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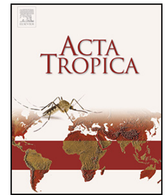

Genetic diversity of *Trypanosoma cruzi* in bats, and multilocus phylogenetic and phylogeographical analyses supporting Tcbat as an independent DTU (discrete typing unit)

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ABSTRACT

Trypanosoma cruzi is a complex of phenotypically and genetically diverse isolates distributed in six discrete typing units (DTUs) designated as TcI–TcVI. Five years ago, *T. cruzi* isolates from Brazilian bats showing unique patterns of traditional ribosomal and spliced leader PCRs not clustering into any of the six DTUs were designated as the Tcbat genotype. In the present study, phylogenies inferred using SSU rRNA (small subunit of ribosomal rRNA), gGAPDH (glycosomal glyceraldehyde 3-phosphate dehydrogenase) and Cytb (cytochrome b) genes strongly supported Tcbat as a monophyletic lineage prevalent in Brazil, Panama and Colombia. Providing strong support for Tcbat, sequences from 37 of 47 nuclear and 12 mitochondrial genes (retrieved from a draft genome of Tcbat) and reference strains of all DTUs available in databanks corroborated Tcbat as an independent DTU. Consistent with previous studies, multilocus analysis of most nuclear genes corroborated the evolution of *T. cruzi* from bat trypanosomes its divergence into two main phylogenetic lineages: the basal TcII; and the lineage clustering TcIV, the clade comprising TcIII and the sister groups TcI–Tcbat. Most likely, the common ancestor of Tcbat and TcI was a bat trypanosome. However, the results of the present analysis did not support Tcbat as the ancestor of all DTUs. Despite the insights provided by reports of TcIII, TcIV and TcII in bats, including Amazonian bats harbouring TcII, further studies are necessary to understand the roles played by bats in the diversification of all DTUs. We also demonstrated that in addition to value as molecular markers for DTU assignment, Cytb, ITS rDNA and the spliced leader (SL) polymorphic sequences suggest spatially structured populations of Tcbat. Phylogenetic and phylogeographical analyses, multiple molecular markers specific to Tcbat, and the degrees of sequence divergence between Tcbat and the accepted DTUs strongly support the definitive classification of Tcbat as a new DTU.

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1. Introduction

Trypanosoma cruzi is a genetically and ecologically heterogeneous zoonotic taxon formally recognised as formed by six Discrete Typing Units (DTUs) designated as TcI–TcVI. The rationale for the recent DTU classification proposed to accommodate the known genetic diversity within *T. cruzi*, as well as biological, eco-epidemiological and pathological features of each DTU have been recently reviewed (Zingales et al., 2012).

Isolates of distinct DTUs circulate in a complex network of transmission cycles involving humans and other mammals of virtually all

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terrestrial orders and triatomine bugs of diverse species and genera inhabiting a range of ecological niches. In contrast to *T. cruzi*, which predominated in domestic transmission cycles, the genetic diversity, geographical and host ranges, vectors and population genetic structure of *T. cruzi* in natural transmission cycles just began to be investigated using molecular phylogenetic approaches. These studies have revealed an increasing genetic diversity and suggested some degrees of association between *T. cruzi* genotypes and preferential vertebrate hosts and vectors, ecological niches and domestic or sylvatic environments as showed for TcIII (Llewellyn et al., 2009; Marcili et al., 2009a; Rocha et al., 2013; Yeo et al., 2011, 2005), TcIV (Herrera et al., 2015; Marcili et al., 2009b; Roellig et al., 2008), TcI (Lima et al., 2014a; Messenger et al., 2015; Ramírez et al., 2012) and, recently, TcII (Herrera et al., 2015; Lima et al., 2014b).

Bat trypanosomes have been implicated in the evolutionary history of *T. cruzi*. Molecular evidence from an increasing number of trypanosomes from the New and Old Worlds suggest that *T. cruzi* evolved from within a broader monophyletic assemblage – the *T. cruzi* clade – that clusters together bat trypanosomes and trypanosomes of other mammals in the New and Old Worlds. According to this evolutionary scenario, called the bat-seeding hypothesis, the common ancestor of *T. cruzi* was a bat trypanosome that switched from bats into terrestrial mammals in the New World, adapted to transmission by triatomine bugs and gave origin to *T. cruzi* (Hamilton et al., 2012a; Lima et al., 2013, 2012a). However, the origin and inter-relationships of the different DTUs and the hypothesis of Tcbat as the common ancestor of all DTUs remain controversial (Diosque et al., 2014; Flores-López and Machado, 2011; Guhl et al., 2014; Marcili et al., 2009c; Tomasini and Diosque, 2015).

Efforts to contribute to unveil the complex zoonotic cycles of *T. cruzi* through assessments of the genetic diversity of *T. cruzi* in bats, revealed the presence of a previously unidentified genotype of *T. cruzi* that did not perfectly match the molecular markers of any of the six DTUs. This new genotype was named Tcbat (Marcili et al., 2009c). Although Tcbat isolates were placed in phylogenetic trees based on SSU rRNA and Cytb as a monophyletic assemblage clearly separated from all other DTUs (Marcili et al., 2009c); this genotype was initially regarded as a rare and irrelevant discovery. Subsequently, Tcbat was reported in bats from Panama (Pinto et al., 2012) and Colombia (Ramírez et al., 2014a; Trejo-Varón et al., 2013). Recently, Tcbat was reported in a child from Colombia and Tcbat DNA was detected in mummies from Chile (Guhl et al., 2014; Ramírez et al., 2014b). These findings were consistent with the ability of bats to infect other mammals besides bats, inducing subpatent infections, as previously suggested by experimental infections of mice (Maeda et al., 2011; Marcili et al., 2009c).

Currently, consistent with the recommendation from the last revision of *T. cruzi* nomenclature (Zingales et al., 2012), Tcbat has been referred to either as a genotype or a new DTU, but not as DTU-VII. At the time of this revision, Tcbat was limited to a few Brazilian isolates, and its characterization was restricted to a few genes. Multilocus sequence typing was recommended to provide additional support for any putative new DTUs (Zingales et al., 2012). Since then, a large number of molecular markers have added support to Tcbat as an independent lineage of *T. cruzi*, closest to TcI (Pinto et al., 2012; Lima et al., 2012a; Ramírez et al., 2014a,b; Cosentino and Agüero, 2012; Caballero et al., 2015; Franco et al., 2015).

In the present study, we report data from surveys of *T. cruzi* in a comprehensive sampling of diverse species of bats obtained from domestic and sylvatic environments in areas endemic or not endemic for Chagas disease. The isolates were all characterized based on DTU classification and the assessment of relevant phylogenetic relationships. To provide data for the definitive classification of Tcbat as a new DTU, the phylogenetic relationships of this genotype with all DTUs (reference-strains of TcI–TcVI) were inferred through multilocus phylogenetic analysis, including 47 nuclear (single copy) genes and 12 mitochondrial genes recovered from genome databanks and a draft genome of Tcbat. To assess the phylogeographical patterns of Tcbat, we compared the Cytb, SL and ITS rDNA sequences of isolates from Brazil, Colombia and Panama. The findings of the present study add strong support to the phylogenetic positioning of Tcbat and clarify its relationship with the currently recognized *T. cruzi* DTUs.

2. Materials and methods

2.1. Studied areas and isolates of *T. cruzi* from bats

The *T. cruzi* isolates characterized in the present study were obtained from 1491 bats captured from 2001 to 2014 in the Brazilian states of Amazonas (AM), Mato Grosso do Sul (MS), Mato Grosso (MT), Rondonia (RO), Pará (PA), São Paulo (SP), Tocantins (TO) and Rio Grande do Norte (RN). In addition, we also examined 251 bats captured in the Andean region of Colombia (CO) (Fig. 1). The bats were captured using mist nets, and after anesthetization, blood samples were collected through cardiac puncture as previously described (Cavazzana et al., 2010). The capture of animals and all ensuing procedures were conducted according to the recommendations and approval of IBAMA (Brazilian Institute for the Environment and Renewable Natural Resources, permit Number 10080-2). In Colombia, the bats were captured according to procedures recommended by the Tolima University (Trejo-Varón et al., 2013). This study was approved by the Committee on the Ethics of Animal Experimentation of the Institute of Biomedical Sciences, University of São Paulo, where all molecular characterization was performed.

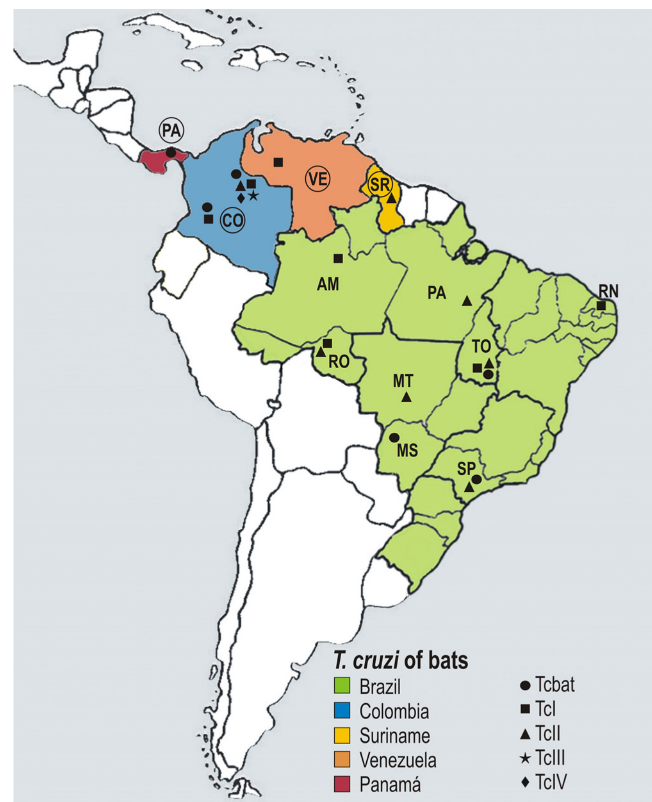

Fig. 1. Geographical origin of the *T. cruzi* isolates from bats characterized in this and in previous studies (Pinto et al., 2012; Ramírez et al., 2014a). Brazil: Amazonas (AM), Rondonia (RO), Pará (PA), Rio Grande do Norte (RN), Mato Grosso do Sul (MS), Mato Grosso (MT), Tocantins (TO), São Paulo (SP).

Table 1
Isolates of *T. cruzi* from bats (cultures and blood samples), host and geographic origin and respective DTU/genotypes.

T. cruzi	Isolate	Host-bats		Feeding	Geographic origin		DTU[d] genotype	year
		Family	Species	habits[c]				
TCC[a] cultures 417		Thyropteridae	*Thyroptera tricolor*	I	Amazonia	BR	TcI	2002
507		Phyllostomidae	*Carollia perspicillata*	F	Rondonia	BR	TcI	2002
640/642		Phyllostomidae	*Carollia perspicillata*	F	Rondonia	BR	TcI	2003
1338		Phyllostomidae	*Carollia perspicillata*	F	–	VE	TcI	–
203		Vespertilionidae	*Myotis ruber*	I	São Paulo	BR	Tcbat	2001
204		Vespertilionidae	*Myotis albescens*	I	São Paulo	BR	Tcbat	2001
294		Vespertilionidae	*Myotis levis*	I	São Paulo	BR	Tcbat	2001
312		Noctilionidae	*Noctilio albiventris*	I	Mato Grosso do Sul	BR	Tcbat	2001
480		Noctilionidae	*Noctilio albiventris*	I	Mato Grosso do Sul	BR	Tcbat	2002
499/597		Vespertilionidae	*Myotis nigricans*	I	Mato Grosso do Sul	BR	Tcbat	2002
1994		Vespertilionidae	*Myotis levis*	I	São Paulo	BR	Tcbat	2004
947/949		Vespertilionidae	*Myotis nigricans*	I	São Paulo	BR	Tcbat	2005
1122		Vespertilionidae	*Myotis albescens*	I	São Paulo	BR	Tcbat	2005
2471/2472/2473 2476/2516		Phyllostomidae	*Artibeus lituratus*	F	Tolima	CO	Tcbat	2012
2474/2477		Phyllostomidae	*Carollia perspicillata*	F	Tolima	CO	Tcbat	2012
2557		Phyllostomidae	*Phyllostomus hastatus*	C	São Paulo	BR	TcII	–
2558		Vespertilionidae	*Myotis nigricans*	I	São Paulo	BR	TcII	–
T. cruzi blood samples[b]								
–	RNMO 10	Emballonuridae	*Peropteryx macrotis*	I	Rio Grande do Norte	BR	TcI	2012
–	RNMO 37/RNMO 43	Molossidae	*Molossus molossus*	I	Rio Grande do Norte	BR	TcI	2012
–	APC 1659	Phyllostomidae	*Platyrrhinus lineatus*	F	Tocantins	BR	TcI	2008
–	BatCha 64	Phyllostomidae	*Micronycteris megalotis*	F	Tolima	CO	TcI	2013
–	BatCha 68	Vespertilionidae	*Myotis riparius*	I	Tolima	CO	TcI	2013
–	BatCha 70	Phyllostomidae	*Artibeus planirostris*	F	Tolima	CO	TcI	2013
–	APC 1621	Phyllostomidae	*Platyrrhinus lineatus*	F	Tocantins	BR	Tcbat	2008
–	APC 1623	Phyllostomidae	*Glossophaga soricina*	N	Tocantins	BR	Tcbat	2008
–	Q 07CHA/Q 10CHA	Phyllostomidae	*Artibeus lituratus*	F	Tolima	CO	Tcbat	2012
–	BatPra 77/BatPra 80 BatPra 88/BatPra 96 BatPra 99	Phyllostomidae	*Artibeus planirostris*	F	Tolima	CO	Tcbat	2013
–	BatCha 67	Phyllostomidae	*Glossophaga soricina*	N	Tolima	CO	Tcbat	2013
–	BatCha 69	Phyllostomidae	*Carollia perspicillata*	F	Tolima	CO	Tcbat	2013
–	BatPra 92	Phyllostomidae	*Carollia perspicillata*	F	Tolima	CO	Tcbat	2013
–	MN7-37	Mormoopidae	*Pteronotus parnellii*	I	Mato Grosso	BR	TcII	–
–	APC 1640	Phyllostomidae	*Platyrrhinus lineatus*	F	Tocantins	BR	TcII	2008
–	MOL174	Mormoopidae	*Pteronotus parnellii*	I	Tocantins	BR	TcII	–
–	VCT1103/VCT3880	Mormoopidae	*Pteronotus parnellii*	I	Pará	BR	TcII	–
–	JRMO 4	Mormoopidae	*Pteronotus parnellii*	I	Rondonia	BR	TcII	2010
–	HMO117	Phyllostomidae	*Carollia perspicillata*	F	Rondonia	BR	TcII	2001
	ROM116943 ROM117028	Mormoopidae	*Pteronotus parnellii*	I	Nickerie	SR	TcII	–
	ROM 113,978 ROM 114,045	Mormoopidae	*Pteronotus parnellii*	I	Brokopondo	SR	TcII	–
T. cruzi marinkellei								
	B7	Phyllostomidae	*Phyllostomus discolor*	F	Bahia	BR	–	
TCC344		Phyllostomidae	*Carollia perspicillata*	F	Rondonia	BR	–	2001
TCC1093		Phyllostomidae	*Artibeus planirostris*	F	Mato Grosso do Sul	BR	–	2005

BR, Brazil; CO, Colombia; SR, Suriname; PA, Panamá.

[a] Cultures of bat trypanosomes cryopreserved in the Trypanosomatid Culture Collection of the Department of Parasitology, University of São Paulo, São Paulo, Brazil. TCC correspond to codes of cultures deposited in this collection.

[b] Bat blood samples deposited at the TCCU-USP

[c] Preferential feeding habits of bats: I, insectivorous; F, frugivorous; N, nectarivorous; C, carnivorous.

[d] Lineages determined based on mini-exon markers and phylogenetic analyses inferred in this study.

The identification of bats were performed using conventional morphological keys, and molecular methods using DNA from liver or blood samples preserved in ethanol (v/v) through barcodes of Cytb and Cytochrome c oxidase subunit I (COI) genes (Clare et al., 2013; Cui et al., 2007; Folmer et al., 1994).

To detect and isolate bat trypanosomes, the blood samples were subjected to the hemoculture method as previously described (Maia da Silva et al., 2004). All obtained isolates were cryopreserved in the Trypanosomatid Culture Collection (TCC) of the Department of Parasitology, University of São Paulo, Brazil. A small blood sample from each captured bat was preserved in ethanol (v/v) for DNA preparation for subsequent molecular characterization and was included in the blood sample collection (BSC) and DNA sample collection complementing the TCC collection (Table 1).

2.2. Nested-PCR for detection and identification of trypanosomes in bat blood samples

To detect the presence of trypanosomes in bat blood samples, we employed nested PCR targeting a small region of the SSU rRNA gene (∼561 bp), which corresponds to the 3′ portion of the variable V7V8 region of SSU rRNA, according to Noyes et al. (1999). In general, blood samples and cultures containing more than one species/genotype and hybrid isolates of *T. cruzi* were identified according to the chromatogram patterns resulting from the direct sequencing of PCR-amplified products, and after cloning the PCR-amplified DNA sequences, different cloned sequences were detected. At least 5 clones were sequenced from each sample, except those from hybrid strains and previously detected mixed infections that had at least 10–15 sequences determined for each sample.

2.3. Phylogenetic analyses of SSU rRNA, gGAPDH, Cytb, ITS1 rDNA and SL sequences

DNA was extracted from cultured trypanosomes by classical phenol–chloroform method and used for PCR amplification of V7V8 SSU rRNA (∼800 bp) and gGAPDH (∼770 bp) genes using primers and PCR conditions described previously (Borghesan et al., 2013). PCR amplification of *Cytb* gene was performed as before (Marcili et al., 2009c). PCR-amplifications and sequencing of ITS1 rDNA and SL sequences were done as described previously (Maia da Silva et al., 2004).

The sequences determined in this study (Table S1 - Supplementary material) were aligned with corresponding sequences from *T. cruzi* strains of all *T. cruzi* DTUs and all available sequences from Tcbat, including SSU rRNA sequences from Panamanian samples (Pinto et al., 2012) and Cytb sequences from Colombian bats (Ramírez et al., 2014a), which were retrieved from GenBank (Table S2—Supplementary material).

2.4. *T. cruzi* genomes and the nuclear and mitochondrial genes used for multilocus phylogenetic analyses

Searches of selected genes for multilocus analyses were performed using BLAST against draft and annotated genomes, freely available in TriTrypDB and/or NCBI databanks for the following references of *T. cruzi*: JRcl4 and Sylvio X10/1 (TcI); Esmeraldo cl3 (TcII); CL Brener (Esmeraldo-like and Non-Esmeraldo-like haplotypes) and Tulahuen cl2 (TcVI); M6241 cl6 (TcIII) and CANIII (TcIV) and *Trypanosoma cruzi marinkellei* (B7). We also searched for genes in the draft genomes of *T. cruzi* Tcbat (TCC1994) and *T. cruzi marinkellei* (TCC344), which were generated in our laboratory (Assembling the Tree of Life, NSF-USA) using standard pyrosequencing shotgun methodology according to Roche 454 protocols and assembled using Newbler software (version 2.3), as previously described (Alves et al., 2011). For access to the unpublished draft and ongoing genomes analyzed in the present study, contact the corresponding author. The sequences retrieved from unpublished genomes were deposited in GenBank (Table S3—Supplementary material).

For multilocus phylogenetic analyses, we selected 59 genes, 47 nuclear genes and 12 mitochondrial genes, and we examined these loci for concatenation fitness in previous studies and as useful markers for phylogenetic inferences of the DTUs (Flores-López and Machado, 2011; Yeo et al., 2011; Lauthier et al., 2012; Messenger et al., 2012; Diosque et al., 2014; Tomasini and Diosque, 2015). The genes analyzed in the present study are listed in Tables S2 and S3 Supplementary materials. The genes retrieved from the genomes of each DTU and Tcbat were individually aligned using Clustal X, and the alignments were manually refined. We created concatenated alignments with different combinations of nuclear genes. For phylogenetic inferences using mitochondrial genes, we examined 12 genes (9S, ND9, MURF5, ND7, COIII, Cytb, ATPase6, MURF1, COII, MURF2, COI and RPS12), individually and in different combinations.

2.5. Phylogenetic analyses

The sequences were aligned using Clustal X and the alignments were manually refined. The phylogenies were inferred using Parsimony (P), Maximum likelihood (ML) and Bayesian inference (BI) analyses. Parsimony and bootstrap analyses were performed using PAUP* version 4.0b10 (Swofford, 2002), with 500 random-sequence-addition through branch swapping (RAS-TBR). The ML analysis was performed using RAxML v.7.0.0 (Stamatakis, 2006), and BI analyses were performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Nodal support was estimated with 500 bootstrap replicates in RAxML using GTRGAMMA and maximum parsimony starting trees as previously described (Borghesan et al., 2013). BI analyses was performed in MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001) with the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. The first 25% of the trees from 1.000.000 generations were discarded as burn-in.

Network genealogy was inferred with SplitsTree v4.11.3 using the neighbor-net method (Huson and Bryant, 2006). Internode supports were estimated by performing 100 bootstrap replicates using the same parameters optimized for network inferences.

3. Results and discussion

3.1. Phylogenetic inferences based on SSU rRNA and gGAPDH sequences of Tcbat and TcI–TcVI isolates

Currently, the identification and phylogenetic positioning of any new trypanosome is based on SSU rRNA and gGAPDH gene sequences. Generic identification methods based on these genes, able to identify any trypanosome species, as well as to assess genetic diversity within species and infer phylogenetic relationships, have been employed in surveys for trypanosomes in blood samples and vectors (Adams et al., 2010; Fermino et al., 2013; Garcia et al., 2011; Hamilton et al., 2009, 2007; Lima et al., 2013, 2012a; Noyes et al., 1999; Paparini et al., 2014). Despite the close phylogenetic relationships between *T. cruzi* and all other *Schizotrypanum* species (*T. cruzi marinkellei*, *Trypanosoma dionisii* and *Trypanosoma erneyi*), SSU rRNA and gGAPDH sequences clearly separated all these species and revealed intra-specific polymorphisms (Cavazzana et al., 2010; Hamilton et al., 2012a,b; Lima et al., 2013, 2012a; Marcili et al., 2009a,b,c).

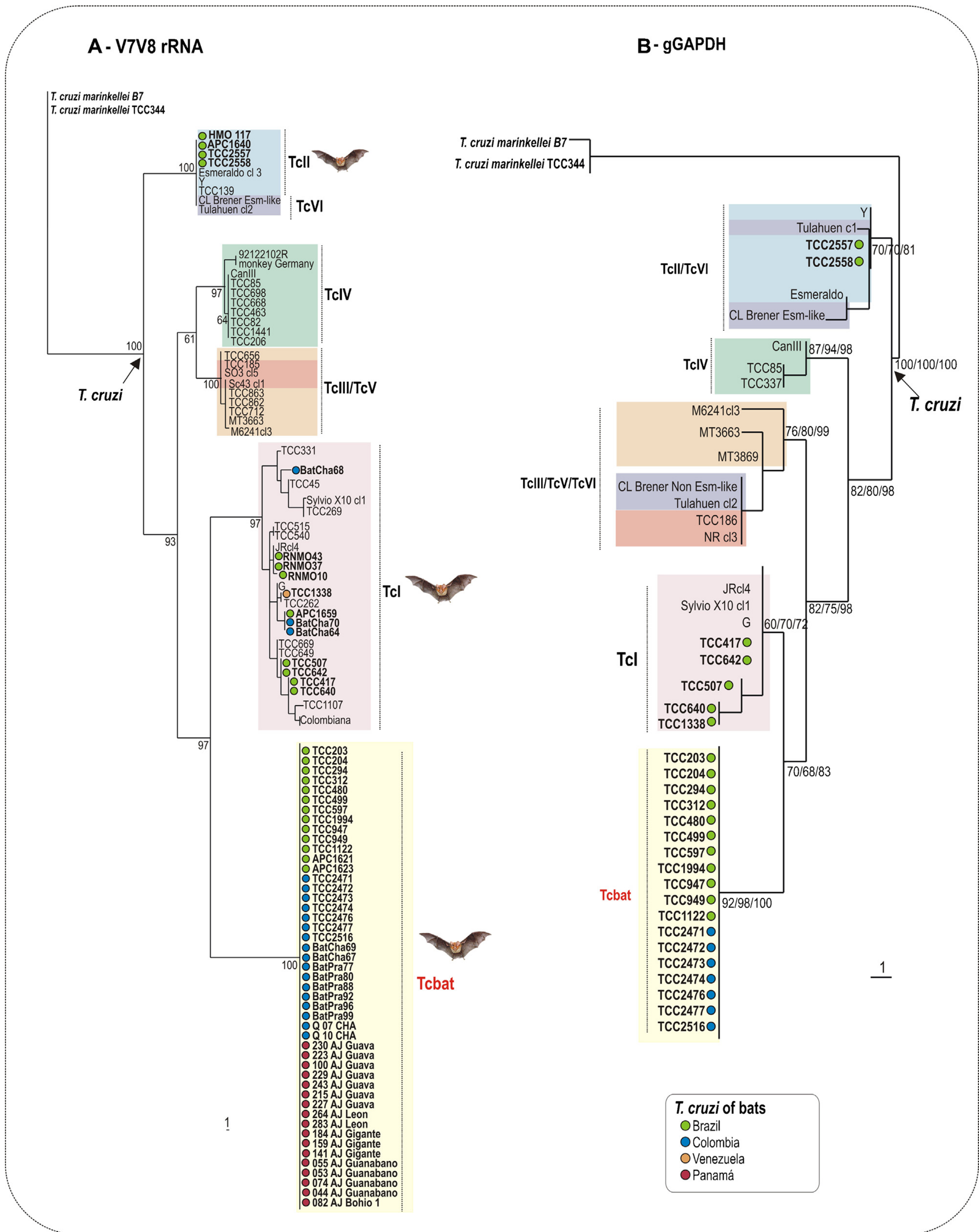

Fig. 2. Phylogenetic analysis of *T. cruzi*. (A) Phylogenetic relationships based on the V7V8 SSU rRNA (799 characters) sequences from 63 *T. cruzi* isolates from bats (bold) inferred by parsimony. Numbers at nodes are bootstrap values derived from 500 replicates. (B) Phylogenetic analyses (ML) inferred using gGAPDH sequences from 25 *T. cruzi* isolates from bats (770 characters, −Ln = 1351.018004). Numbers at the nodes correspond respectively to P, ML (500 replicates) and BI support values.

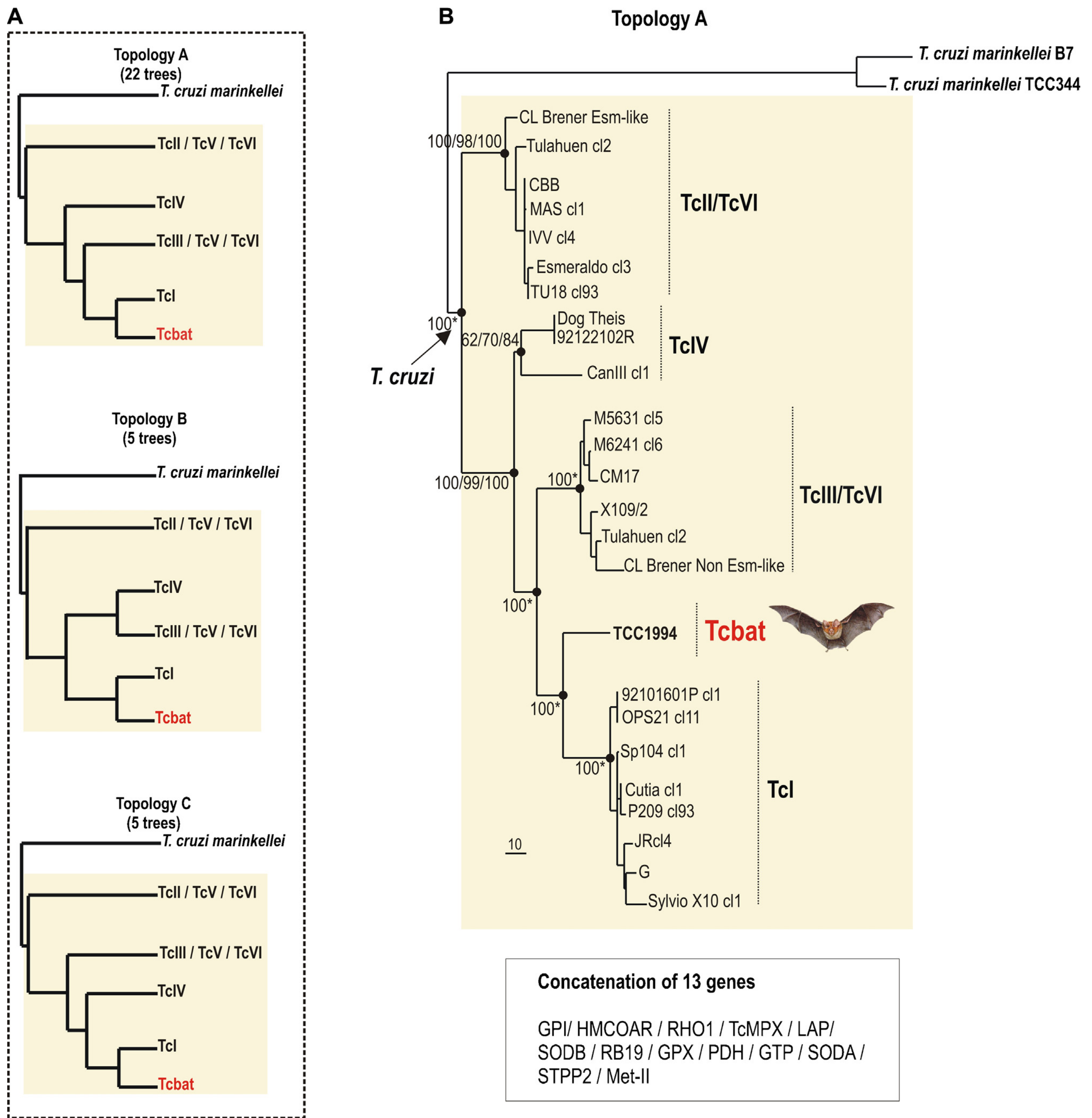

Fig. 3. Phylogenetic relationships of Tcbat with all DTUs based on nuclear genes. (A) The commonest phylogenetic topologies (ML) of individual or concatenated nuclear gene sequences (including 30 genes previously selected by Flores-López and Machado, 2011); (B) Phylogenetic tree (ML) using 13 genes (5.260 characters) selected by Diosque et al., 2014. Numbers at the nodes correspond respectively to P, ML and BI support values.

In the first report on Tcbat, phylogenetic trees based on the V7V8 SSU rRNA and Cytb genes placed 11 isolates of Tcbat closest to TcI, which was clearly separated from all known DTUs (Marcili et al., 2009c). Here, the V7V8 SSU rRNA sequences from 30 isolates of Tcbat, 12 of TcI and 13 of TcII from bats (cultures and blood samples) (Table 1) were genotyped according to SSU rRNA barcoding, representative of the broad sampling used for phylogenetic analysis of *T. cruzi* strains of all DTUs. In all analyses (P, ML and BI), Tcbat was placed sister to TcI, forming a monophyletic assemblage (Fig. 2A).

Phylogenetic studies of trypanosomes have demonstrated that the gene gGAPDH allow the accurate placement of novel species and genotypes. Sequences of this gene are easily and non-ambiguously aligned without gaps and in general are also more polymorphic than SSU rRNA; hence, these gene sequences are suitable to assess the phylogenetic relationships of distant species as well as lineages and genotypes within a given species (Fermino et al., 2013; Hamilton et al., 2009, 2007; Lima et al., 2013, 2012a). Herein, we investigated the suitability of the gGAPDH gene to distinguish Tcbat and resolve the relationships between the DTUs of *T. cruzi*. *T. c. marinkellei* was employed as an outgroup. The results demonstrated that isolates of Tcbat, TcI–TcIV have DTU-specific sequences that cluster in well-resolved clades. The branching pattern of gGAPDH derived-trees supported two major phylogenetic lineages: one lineage comprising the basal TcII and the other lineage comprising TcIV and a clade formed by TcIII and the sister groups

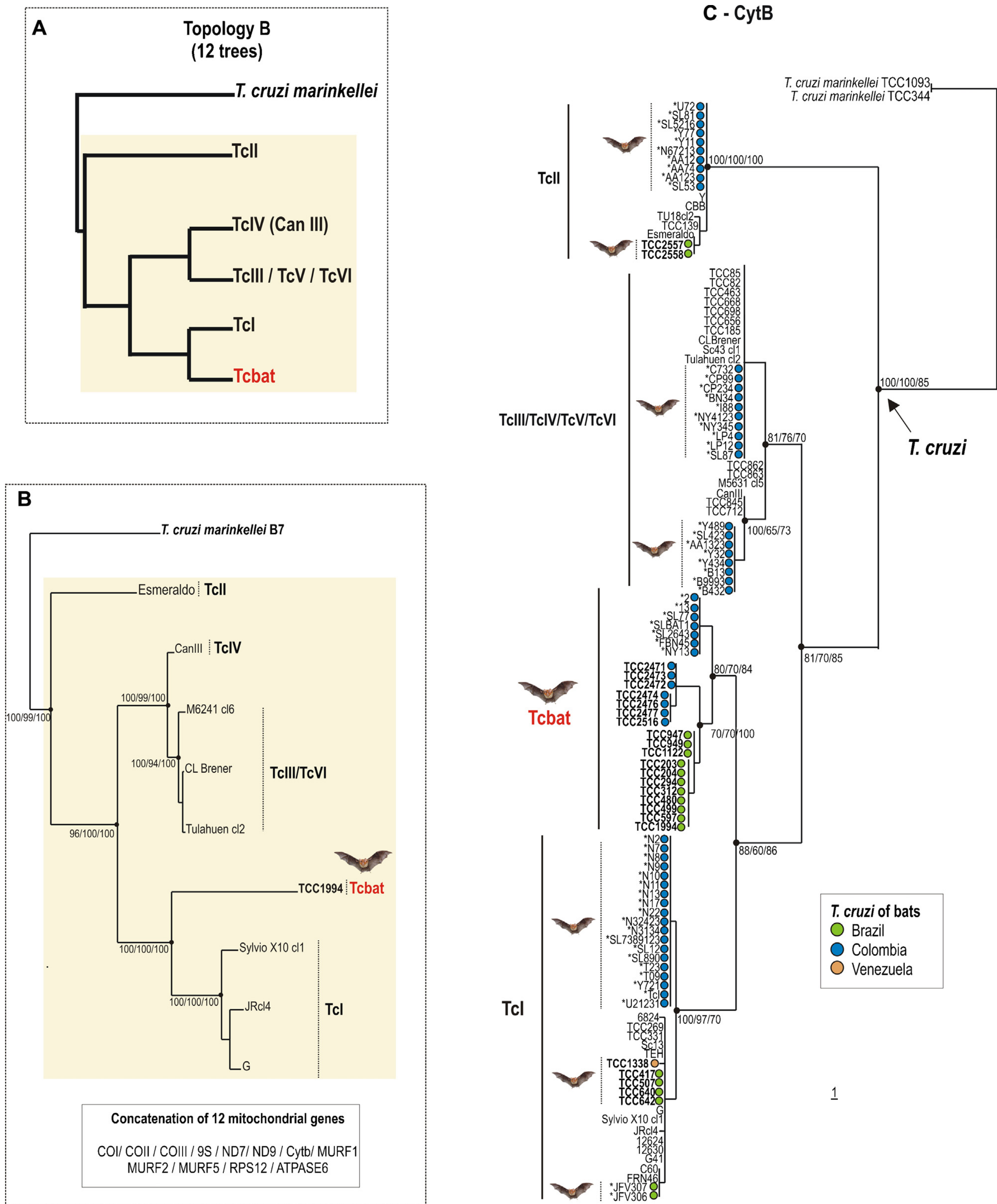

Fig. 4. Phylogenetic relationships among Tcbat and all DTUs inferred using mitochondrial genes. (A) Phylogenetic trees (P) generated by individual or combined (12 genes) mitochondrial genes. (C) Phylogenetic tree (P) based on partial Cytb gene sequences (471 characters) from 81 isolates of *T. cruzi* from bats. (*) sequences obtained by Ramírez et al. (2014a) and Lima et al. (2014a). The numbers at the nodes correspond respectively to P, ML and BI support values.

TcI–Tcbat (Fig. 2B). As expected, the sequences of the heterozygous hybrid TcV and TcVI strains clustered together with those of the parental groups TcII and TcIII (Fig. 2A and B), consistent with most single-copy nuclear loci (Fig. 3A). These findings reinforced the recent emergence of TcV/TcVI by TcII and TcIII hybridization events (Westenberger et al., 2005; Flores-López and Machado, 2011). The existence of polymorphisms exclusive of either TcV or TcVI requires further analysis beyond the scope of this study.

The gGAPDH-derived phylogenetic tree showed better resolved branching patterns than SSU rRNA (Fig. 2A, B). The inferred topology of gGAPDH was consistent with the most common and well-supported phylogenies inferred using a multilocus approach

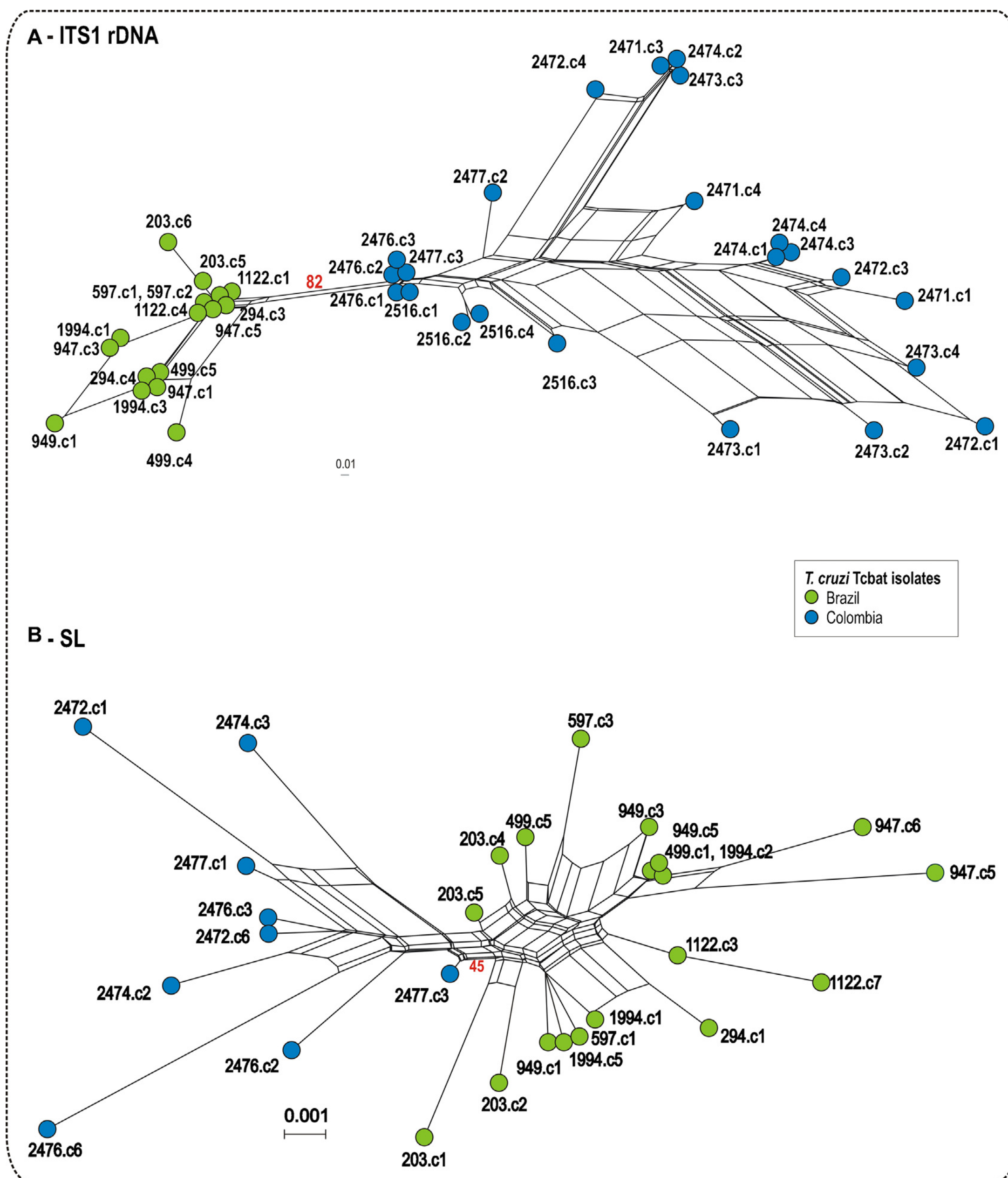

Fig. 5. Network genealogies of Tcbat isolates from Brazil and Colombia using Neighbor-Net. (A) ITS1 rDNA sequences from Tcbat isolates from Brazil (8 isolates) and Colombia (7); (B) Network of SL sequences of Tcbat isolates from Brazil (8) and Colombia (4). Numbers in nodes correspond to bootstrap values estimated by 500 replicates using the same parameters optimized for network inferences.

(Flores-López and Machado, 2011; Diosque et al., 2014; Tomasini and Diosque, 2015). Therefore, gGAPDH was valuable for the identification and inference of phylogenetic relationships among *T. cruzi* DTUs excepting the hybrids TcV and TcVI.

The possibility that one gene can identify species and lineages/genotypes within trypanosome species, as previously demonstrated for *T. cruzi* and allied species (Cavazzana et al., 2010; Hamilton et al., 2012a; Lima et al., 2012a), and suggested in the present study for *T. cruzi* genotypes, greatly simplifies the use of phylogenetic approaches for the taxonomy and phylogeny of trypanosomes. Incontestably, after this first step, other molecular markers and approaches must be employed for a deeper understanding of the genetic diversity, relationships inter- and intra-DTUs and for population genetics. For these purposes, multilocus sequence typing (MLST) of nuclear and mitochondrial loci, microsatellites and Next Generation Sequencing approaches have been used and were recently revised (Messenger et al., 2015).

3.2. Multilocus phylogenetic analyses of Tcbat based on nuclear and mitochondrial genes

To date, the sequences of the genes SSU rRNA, Cytb and H2B (Marcili et al., 2009c), and homologs of cruzipain (Lima et al., 2012b), *Tc*SC5D (Cosentino and Agüero, 2012), *Tc*ABCG1 transporter (Franco et al., 2015) and the proline racemase *Tc*PRAC enzyme (Caballero et al., 2015) were successfully used as molecular markers to identify Tcbat. In the present study, we inferred the phylogenetic relationships of Tcbat with all DTUs using the genes recommended in previous multilocus analyses (Diosque et al., 2014; Flores-López and Machado, 2011; Lauthier et al., 2012;

Messenger et al., 2012; Tomasini and Diosque, 2015; Yeo et al., 2011).

Herein, multilocus phylogenetic analyses including Tcbat homologs of previously selected nuclear genes in *T. cruzi* phylogenies corroborated Tcbat sister to TcI and clustering closer to TcIII than to TcIV, all forming a strongly supported monophyletic lineage separated from the basal lineage TcII (topology A) (Fig. 3A). Topology A was most commonly observed in the present study (22 loci) (Fig. 3A) and in a previous analysis of 31 nuclear loci (30 included in the present study) (Flores-López and Machado, 2011). Topologies B (=G in Flores-López and Machado, 2011) and C (first reported in the present study) were revealed by 5 nuclear loci (Fig. 3A). Alternative topologies (15 of 59 loci), in general, were unresolved or weakly supported (data not shown). Although these multilocus analyses were restricted to one isolate from each DTU (genome data), consistent with Flores-López and Machado (2011), the results from single or combined loci were highly consistent with phylogenies using a set of Tcbat isolates inferred by gGAPDH (Fig. 2B). Additional support for this topology was the congruent pattern obtained by the concatenation of 13 nuclear loci consistent with previous studies (Diosque et al., 2014; Tomasini and Diosque, 2015). We included Tcbat in alignments created with concatenated sequences from all DTUs of the 4, 7 and 13 nuclear genes selected by Diosque et al. (2014). Resulting phylogenetic trees using the combination of 13 genes, but not of 7 or 4 genes (data not shown), yielded the topology A strongly supported in P, ML and BI analyses (Fig. 3B). The inclusion of sequences from hybrid lineages did not disturb the topology of the phylogenetic trees, which was compatible with TcII and TcIII as parents. Accordingly, two main phylogenetic lineages were strongly supported: one lineage clustering TcII isolates (and sequences from TcV and TcVI) and other lineage comprising TcIV, TcIII (plus TcV and TcVI) and TcI–Tcbat (Fig. 3B). Therefore, the phylogenetic trees (Topology A) obtained in the present analysis were consistent with those generated using the same set of 13 nuclear genes from all DTUs excepting Tcbat (Tomasini and Diosque, 2015).

In the first description of Tcbat (Marcili et al., 2009c), Cytb sequences from 7 isolates of Tcbat and isolates of all DTUs resulted in a phylogenetic tree supporting Tcbat closest to TcI (sister groups), whereas TcII was corroborated as the most basal lineage and TcIII–TcVI clustered together in a clade with a complex branching pattern—the topology B (Fig. 4A). The Cytb sequences of Tcbat from Panama corroborated the topology B (Pinto et al., 2012). Similarly, a recent study encompassing Cytb sequences from a larger number of isolates of all DTUs and a few Tcbat sequences also corroborated the topology B (Tomasini and Diosque, 2015). We also assessed the relationships between Tcbat and other DTUs using other 11 mitochondrial genes, and individual or concatenated sequences always yielded the topology B (Fig. 4B). Incongruent phylogenies inferred using nuclear (topology A) and mitochondrial (topology B) genes might reflect genetic exchange and mitochondrial introgressions, which have been demonstrated as common events in the evolution of *T. cruzi* (Yeo et al., 2011; Messenger et al., 2012, 2015; Tomasini and Diosque, 2015). The results of the present study did not suggest any putative event of recombination/introgression involving Tcbat. However, studies of additional *T. cruzi* isolates from bats, especially TcI and other DTUs sharing bat hosts with Tcbat, are necessary to undertand the origin of Tcbat.

Cytb sequences have been confirmed as valuable, highly polymorphic markers for the assessment of the genetic variability of bat trypanosome species and within *T. cruzi* (Marcili et al., 2009a; Pinto et al., 2012). In the present study, the analysis of bat isolates of *T. cruzi*, Tcbat, TcI and even TcII emerged as complexes of genotypes. In a recent study based on the polymorphism of microsatellite and mitochondrial loci, a new clade of TcI was associated with bats and placed outside the main cluster of TcI (Lima et al., 2014a). In the Cytb analysis conducted herein, two isolates of this clade were confirmed to be TcI, clearly separated from Tcbat (Fig. 4C).

3.3. The repertoire of *T. cruzi* genotypes in bats

Bats are known hosts of *T. cruzi* and closely related species of the subgenus *Schizotrypanum*, which are all morphologically indistinguishable from *T. cruzi* and restricted to bats, although their development within mammalian cells in vitro is similar to *T. cruzi* (Cavazzana et al., 2010; Franzen et al., 2012; Lima et al., 2012a). There are an increasing number of studies using molecular diagnosis and phylogenetic analysis to assess trypanosome species and DTUs of *T. cruzi* from bats (Cavazzana et al., 2010; Cottontail et al., 2014; Garcia et al., 2012; Hamilton et al., 2012a,b; Lima et al., 2013, 2012a; Lisboa et al., 2008; Maia da Silva et al., 2009; Pinto et al., 2012; Ramírez et al., 2014a).

However, the molecular characterization of *T. cruzi* from bats for classification in DTUs remains limited. We previously reported on Brazilian bats infected with TcI and Tcbat (Table 1). More recently, the detection of trypanosomes in bat blood samples using nested PCR has provided evidence of a high prevalence of Tcbat in bats. In addition to *Artibeus jamaicensis* (Phyllostomidae bats) from Panama (Pinto et al., 2012), a survey of bat trypanosomes in Colombia (eastern region) revealed a range of bat species infected with Tcbat as well as with TcI–TcIV (Ramírez et al., 2014a).

In the present study, in addition to cultured isolates of *T. cruzi* from bats characterized in our previous studies (Marcili et al., 2009; Cavazzana et al., 2010), we characterized additional bat isolates obtained in culture and detected through nested-PCR in blood samples (Noyes et al., 1999) to survey for trypanosomes in Brazilian bats. The analysis of ~350 bats captured across Amazonia, The Pantanal, Cerrado and Caatinga revealed a relatively low prevalence of *T. cruzi* in bats, showing only 30 bats (8.5%) positive for *T. cruzi*. In contrast, these bats showed a high prevalence (~25%) of infections with *T. c. marinkellei*and *T. dionisii*. Results from the present study agreed with our previous study (Cavazzana et al., 2010), and revealed other widespread trypanosome species (Cottontail et al., 2014). In our study, mixed infections with *T. cruzi* and other species were common, whereas bats infected with more than one *T. cruzi* genotype were rare (data not shown).

Bats infected with *T. cruzi* were observed in all Brazilian biomes, and could be detected either in bats captured in either domestic (including bats using roofs of human dwellings as shelters) or sylvatic environments. Most of the 30 samples of *T. cruzi* obtained from Brazilian bats were typed as Tcbat (13), whereas a smaller prevalence of both TcI (8) and TcII (9) was found. Brazilian surveys revealed Tcbat in MS, SP and TO; TcI in bats from AM, RO, RN and TO, and TcII in bats from SP, TO, MT, RO and PA (Table 1).

Here, we compared Brazilian and Colombian *T. cruzi* isolates from bats, including cultures obtained in our previous study (Trejo-Varón et al., 2013) and recently, both in the state of Tolima at the Andean region (Table 1). All cultures of *T. cruzi* from Colombian bats were assigned to Tcbat. In addition, through nested-PCR of additional 100 bats we found three bats infected with TcI and 10 with Tcbat (Table 1). In our surveys for bat trypanosomes in Brazil and Colombia we never detected TcIII-TcVI. In contrast, Colombian bats captured in Casanare, an area endemic for Chagas disease in the Orinoquia region showed higher prevalence and diversity of *T. cruzi* including Tcbat, TcI–TcIV (Ramírez et al., 2014a).

Similarly to other sylvatic hosts, TcI occurs in bats across South America. Here, we reported TcII in bats from The Atlantic Forest (SP) and Cerrado (TO and MT) biomes, areas formally endemic for Chagas disease disease with known circulation of TcII. However, to our knowledge, this is the first study demonstrating that Amazonian bats are infected with TcII. In addition, autochthone TcII was

recently reported in this region, detected by PCR in dogs and *Rhodnius pictipes* in the State of Para in eastern Brazilian Amazonia (Lima et al., 2014b).

In Brazil, Tcbat was identified in insectivorous bats of the families Vespertilionidae (*Myotis ruber*, *Myotis albescens*, *Myotis levis*, *Myotis nigricans*) and Noctilionidae (*Noctilio albiventris*), and in two species of Phyllostomidae (*Glossophaga soricina* – a nectar feeding bat, and *Platyrrhinus l ineatus* – a frugivorous bat). In Panama, *A. jamaicensis* (frugivorous) of Phyllostomidae harbour Tcbat (Pinto et al., 2012). In eastern Colombia, Tcbat was reported in insectivorous bats of Vespertilionidae (*M. oxyotus*) and Emballonuridae (*Rhynchonycteris naso*), and in the frugivorous Phyllostomidae bat *Carollia perspicillata* (Ramírez et al., 2014a). In the surveys conducted in the present study in Colombia, we identified Tcbat in the following Phyllostomidae species: *Artibeus lituratus*, *Artibeus planirostris*, *G. soricina,* and *C. perspicillata* (Table 1).

3.4. Phylogeographical analysis of Tcbat in Brazilian, Colombian and Panamanian bats

Aiming to evaluate the genetic diversity within Tcbat, we compared isolates from Brazil and Colombia using SSU rRNA, gGAPDH, Cytb, SL and ITS rDNA. The SSU rRNA and gGAPDH sequences were highly similar. However, the comparison of Cytb sequences of a broad sampling of *T. cruzi* corroborated this gene as highly intra-DTU polymorphic as suggested before (Marcili et al., 2009c; Ramírez et al., 2014a) revealing three groups within Tcbat (Fig. 4C). All Brazilian isolates clustered together and separated from clusters exclusively comprising Tcbat from Panama and Colombia. The Colombian isolates of Tcbat were separated into two clusters; samples from the eastern region (Ramírez et al., 2014a,b) were separated from those of bats from the Andean region (Trejo-Varón et al., 2013). In addition, the branching pattern of Tcbat isolates suggested additional subpopulations within Tcbat (Fig. 4C).

To further evaluate the genetic variability within Tcbat, we compared isolates from Brazilian and Colombian bats through network analysis of the sequences from the highly polymorphic ITS1 rDNA (Fig. 5A) and SL gene (Fig. 5B). ITS rDNA was valuable for unraveling cryptic diversity among the isolates of diverse trypanosome species such as *Trypanosoma rangeli* (Maia da Silva et al., 2004), *Trypanosoma theileri* (Garcia et al., 2011) and *T. cruzi* (Marcili et al., 2009a,b,c). We previously demonstrated the existence of ITS1 rDNA polymorphisms disclosing genotypes within TcIII (Marcili et al., 2009b) and polymorphisms among the DTUs, with unique pattern facilitating the identification of Tcbat (Marcili et al., 2009a). Herein, the network analysis of ITS1 rDNA sequences from the isolates of Tcbat corroborated the separation between Brazilian and Colombian (isolates from the Andean region) isolates and identified relevant polymorphisms within both main clusters (Fig. 5A).

We also investigated whether sequences from the highly polymorphic intergenic region of the SL gene, traditionally employed for assessing genotypes within TcI (Cura et al., 2010) could be useful as an additional marker to assess polymorphisms within Tcbat. Results showing high inter- and intra-isolates polymorphisms, the network of SL sequences confirmed the separation between Brazilian and Colombian Tcbat isolates (Fig. 5B), regardless of whether the isolates were obtained from bats captured in domestic shelters or sylvatic environments.

4. Conclusions

Phylogenetic inferences based on SSU rRNA, gGAPDH and Cytb genes strongly supported a monophyletic lineage exclusively comprising the identified Tcbat isolates from Brazil, Colombia and Panama. In addition, the analyses of 47 nuclear and 12 mitochondrial loci, individually or in different combinations, also supported Tcbat as an independent phylogenetic lineage highly associated with bats. Phylogenetic and phylogeographical analyses and multiple Tcbat specific molecular markers strongly support Tcbat as a new DTU. The DTUs represent groups of *T. cruzi* isolates, distinguishable by common molecular markers, which are genetically more similar to each other than to the isolates of other groups (Zingales et al., 2012).

The data from this and previous studies confirmed Tcbat as a widespread genotype of a range of bat species. Phylogeographical analysis revealed that intra-Tcbat genetic diversity is apparently spatially structured. In addition to the great contribution to the knowledge of Tcbat, surveys of bat trypanosomes have revealed an increasing complexity of trypanosome species and genotypes. The repertoire of *T. cruzi* genotypes in bats is currently much larger than previously envisaged prior to the advances of molecular phylogenetic approaches and the use of molecular tools to detect trypanosomes in field blood samples.

In the recently hypothesized bat-seeding scenario, the common ancestor of *T. cruzi* was a bat trypanosome, and the closest living relative is *T. c. marinkellei,*a bat-restricted trypanosome (Cavazzana et al., 2010; Franzen et al., 2012; Hamilton et al., 2012a). However, none of the genes investigated in the present study support Tcbat as the common ancestor of all DTUs. Indeed, Tcbat was corroborated as a recently diverged genotype sharing last common ancestor with TcI. Regardless nuclear or mitochondrial loci or methods used for phylogenetic inferences, in most analyses Tcbat and TcI were placed as sister groups, and TcII as the first lineage to diverge from the common ancestor of all *T. cruzi*. In addition, well-supported phylogenies based on nuclear genes positioned TcI–Tcbat more closely related to TcIII than to TcIV, which was the first to diverge from a common ancestor of TcI, TcIII, TcIV and Tcbat. The unraveling of genetically and ecologically complex DTUs is one main challenge in understanding the origin of each DTU and the role played by bats and terrestrial mammals in the divergence within *T. cruzi*.

In a putative evolutionary scenario, we can hypothesized successive events of lineage divergence and host switches of bat trypanosomes into terrestrial mammals given origin to distinct DTUs rather than multiple genotypes derived from terrestrial mammals jumping into bats. Vectors sharing niches with bats and terrestrial mammals could have mediated host switches via contamination of terrestrial mammals with faeces of infected bugs, mechanical transmission by a range of hematophagous arthropods or the ingestion of infected insects. In addition, predation on bats by terrestrial mammals could also have played an important role in host switching. According to recent studies, the successive events facilitating the emergence of *T. cruzi* from an ancestor bat trypanosome and subsequent divergences that gave rise to DTUs all occurred much more recently than previously imagined. However, the time of lineage and genotype divergence remains controversial (Flores-López and Machado, 2011; Hamilton et al., 2012b; Tomasini and Diosque, 2015).

Indeed, further analysis of *T. cruzi*, particularly from unexplored settings, vertebrate hosts, vectors and ecological niches will reveal new genotypes. The understanding of the detailed genetic diversity, phylogenetic relationships and natural history of *T. cruzi* is crucial for the studies about genetic, population structure, ecology, epidemiology and the emergence of this important human pathogen.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

LL participated of bat captures for sample collection, trypanosome species and genotype identification and performed all phylogenetic analyses. OEA, PAO, JATV, JCC, MP assisted with sample collection, DNA sequences and data interpretation. MGS and GB was responsible for Tcbat genome sequencing. LL, EPC, MMGT designed and coordinated the study and drafted the manuscript. All authors have read, revised and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.07.015

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