standard error, calculated in FSTAT; D_{AS} = mean pair-wise shared allele distance ± standard deviation calculated in FSTAT; Observed (Ho) and expected (He) heterozygosity in each infrapopulation under study.

Population	Metapopulation	Infra-population	MLG/N	MLG diversity (d)	A_r ± SD	D_{AS} ± SD	Number of private alleles	Mean of private alleles per locus (± SD)	Ho	He	G_{IS}	*P*
Peridomestic	*D. marsupialis*	D1	5/15	0.33	1.70 ± 0.12	0.42 ± 0.02	0	0.0	0.5	0.3	−0.2	0.90
		D2	9/9	1	2.1 ± 0.14	0.85 ± 0.05	2	0.22 ± 0.22	0.5	0.5	0.2	0.20
		D3	10/10	1	2.4 ± 0.30	0.76 ± 0.04	3	0.33 ± 0.24	0.6	0.5	0.4	0.02*
		D5	7/8	0.88	1.7 ± 0.17	0.38 ± 0.03	2	0.22 ± 0.15	0.4	0.3	0.4	0.04*
	Total	–	31/42	0.74	2.3 ± 0.069	0.56 ± 0.04	7	0.89 ± 0.31	0.5	0.5	0.1	0.30
	T. maculata	M1	10/11	0.91	2.3 ± 0.32	0.97 ± 0.06	0	0	0.6	0.5	−0.6	0.90
		M2	3/10	0.3	2.0 ± 0.23	0.74 ± 0.07	0	0	0.7	0.4	−0.9	0.90
	Total	–	13/21	0.62	2.6 ± 0.065	0.86 ± 0.06	0	0	0.6	0.5	−0.4	0.20
Total		**–**	**44/63**	**0.70**	**2.5 ± 0.294**	**0.67 ± 0.05**	**0**	**0.89 ± 0.31**	**0.55**	**0.52**	**0.04**	**0.30**
Sylvatic	*R. pallescens*	P1	3/5	0.6	1.8 ± 0.20	0.54 ± 0.05	0	0.0	0.5	0.4	−0.4	0.10
		P2	5/10	0.5	1.8 ± 0.16	0.70 ± 0.05	0	0.0	0.6	0.4	−0.4	0.90
		P3	6/12	0.5	2.0 ± 0.14	0.51 ± 0.03	6	0.67 ± 0.29	0.8	0.5	−0.9	1.00
		P4	8/8	1	2.5 ± 0.42	0.57 ± 0.04	1	0.11 ± 0.11	0.8	0.6	−0.3	0.80
		P5	4/10	0.4	1.7 ± 0.19	0.74 ± 0.05	3	0.33 ± 0.17	0.5	0.4	−0.3	0.90
		P6	5/6	0.83	2.1 ± 0.20	0.62 ± 0.05	2	0.22 ± 0.15	0.7	0.5	−0.3	0.90
	Total	–	31/51	0.61	2.9 ± 0.362	0.62 ± 0.05	12	1.67 ± 0.33	0.65	0.58	−0,0.04	0.80
Total		**–**	**31/51**	**0.61**	**2.9 ± 0.06**	**0.62 ± 0.05**	**19**	**1.28 ± 0.32**	**0.65**	**0.58**	**−0.04**	**0.80**

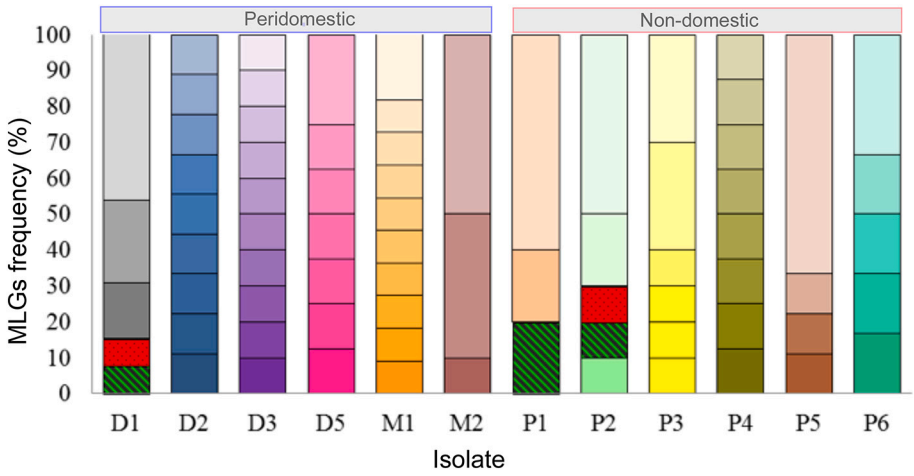

Fig. 2. Multilocus-genotype (MLG) composition discriminated by *T. cruzi* isolate. Each colour indicates a different MLG. Only two MLGs (green stripes and dotted red) were shared between isolates from *R. pallescens* and *D. marsupialis.* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

population genetic analyses for these *T. cruzi* populations.

Hierarchical AMOVA based on MLGs revealed that 80.2% of the observed genetic variation was attributed to differences among clones, while 19.8% was associated with variation between infrapopulations (among clones from each isolate) (Table S3). No significant genetic structure was detected based on host type (metapopulation, $R_{ST} = 0.003$, $p = 0.002$) or ecotope (population, $R_{ST} =$

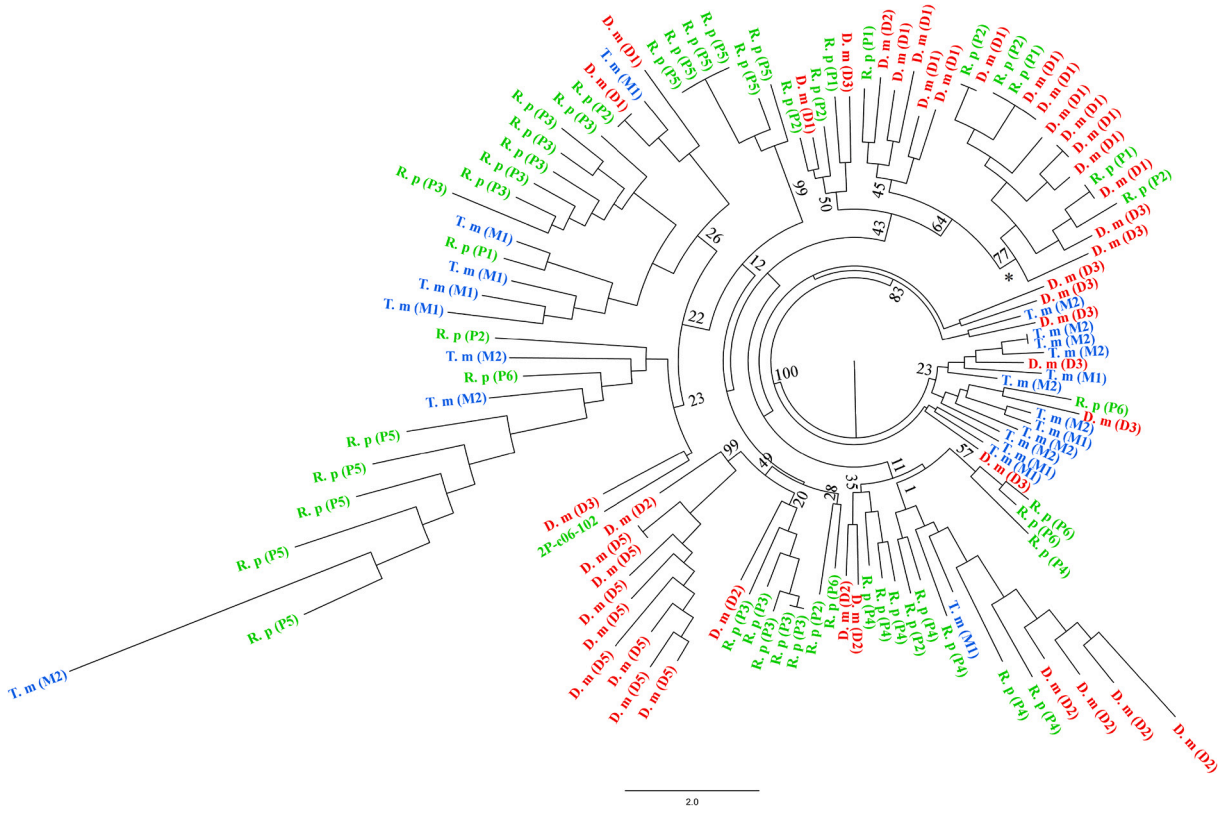

Fig. 3. Neighbor-joining tree constructed from pair-wise genetic distance values derived from MLGs analysis in clones from Margarita Island. Blue, green, and red colors represent clones derived from *T. maculata, R. pallescens,* and *D. marsupialis*, respectively. Asterisk (*) denotes a well-supported cluster harboring clones from infrapopulations isolated from distinct metapopulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0.03, $p = 0.64$) in this study.

The neighbor-joining tree, constructed from pairwise genetic distance values between MLGs, exhibited clustering of similar clones from the same isolate (i.e., infrapopulation). This clustering pattern reflected the diversity of clones within each host type (i.e., metapopulation), particularly among clones from triatomine vectors. While there was no strong evidence for subdivision between clones from peridomestic and sylvatic habitats (i.e., population), a well-supported cluster (bootstrap >70) containing clones from infrapopulations isolated from distinct metapopulations such as *R. pallescens* and *D. marsupialis* was identified (Fig. 3).

4. Discussion

The genetic characterization of *T. cruzi* isolates plays a crucial role in understanding the interactions between vectors and hosts, shedding light on the components and roles of each in transmission cycles within specific epidemiological contexts (Hernandez et al., 2016). During this study, besides *D. marsupialis*, 10 specimens of the species *R. rattus* were collected in Talaigua Nuevo, which were evaluated by blood culture and PCR, and were negative for *T. cruzi* infection as reported elsewhere (Cantillo-Barraza et al., 2015).

Evidence on the routes of *T. cruzi* transmission the island suggests that transmission occurs via the classical vectorial route, through the intrusion of peridomestic colonies of *T. maculata* (Cortés and Suárez, 2005; Cantillo-Barraza et al., 2014), as well as potential intrusion of *R. pallescens* from palms (Cantillo-Barraza et al., 2014, 2015). Although there have been outbreaks of acute Chagas disease due to oral transmission of *T. cruzi* in the neighboring department of Cesar over the past 10 years (Soto et al., 2014), there are no records of such outbreaks in the area where the present study was conducted.

Our study provides further evidence supporting the circulation of non-domestic *T. cruzi* (TcI_{Dom}) into peridomestic transmission within an endemic area lacking domestic triatomine bugs, yet with recent Chagas disease cases in humans and dogs (Cantillo-Barraza et al., 2015). This is evidenced by infection prevalence (1.7%; CI = 0.68–2.15%), comparable to areas where *R. prolixus* predominates and is domestic (Cantillo-Barraza et al., 2015; Cantillo-Barraza et al., 2014; Olivera et al., 2019). Our findings regarding *T. cruzi* genotyping on Margarita Island suggest that the strains circulating in the area form a single polymorphic population, with sustained local gene flow between non-domestic and peridomestic habitats facilitated by opportunistic triatomine vectors and synanthropic hosts. This scenario reveals a complex transmission cycle in the region, where diverse parasite populations from sylvatic and peridomestic environments converge, posing a significant infection risk to humans. Despite robust results, the cloning methodology can influence strain selection, particularly with isolates from insects previously exposed to mice, impacting *T. cruzi* strain diversity. Further studies, such as single-cell sequencing, are needed for a comprehensive understanding of transmission dynamics in diverse ecotopes of endemic Chagas disease scenarios.

All isolates from Margarita Island correspond to the TcI_{Dom} genotype, previously reported in isolates from humans and domestic vectors such as *R. prolixus* (Cura et al., 2010; Falla et al., 2009; Hernandez et al., 2016; León et al., 2019; Ramírez et al., 2017, 2013, 2012; Ramirez-Sierra et al., 2010), as well as in several non-domestic vectors like *R. pallescens*, *Psamolestes arthury*, and *T. venosa,* among others (Hernandez et al., 2016; Velásquez-Ortiz et al., 2022; Velásquez-Ortiz et al., 2019). Our findings, along with our studies in the area, which have been designed under strict inclusion criteria, led us to estimate that children under 14 years of age diagnosed as seropositive in Talaigua Nuevo and Mompós-La Loma had autochthonous and recent infections (Cantillo-Barraza et al., 2015; Cantillo-Barraza et al., 2014). Furthermore, we provide new evidence for the presence of TcI_{Dom} circulating in non-domestic *T. maculata* and *R. pallescens*, as well as in synanthropic *D. marsupialis* role. Despite the majority of genetic variation being found among clones, the presence of several genetically similar clones and the absence of significant genetic structure among infrapopulations (clones from each isolate) or among metapopulations (strains from hosts) reveal active *T. cruzi* transmission between human dwellings and non-domestic habitats. Moreover, the shared multilocus genotypes (MLGs) and SL-IR haplotypes between non-domestic *R. pallescens* and synanthropic *D. marsupialis* collected near dwellings suggest that *T. cruzi* transmission involves *D. marsupialis* as a carrier of isolates between non-domestic and peridomestic environments. Additionally, similar SL-IR haplotypes were found in clones from different hosts or habitats, and no significant population genetic structure was defined in the AMOVA analysis and R_{ST} values. Instead, high genetic diversity was observed among all clones, as evidenced by both haplotype and multilocus-based gene diversity parameters.

The high genetic diversity observed in *T. cruzi* populations has been linked to population size increases resulting from intense local parasite transmission among diverse hosts and natural habitats in several endemic regions of northern South (Gomez-Palacio et al., 2016; Llewellyn et al., 2011; Ramírez et al., 2012). Intensive *T. cruzi* transmission dynamics have been documented in the Orinoco river-plains ecoregion, spanning Colombia and Venezuela, where sylvatic *R. prolixus* populations coexist with diverse hosts and preserved environments (Llewellyn et al., 2011; Ramírez et al., 2012). Our study reveals a similar landscape on Margarita Island, referring to the environmental and ecological context. This includes factors such as the physical geography (e.g., plains land), vegetation types (e.g., palms), human settlement patterns (e.g., roofs of palm leaves), and other environmental characteristics (e.g., humidity, altitude, temperature, etc.) that influence the transmission dynamics of *T. cruzi*. However, unlike the Orinoco river-plains ecoregion, Margarita Island possesses a more complex transmission scenario involving distinct triatomine species such as non-domestic *R. pallescens*, peridomestic *T. maculata*, and a key intermediary host, *D. marsupialis.*

The crucial role of *D. marsupialis* as a reservoir and potential vector for *T. cruzi* has been extensively documented (Cantillo-Barraza et al., 2020). Previous observations of infected individuals visiting dwellings in Margarita Island have hinted at the significant role of *D. marsupialis* in the region's epidemiology (Cantillo-Barraza et al., 2020; Cantillo-Barraza et al., 2015, 2014). Our findings further support this notion, revealing evidence that *D. marsupialis* harbors parasites genetically similar to those found in non-domestic *R. pallescens*. This suggests that the intensity of circulation of parasites in both ecotopes increases the risk of *T. cruzi* infection for residents.

The configuration of this *T. cruzi* transmission scenario in an endemic region for Chagas disease, characterized by the absence of

domestic triatomines and the active involvement of *D. marsupialis,* challenges the current paradigm of disease transmission in Colombia, which typically involves the coexistence of independent sylvatic and domiciliary cycles (Coura et al., 2014; Guhl and Ramírez, 2013; Ramírez et al., 2017). Recognizing the epidemiological importance of *D. marsupialis* in endemic areas of South America due to its synanthropic behavior and role as a *T. cruzi* reservoir (Cantillo-Barraza et al., 2015; Jansen et al., 2015; Ocaña-Mayorga et al., 2010), we propose the adoption of environmental management strategies such as improved waste management or targeted interventions to reduce human-*D. marsupialis* contact in the study area.

In conclusion, this study provides new information about *T. cruzi* population transmission dynamics in an area with non-domestic vectors in the Colombia Caribbean region. The presence of a single polymorphic *T. cruzi* population indicates sustainable local transmission dynamics between triatomines adapted to *A. butyracea* forests to peridomestic areas coexisting with synanthropic mammal reservoirs as *D. marsupialis.* This scenario highlights the need for an alternative eco-health approach that complements the traditional chemical vector control management used so far, so likely to be more effective for sustainable interruption of transmission.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parepi.2024.e00364.

Authors' contributions

OCB, OTC and AGP designed the research; OCB and PLM performed the experiments; OCB, JJD, PLM, AGP analyzed the data; OCB, JJD, OTC, PLM, AGP wrote the paper. All authors approved the final manuscript.

CRediT authorship contribution statement

Omar Cantillo-Barraza: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Jeiczon Jaimes-Dueñez:** Writing – review & editing, Formal analysis. **Paula L. Marcet:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Omar Triana-Chavez:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization. **Andrés Gómez-Palacio:** Writing – review & editing, Formal analysis, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used the assistance of ChatGPT 4.0 by OpenAI, in order to providing helpful insights and corrections during the process of improving the drafting and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no conflict of interests. The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the Centers for Disease Control and Prevention (CDC).

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