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Doutorado em Biologia Parasitária

Diversidade genética de isolados de *Trypanosoma cruzi* DTU I obtidos de mamíferos silvestres e triatomíneos de biomas brasileiros, avaliada por genes variáveis e constitutivos.

IRENE FABIOLA ROMAN MALDONADO

Rio de Janeiro
Junho de 2018

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Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitaria

Orientadora: Prof. Dr[a.] Ana Maria Jansen.

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Rio de Janeiro, 05 de junho de 2018

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Ata da defesa de tese de doutorado em Biologia Parasitária de **Irene Fabiola Roman Maldonado**, sob orientação da Drª. Ana Maria Jansen-Franken. Ao quinto dia do mês de junho de dois mil e dezoito, realizou-se às dez horas, no Auditório Maria Deane/FIOCRUZ, o exame da tese de doutorado intitulada: **"Diversidade genética de isolados de *Trypanosoma cruzi* DTU I obtidos de mamíferos silvestres e triatomíneos de biomas Brasileiros, avaliada por genes variáveis e constitutivos."** No programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutora em Ciências - área de concentração: Genética e Bioquímica, na linha de pesquisa: Variabilidade Genética de Parasita, Vetores e Hospedeiros. A banca examinadora foi constituída pelos Professores: Dr. Reginaldo Peçanha Brazil - IOC/FIOCRUZ (Presidente), Drª. Marta Maria Geraldes Teixeira - USP/SP, Drª. Constança Felicia de Paoli de Carvalho Britto - IOC/FIOCRUZ e como suplentes: Dr. Marcelo Salabert Gonzalez – UFF/RJ e Dr. Edson Oliveira Delatorre – IOC/FIOCRUZ. Após arguir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela Aprovação da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutora em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 06 dejunho de 2018.

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A meu esposo Carlos
Com quem comparto a mesma paixão pela ciência

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Science and everyday life cannot and should not be separated.

Rosalind Franklin

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RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Irene Fabiola Roman Maldonado

Trypanosoma cruzi, parasita hemoflagelado, é geneticamente heterogêneo e atualmente representado por seis linhagens (Discrete Typing Units, DTUs) designados TcI-TcVI. TcI é a DTU mais amplamente distribuída e é capaz de infectar centenas de espécies de mamíferos em todos os estratos florestais. Embora várias investigações sobre a diversidade genética e distribuição de genótipos de TcI tenham sido realizadas no norte de América do Sul, pouco se sabe sobre essa diversidade no Brasil. Nosso objetivo foi contribuir com entendimento da diversidade de TcI utilizando marcadores de evolução rápida e lenta para estudar isolados de TcI obtidos de animais silvestres de cinco biomas brasileiros. Este constitui o primeiro trabalho em que a análise de diversidade por multilocus sequence typing (MLST) foi combinada com análise por multilocus microsatélite typing (MLMT) e por maxicírculo. Os resultados demonstraram a existência de alta diversidade genética e de ocorrência de eventos de introgressão mitocondrial. Não foram observadas evidências robustas de intercâmbio genético em isolados de TcI do Brasil. A ocorrência de fluxo genético entre os isolados foi evidenciada em todos os biomas, excetuando-se a Amazônia, onde se observou isolamento genético dos isolados TcI, e a mais alta heterogeneidade genética. Foi observada ausência de associações estritas de genótipos TcI com áreas geográficas e/ou espécies hospedeiras, no entanto essas associações não podem ser descartadas totalmente. Adicionalmente, evidenciou-se que *Didelphis marsupialis* pode desempenhar um papel como o principal bioacumulador e dispersor de TcI.

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Genetic diversity of Trypanosoma cruzi DTU I isolates obtained from wild and triatomine mammals of Brazilian biomes, evaluated by variable and constitutive genes.

ABSTRACT

PHD THESIS IN PARASITE BIOLOGY

Irene Fabiola Roman Maldonado

Trypanosoma cruzi, is a is genetically heterogeneous hemoflagellate parasite,and is currently represented by six lineages (Discrete Typing Units, DTU) called TcI-TcVI. TcI is the most widely distributed DTU and is able to infect hundreds mammalian species in all forest strata. Although several investigations into the genetic diversity and distribution of TcI genotypes have been conducted in northern South America, little is known about this diversity in Brazil. Our objective was to contribute with an understanding of TcI diversity using fast and slow evolution markers to study TcI isolates obtained from wild animals from five Brazilian biomes. This is the first work in which diversity analysis based on multilocus sequence typing (MLST) was combined with multilocus microsatellite typing (MLMT) and maxicircle analysis. The results demonstrated the existence of high genetic diversity and the occurrence of mitochondrial introgression events. No robust evidence of genetic exchange was observed in isolates of TcI from Brazil; the occurrence of genetic flow among the isolates was evidenced in all biomes, except for the Amazon, where genetic isolation of the TcI isolates was observed, and the highest genetic heterogeneity. We observed the absence of strict associations of TcI genotypes with geographical areas and/or host species, although these associations cannot be completely ruled out. In addition, it has been shown that Didelphis marsupialis may play a role as the main bioaccumulator and dispersant of TcI.

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LISTA DE SIGLAS E ABREVIATURAS

COAR	3-*Hidroxi*-3-*metilglutaril*-CoA *reductase*
DC	Doença de Chagas
DCA	Doença de Chagas Aguda
DNA	Ácido desoxirribonucleico
DHFR-TS	Dihydrofolate Reductase Thymidylate Synthase
kDNA	DNA do cinetoplasto
DTU	*Discrete Typing Units*
GTP	Small GTP-binding protein Rab7
LAP	*Leucine Aminopeptidase*
LYT	*Lytic Pathway Protein*
MET III	*Metacyclin*-III
MLEE	*Multilocus Enzyme Electroforesis*
MLMT	*Multilocus Microsatélite Typing*
MLST	*Multilocus Sequence Typing*
OPAS	Organização Panamericana da Saúde
PCE	*Predominant Clonal Evolution*
PCR	*Polymerase Chain Reaction*
PDH	*Piruvate dehydrogenase component* E1 *subunit alfa*
RAPD	Randomly Amplified Polymorphic DNA
RFLP	*Restriction Fragment Length Polymorphism*
RB19	RNA-binding protein-19
RHO1	Rho-like GTP binding protein
rRNA 24S	Gene RNA ribosomal 24S
SSU	S*mall Subunit*
TR	*Trypanothione Reductase*

1 INTRODUÇÃO

1.1 *Trypanosoma cruzi*

Trypanosoma cruzi (*T. cruzi*) é um protista flagelado da família Trypanosomatidae, Ordem Trypanosomatida, Classe Kinetoplastea (Cavalier-Smith, 1981, Simpson *et al.*, 2006, Kaufer *et al.*, 2017) descrito por Carlos Justiniano Ribeiro Chagas em 1909. *Trypanosoma cruzi* está amplamente distribuído no continente americano desde a região sul dos Estados Unidos até o sul da Argentina. É um parasito heterogêneo, capaz de infectar centenas de espécies de hospedeiros mamíferos de oito ordens (Jansen *et al.,* 2015). O ecletismo de hospedeiros e habitats se expressa na complexidade dos ciclos de transmissão na natureza, que podem ou não acontecer de modo independente nos diferentes estratos florestais, mesmo considerando um mesmo fragmento (Pinho *et. al.*, 2000; Lisboa *et. al.*, 2006). No homem, a infecção pelo parasito pode resultar na doença de Chagas (DC), uma complexa zoonose que está amplamente distribuída no continente americano.

Uma das principais características deste táxon é a alta diversidade morfológica, bioquímica, biológica e genética a qual vem sendo observada em cada vez maior detalhamento, na medida em que o poder resolutivo das técnicas moleculares aumenta. Atualmente, são reconhecidas seis linhagens (TcI-TcVI) ou Unidades Discretas de Tipagem (DTU) além de TcBat, que é um genótipo descrito pela primeira vez em morcegos, candidato a ser a linhagem TcVII (Zingales *et al.*, 2009, Hamilton *et al*., 2012).

Outra característica em destaque é a presença de uma estrutura proeminente denominada cinetoplasto (kDNA), peculiaridade da classe Kinetoplastea. Este corresponde ao DNA condensado, extra nuclear, encontrado no interior da única mitocôndria. Dois tipos de DNA circular estão presentes no cinetoplasto: minicírculos e maxicírculos. Existem vários milhares de minicírculos, que variam em tamanho de cerca de 0,5 a 2,5 kb, e algumas dúzias de maxicírculos, que variam de 20 a 40 kb (Shapiro *et al*., 1995). Juntos, maxicírculos e minicírculos representam aproximadamente 30% do total do genoma celular (Fidalgo *et al.,* 2011). Os minicírculos apresentam alta heterogeneidade, mas também possuem quatro regiões conservadas, onde os locais de origem de replicação são localizados. Os maxicírculos são estrutural e funcionalmente análogos ao DNA mitocondrial.

Trypanosoma cruzi apresenta uma estrutura populacional predominantemente clonal (PCE), como proposto por Tibayrenc e Ayala (Tibayrenc *et al.*, 1990; Tibayrenc e Ayala

2012, 2015a e 2015b). A PCE considera a progênie idêntica ou muito similar à linhagem parental. Este modelo, no entanto, reconhece também eventos de intercâmbio genético, que deram origem às linhagens híbridas (TcV e TcVI), as quais foram estabilizadas na natureza por propagação clonal (Tibayrenc *et al.*, 2012). Alguns autores têm questionado a frequência com que a recombinação genética ocorre em *T. cruzi*, levando a intensos debates (Tomasini *et al.*, 2014, Ramirez e Llewellyn *et al.*, 2014).

1.2 Ciclo de vida de *Trypanosoma cruzi*

Morfologicamente, *T. cruzi* apresenta quatro principais formas ou estágios do desenvolvimento. A forma amastigota, que se replica por fissão binária dentro das células fagocíticas e não fagocíticas do mamífero; a epimastigota, que se replica no intestino posterior do inseto vetor; e a forma tripomastigota, que é não replicativa e está presente no sangue do hospedeiro mamífero (formas tripomastigotas sanguícolas), no reto e fezes dos vetores triatomíneos (tripomastigotas metacíclicos) (Figura 1). Vale mencionar que as quatro formas evolutivas têm competência para infectar mamíferos, uma característica que explica parte do amplo potencial de dispersão do parasito (Dias 2006; Coura 2015; Kessler et al 2017).

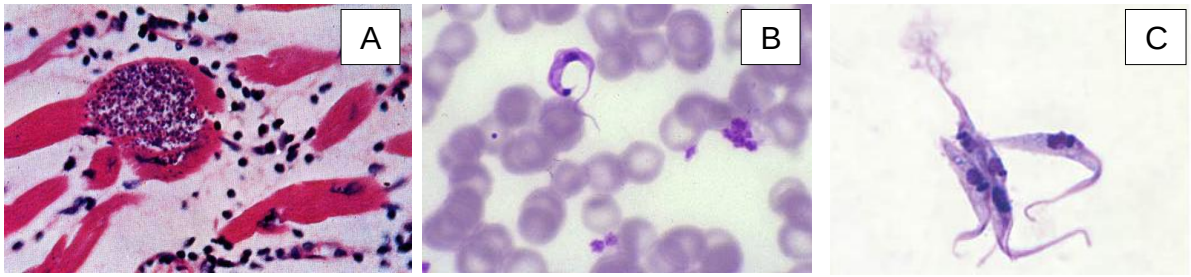

Figura 1: Formas morfológicas de *Trypanosoma cruzi*. A) amastigotas intracelulares. B) tripomastigotas no sangue. C) epimastigotas em cultura axênica. Fonte: http://www.stanford.edu,http://winona.edu,http://www.dpd.cdc.gov

A via vetorial contaminativa é descrita como a forma clássica de transmissão de *T. cruzi* (Figura 2). Esta ocorre quando o inseto vetor elimina as formas tripomastigotas metacíclicas junto com as fezes, enquanto realiza o repasto sanguíneo. Os parasitos penetram através de solução de continuidade da pele ou pela mucosa quando hospedeiro mamífero se esfrega ou coça. No hospedeiro mamífero, os parasitas ingressam nas células nucleadas de praticamente todos os tecidos e se multiplicam na forma amastigota. Seguidamente, uma nova diferenciação ocorre, e as formas agora tripomastigotas nas células nucleadas, produzem o rompimento das mesmas o que resulta na liberação dos parasitos na corrente sanguínea. A transmissão para o inseto vetor ocorre quando este novamente se alimenta do sangue e ingere

as formas tripomastigotas circulantes no sangue do hospedeiro mamífero (Brener 1971; Tyler e Engman 2003; Rassi *et al.*, 2010).

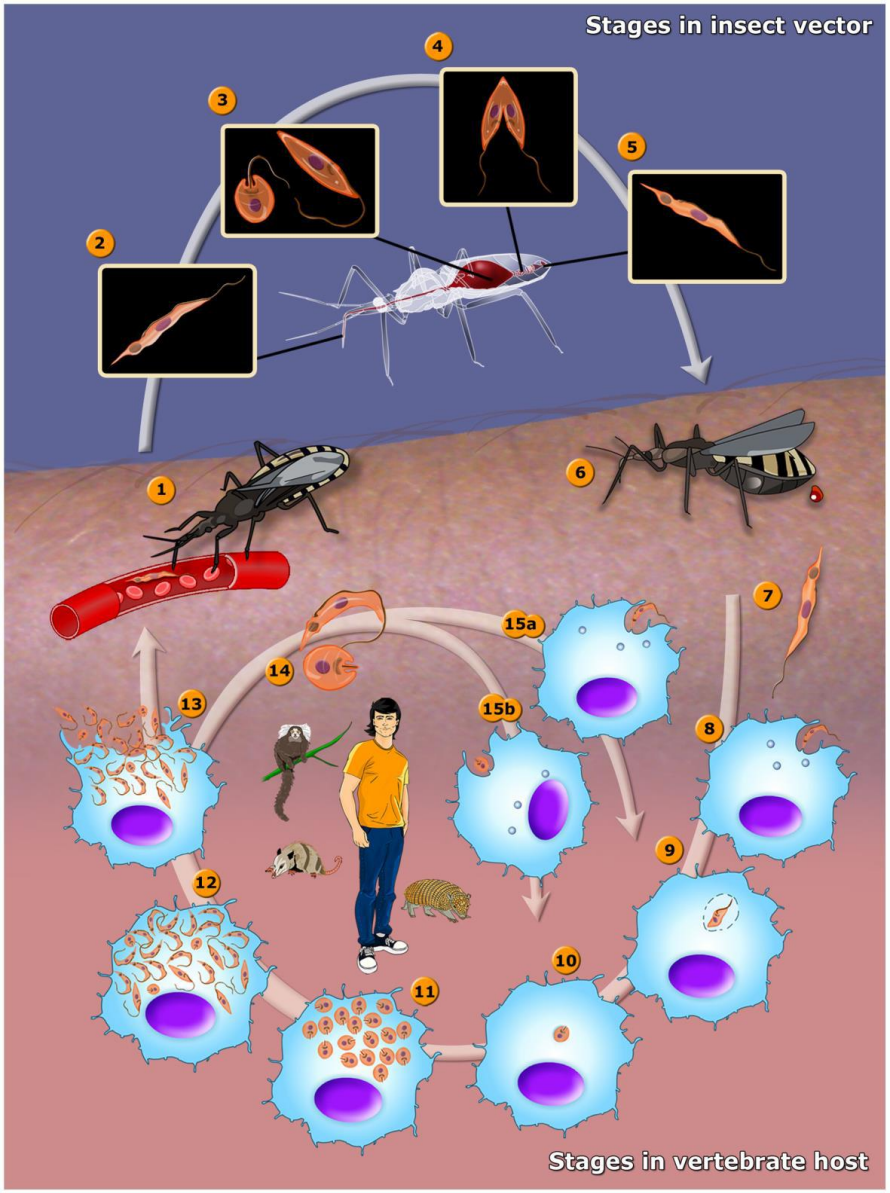

Figura 2: Ciclo de vida do *Trypanosoma cruzi* e seus estágios de desenvolvimento no hospedeiro mamífero e no inseto vetor, o triatomíneo. Fonte: Teixeira *et al.*, 2012.

A transmissão intradomiciliar do parasito para o homem tem sido controlada em alguns países (WHO, 2002; Yamagata e Nakagawa, 2006; Abad-Franch *et al.*, 2013; Salvatella *et al.*, 2014), no entanto, a reemergência da doença de Chagas por transmissão do parasito pela via oral resultou em que esta seja atualmente a principal forma de transmissão na Bacia Amazônica (Shikanai, 2012). A transmissão pela via oral é bem documentada (Coura, 2002; Rios *et al.*, 2011; Santalla *et al.*, 2011) e provavelmente representa a principal via de infecção de mamíferos domésticos e silvestres (Yoshida 2008, 2009; Jansen *et al.*, 2015). A

transmissão pela via oral de *T. cruzi* é extremamente eficiente (Noireau *et al.*, 2009) e tem sido responsável por um alto número de infecções humanas nos últimos anos não apenas no Brasil, mas também na Venezuela e Colômbia (Dias 2006, de Noya *et al.*, 2015).

Os surtos por via oral ocorridos no Brasil aconteceram a partir da ingestão de diversos alimentos contaminados com formas metacíclicas, tais como caldo de cana, açaí, palmito, bacaba ou até sopa contaminada com triatomíneos (Shaw *et al.*, 1969; Dias 2008; Bastos *et al.*, 2010; Steindel *et al.*,2008; Pinto AY; Nóbrega *et al.*, 2009).

Outras formas de infecção dos hospedeiros mamíferos incluem a transmissão vertical congênita, de mãe a filho; de transfusões de sangue ou transplantes de órgãos (Gascon *et al.*, 2010; Basile *et al.*, 2011).

Na natureza, *T. cruzi* é mantido através de distintos e complexos ciclos de transmissão que envolvem hospedeiro e vetores em todos os ambientes (Araujo *et al.*, 2009; Noireau *et al.*, 2009). Classicamente, os ciclos de transmissão foram descritos como domésticos, peridomésticos e silvestres. No entanto nem sempre é fácil estabelecer os limites entre estes três ecótopos. O ciclo doméstico envolve triatomíneos que colonizam o peridomicílio e/ou o domicílio, além de humanos e animais domésticos; o ciclo peridoméstico envolve mamíferos sinantrópicos, roedores, morcegos e, principalmente, marsupiais; o ciclo silvestre ou enzoótico está relacionado aos triatomíneos vetores e mamíferos silvestres de vida livre com a ocasional ocorrência de casos humanos (de Freitas *et al.*, 2006; Rassi *et al.*, 2010). No entanto, a expressiva plasticidade biológica de *T. cruzi* pode resultar em ciclos de transmissão superpostos e interconectados em diferentes habitats e estratos florestais. Isto reflete um sistema complexo na natureza (Rozas *et al.*, 2007; Diotaiuti *et al.*, 1995)

1.3 Diversidade genética e populacional de *T. cruzi*

A origem do clado *T. cruzi* é ainda muito discutida e duas hipóteses têm sido propostas. A teoria do supercontinente sul sugere que as espécies de tripanosomatídeos apareceram a partir de uma espécie de Trypanosoma presente em marsupial, quando América do Sul, Antártica e Austrália formavam um único continente. Uma vez que espécies de tripanosomatídeos de marsupiais australianos foram incluídas dentro do clado *T. cruzi*, essa teoria ganhou força (Stevens *et al.*, 1998, 1999, 2001). Por outro lado, a descrição de espécies de tripanosomatídeos de mamíferos africanos e de outras espécies de tripanosomatídeos em morcegos africanos e americanos dentro do clado de *T. cruzi* (Hamilton *et al.*, 2009; Lima *et al.*, 2012, 2013, 2015), sugere que as espécies do clado *T. cruzi* tiveram origem a partir de um tripanosomatídeo de morcego, e que este foi se adaptando a outras espécies de mamíferos em

vários eventos distintos. Essa teoria é conhecida como *Bat-seeding hypotese* (Hamilton *et al.,* 2012).

Sendo um parasito muito antigo e disperso em diferentes ambientes, não resulta estranha a presença de uma alta heterogeneidade, tanto morfológica, bioquímica e genética (Revollo *et al.*, 1998, Macedo *et al.*, 2004; de Freitas *et al.*, 2006). A heterogeneidade de *T. cruzi* já havia sido observada por Chagas e Brumpt (formas largas e finas) e vem sendo discutida até o presente.

Andrade *et al.*, agrupou a população de *T. cruzi* em tipos ou biodemas I-III baseado nas características morfológicas dos parasitas no sangue periférico de camundongos e o comportamento dos isolados nestes hospedeiros (Andrade *et al.* 1974; Andrade *et al.* 1983).

No início dos anos 80, mediante aos métodos bioquímicos, foi possível visualizar a heterogeneidade baseada no perfil eletroforético de enzimas com a mesma função, onde foi possível identificar três grupos de perfis distintos. O Zimodema 1, associado ao ciclo silvestre; o Zimodema 2, associado ao ciclo doméstico; e o Zimodema 3, menos comum e também associado ao ciclo silvestre em áreas restritas (Miles *et al.,* 1980). Novos estudos, ampliando o número de enzimas, demonstraram a diversidade presente entre os isolados, onde foram encontrados 44 zimodemas diferentes. A heterogeneidade de *T. cruzi* foi também observada quando DNA mitocondrial (kDNA) foi analisado utilizando *fragment length polymorphisms* ou RFLP (Morel *et al.,* 1980).

Posteriormente, foram realizados estudos da diversidade de *T. cruzi* utilizando porções de DNA de sequência e localização conhecidas, denominados marcadores genéticos, os quais mostraram uma alta diversidade. Por outro lado, a amplificação pela reação em cadeia da polimerase (PCR) dos genes rRNA 24S e da região intergênica dos genes mini-exon, mostraram um claro dimorfismo entre os isolados de *T. cruzi* (Souto *et al.,* 1996; Fernandes *et al.,* 1999). Assim, no simpósio realizado no ano 1999, no Rio de Janeiro Brasil, a nomenclatura foi homogeneizada, renomeando as duas maiores linhagens como *T. cruzi I* (TcI) e *T. cruzi* II (TcII) (Anonymous, 1999). Com essa classificação, dentro de TcI se agrupam os equivalentes ao Zimodema 1, e no TcII os equivalentes ao Zimodema 2. Essas duas linhagens não acomodaram Z3, que foi posteriormente definido com marcadores dos genes mini-exon (Fernandes *et al.,* 1998).

Estudos realizados com RAPD (Brisse *et al.,* 2000), sequências do gene SSUrDNA (Brisse *et al.,* 2001), e análises de um grande número de loci de isoenzimas (Breniere *et al.,* 2003) agruparam Z3 com TcII e sugeriram uma grande heterogeneidade da linhagem TcII, que foi subdividida em cinco linhagens TcIIa-TcIIe. A confirmação desses agrupamentos foi

obtida com análises de outros genes nucleares e mitocondriais (Brisse *et al.,* 2003; Sturm *et al.,* 2003; Westenberger *et al.,* 2005, 2006; Freitas *et al.,* 2006).

No ano 2003, o conceito de Unidades Discretas de Tipagem (DTU) foi introduzido. Estas unidades designavam a um conjunto de parasitas que são geneticamente mais similares. Assim, desde 2009, e com base em diferentes marcadores moleculares e características biológicas, foi adotada a nomenclatura que inclui seis DTUs, a saber: *T. cruzi* I (TcI), *T. cruzi* II (TcII), *T. cruzi* III (TcIII), *T. cruzi* IV (TcIV), *T. cruzi* V (TcV), and *T. cruzi* VI (TcVI) (Zingales *et al.,* 2009). Adicionalmente, com base em análises filogenéticas e filogeográficas, e mediante múltiplos marcadores moleculares, uma linhagem independente às demais DTUs e associada a quiróptera (TcBat) tem sido descrita (Marcili *et al.,*2009; Lima *et al.,* 2015).

As DTUs TcI-TcIV compõem clados monofiléticos, em tanto que TcV e TcVI são conhecidos como híbridos inter-linhagens naturais recentes (Machado & Ayala, 2001; Lewis *et al.,* 2011). As DTUs TcI e TcII são consideradas as linhagens ancestrais mais distantes, no entanto, o tempo de divergência ainda é objeto de debate. Os primeiros estudos estimaram uma origem de entre 88 e 37 milhões de anos sobre a base de estudos com a subunidade pequena de rDNA (Briones *et al.,* 1999; Kawashita *et al.,* 2001). Mais recentemente, estudos baseados em 22 loci nucleares concatenados, sugerem uma origem de aproximadamente 3 milhões de anos atrás (Flores López e Machado 2011).

Em relação à origem das DTUs TcIII e TcIV, os resultados são ainda controversos. Alguns estudos apontam a uma origem em eventos ancestrais de hibridação entre TcI e TcII (Lewis *et al.,* 2011; Westenberger *et al.,* 2005). Por outro lado, de Freitas *et al.* (2006) sugerem que a DTU III corresponde a um grupo ancestral, junto com TcI e TcII, baseado no fato de que elas apresentam um genoma mitocondrial totalmente distinto de TcI e TcII (Freitas *et al.,* 2006).

Existe um consenso em relação à natureza híbrida das DTUs TcV e TcVI, como resultante de pelo menos um evento de hibridação entre as DTUs TcII e TcIII (Sturm & Campbell, 2010; de Freitas *et al.,* 2006; Westenberg *et al.,* 2005; Machado & Ayala 2001; Flores-Lopes *et al.,* 2011). Na Figura 3 se mostram as duas propostas de origem das DTUs híbridas, incluindo as demais DTUs.

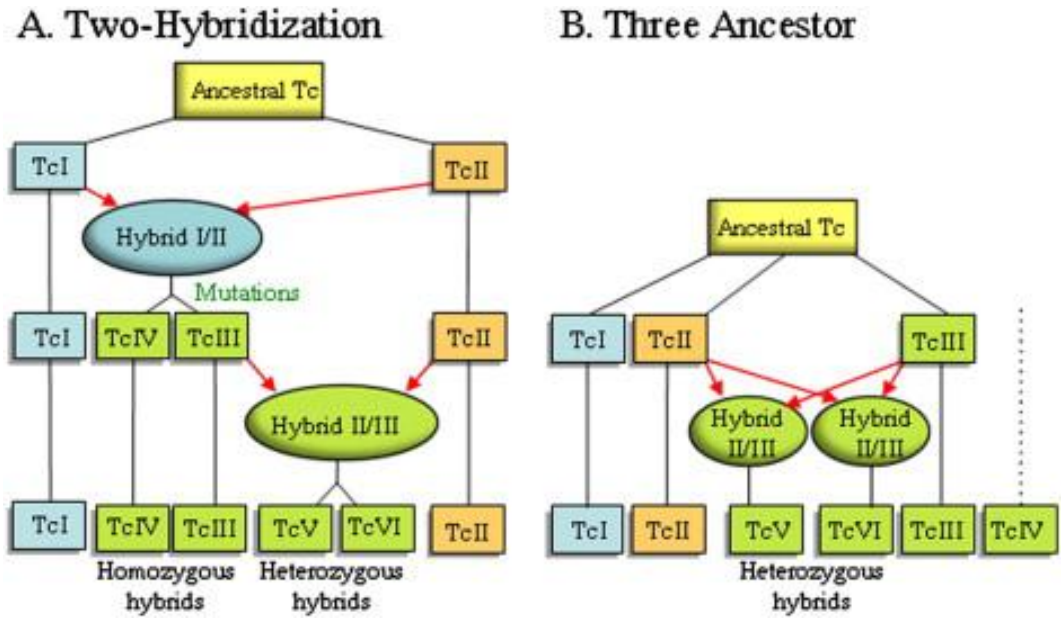

Figura 3*:* Comparação dos modelos de duas hibridizações (a) duas hibridizações e (b) três ancestrais, que descrevem o papel da troca genética durante a evolução do *T. cruzi*. Fonte: Zingales *et al.*, 2012.

Desde a descrição por Carlos Chagas, os estudiosos têm discutido o significado biológico das diferentes formas do parasito. Ao longo dos anos, associações das DTUs com ciclos de transmissão e/ou hospedeiros foram propostos. Classicamente, a DTU TcI foi associada ao ciclo de transmissão silvestre. Isto provavelmente se deve a que TcI prevalece entre isolados obtidos de mamíferos silvestres (Fernandes *et al.*, 1998). A linhagem TcII foi classicamente associada ao ciclo de transmissão doméstico, incluindo infecções humanas (Zingales *et al.*, 2012). No entanto, na medida que os estudos são mais abrangentes em termos de número de excursões e espectro de espécies de mamíferos silvestres analisadas, essas supostas associações têm sido questionadas (Jansen *et al.*, 2015).

Na natureza, os animais são expostos à infecção por diferentes DTUs de *T. cruzi* que pode ocorrer uma ou repetidas vezes de modo simultâneo ou sequencial. Além disso, essas infecções acontecem em animais portadores de outras taxa de parasitas, o que faz deste, um sistema intrincado e complexo.

1.4 Reservatórios mamíferos de *T. cruzi*

O conceito de reservatório vem sendo reavaliado periodicamente. Cada vez mais vem sendo abandonada a ideia de reservatório como fonte de infecção para uma determinada espécie alvo, que em geral era o homem e os animais domésticos. Também, o conceito de ausência de patogenicidade ou virulência para o hospedeiro, vem sendo retirada da definição

de reservatório, uma vez que estes dois atributos podem ser, e muitas vezes são, um traço que aumenta as chances de dispersão de parasitos. A definição proposta por Ashford (1996) considera reservatório como um conjunto de espécies que garantem a dispersão e manutenção de um determinado parasita na natureza, e é o que temos adotado em nossos trabalhos.

A interação parasito-reservatório é considerada um sistema complexo, dinâmico, imprevisível que envolve vários fatores (Jansen e Roque, 2010) e que acontece em um determinado recorte tempo-espacial, o que resulta em que a interação parasito-reservatório apresenta diferentes perfis e esteja em constante mudança (Jansen e Roque 2010)

Um dos mais antigos e importantes reservatórios de *T. cruzi* são os didelfídeos. Os didelfídeos estão distribuídos amplamente no continente americano, do sudeste de Canadá ao sul da Argentina. Os didelfídeos são nômades, solitários e excelentes escaladores. Eles podem ser encontrados no topo e nos buracos de árvores, como também no solo e outros refúgios naturais (Jansen *et al.*, 2010) São considerados mamíferos generalistas, se adaptam facilmente aos domicílios de seres humanos e têm sido reconhecidos como sinalizadores de ambiente perturbado (Austad, 1988; Olifiers *et al.* 2005).

O termo bioacumulador se refere originalmente à possibilidade de concentração de substâncias por um organismo vivo (Streit B, 1998). Este termo foi adaptado ao contexto ecológico passando a ser utilizado por diversos autores para indicar a acumulação de parasitos em animais (Diaz Alcaraz 2015; Rocha *et al.*, 2013[a]; Rocha *et al.*, 2013[b]).

As espécies do gênero *Didelphis* spp. tem sido propostas como bioacumuladores e provavelmente dispersores das DTUs de *T. cruzi* na natureza (Jansen *et al.*, 2015), uma vez que são capazes de manter uma alta taxa de infecções mistas.

1.5 Brasil e o cenário atual da doença de Chagas

O Brasil apresenta uma enorme diversidade de ambientes e de fauna, composto por seis distintos biomas ou ecorregiões (Costa *et al.*, 2003). O bioma Amazônia é o maior deles ao norte, fazendo fronteira ao leste com o Cerrado e Pantanal ao sul. O nordeste do país é dominado pelo bioma Caatinga, composto principalmente por vegetação xérica; o bioma Mata Atlântica se estende do sul de Pernambuco até o sul do Rio Grande do Sul, ao longo da costa brasileira e caracterizado por uma floresta tropical úmida. De acordo com Paglia *et al.*, (2012), a diversidade de mamíferos silvestres, potenciais hospedeiros de *T. cruzi*, ao longo desse mosaico paisagístico é maior na Amazónia, seguida da Mata Atlântica, Cerrado, Caatinga e Pampa.

Após a bem sucedida campanha para controle das populações intradomiciliares de *Triatoma infestans*, a organização Pan Americana da Saúde (OPAS) certificou ao Brasil como livre de transmissão domiciliar vetorial de *T. cruzi* por espécies de triatomíneos. Isto foi possível mediante as campanhas de borrifado com inseticidas associados com o melhoramento dos domicílios.

Atualmente, são menos frequentes as notificações por casos novos de infecção pela via contaminativa, prevalecendo o atual perfil epidemiológico da doença de Chagas principalmente na transmissão oral. O número de casos atribuídos à infecção oral tem aumentado nos últimos anos, especialmente na região norte do país, classicamente considerada indene. Há relatos de casos e surtos por via oral no Acre, Amazonas, Pará. Na região nordeste, casos e surtos foram descritos no Maranhão, Rio Grande do Norte sem contar casos esporádicos no Sudeste (Roque *et al.*, 2008; Shikanai-Yasuda e Carvalho, 2012).

Embora já vinham ocorrendo há mais de duas décadas, medidas efetivas de controle da transmissão de *T. cruzi*, no atual cenário, ainda estão longe de ser definidas, o que sinaliza que esta zoonose deve ser analisada sob uma nova perspectiva epidemiológica, sendo infrutífero tentar adaptar à situação atual, medidas de controle utilizadas no passado.

1.6 Diversidade genética em *T. cruzi* I

A antiguidade da DTU I de *T. cruzi* é atestada pelo expressivo número de mutações em genes constitutivos deste genótipo. Vários trabalhos sobre variabilidade genética intra DTU I propuseram associações entre subpopulações de TcI com ciclos de transmissão sem que tenham chegado a um consenso (Herrera *et al*., 2007; O'Connor *et al.*, 2007; Cura *et al.* 2010; Llewellyn *et al.*, 2009b).

Os primeiros trabalhos sobre a variabilidade genética foram baseados em RAPD e MLEE (Saravia *et al*., 1987; Barnabé *et al.*, 2000; Brisse *et al*., 2000; Montilla *et al*., 2002). No ano 2006, mediante a análise da região variável de kDNA, foi proposta uma associação entre subgrupos de TcI e localidade de coleta dos hospedeiros (Salazar *et al.*, 2006; Triana *et al*., 2006).

Análises com os genes mini-exon também foram realizados para o estudo da variabilidade genética de TcI. Herrera *et al.*, (Herrera *et al.*, 2007, 2009) propuseram a existência de quatro genótipos ou haplótipos de TcI (TcIa-TcId), a partir de isolados da Colômbia. Para a análise desses haplótipos foram desenhados oligonucleotídeos para identificar três deles, TcIa, TcIb e TcId, utilizando técnicas de PCR específicas (Falla *et al.*, 2009). Associações das subpopulação de TcI com os ciclos de transmissão foram propostas na

Colômbia, onde TcIa foi associado a infecção humana e a vetores do ciclo doméstico, TcIb associado a infecção humana e a vetores peridomésticos; TcIc, a vetores domésticos e TcId ao ciclo silvestre.

Adicionalmente, estudos realizados através da tipagem de microssatélites multilocus (MLMT) demonstraram alta diversidade genética em isolados de TcI em ciclos de transmissão de diversas regiões geográficas de América Latina (Llewelyn *et al.,* 2009). Nesse mesmo estudo, os autores descreveram um genótipo de TcI que foi proposto como associado à infecção humana intradomiciliar, e por isso denominado como Ven/Dom.

Esses resultados foram corroborados por Cura *et al.,* (2010), que analisaram 105 amostras de TcI de localidades endêmicas de América Latina e do norte. Além disso, o grupo reportou a existência de outro genótipo, TcIe, que foi observado na Bolívia, na Argentina e no Chile (Cura *et al.* 2010). Ocaña-Mayorga *et al.*, (2010) estudando 81 isolados de TcI obtidos de vetores e mamíferos na província de Loja, Equador, observaram duas populações que foram por eles propostas como associadas aos ciclos de transmissão doméstico/peridoméstico e silvestre.

Estudos realizados com um conjunto de 24 marcadores microssatélites aplicados a clones biológicos de Colômbia corroboraram a alta diversidade genética de DTU I de *T. cruzi* e a presença de um grupo denominado TcI_{Dom}, associado à infecção humana e vetores domésticos (Ramírez *et al.,* 2013). Este último, em concordância com o genótipo denominado VenDom por Llewellyn *et al.,* (2009) e também detectado em surtos de doença de Chagas por via oral na Venezuela (Segovia *et al.*, 2013).

Até aqui, as análises aplicadas às subpopulações de TcI utilizaram como marcadores principalmente genes de evolução rápida. Análises baseadas em genes constitutivos ou de evolução lenta tiveram início na Colômbia, onde Ramírez *et al.,* (2013), utilizando um conjunto de 50 clones de TcI, analisaram 14 marcadores de *multilocus sequence typing* ou MLST. Os resultados levaram os autores a concluir à existência de três clados relacionados respectivamente aos ambientes doméstico, peridoméstico e silvestre.

Em comum, todos os estudos sobre a diversidade intra TcI até então, apontaram para a existência de uma subpopulação de TcI associada ao ciclo doméstico e à infecção humana. No entanto, esse suposto cluster TcI_{Dom} carecia de robustez, como apontado por Ramírez e Hernández (2017), em estudo subsequente.

Quanto à diversidade intra DTU TcI de *T. cruzi* no Brasil, pouco se sabe (Lima *et al.* 2014). Assim sendo, este estudo teve o objetivo de avaliar a diversidade intra DTU I de *T. cruzi*, considerando um grande painel de isolados TcI do Brasil.

2 JUSTIFICATIVA

Trypanosoma cruzi I (TcI) é o genótipo mais amplamente disperso em toda a América; é encontrado infectando centenas de espécies de mamíferos silvestres de todos os estratos florestais de todos os biomas, podendo ser considerado um sucesso evolutivo. A diversidade de TcI vem sendo muito estudada em vários países com resultados ainda controversos. Em Colômbia foram descritos inicialmente quatro subgrupos de TcI (TcIa- TcId), o que posteriormente foi reduzido. Estudos posteriores naquele país, mostraram a presença de dois subgrupos de TcI: TcI_{Dom}, associado a ciclos domésticos de transmissão e TcI silvestre, associado a ciclos peridomésticos e silvestres. No Brasil, pouco se sabe sobre a variabilidade genética da DTU TcI, desde que existe apenas um trabalho focado na diversidade de TcI de isolados brasileiros. Na Amazônia, onde a infecção humana ocorre principalmente pelo genótipo TcI, os surtos são recorrentes e as variáveis que influenciam a transmissão ainda são desconhecidas. Uma questão a considerar é se existe algum subgrupo de TcI que esteja associado a áreas de surto e que portanto possa se constituir em um indicador de risco epidemiológico. Além disso, é importante entender se a ecologia e a ampla distribuição do genótipo TcI na natureza são devidas à heterogeneidade genética desta DTU.

3 OBJETIVOS

3.1 Objetivo Geral

Estudar a variabilidade genética e distribuição de subpopulações de isolados TcI de *Trypanosoma cruzi* derivados de animais silvestres de vida livre de biomas do Brasil e a eventual associação destas com áreas de surtos, espécies hospedeiras, habitats ou área geográfica.

3.2 Objetivos Específicos

- Avaliar a diversidade de TcI utilizando os seguintes alvos:

  a. Genes nucleares constitutivos:
  b. Loci de microsatélite.
  c. Gene de maxicírculo.
- Examinar mediante árvores filogenéticas
  a. A diversidade intra DTU I.
  b. A distribuição geográfica dos subgrupos.
  c. A existência de associação entre eventuais subgrupo de TcI e áreas de surto ou casos de doença de Chagas.
- Analisar a existência de eventos de intercâmbio genético em TcI.
- Examinar a presença de eventos de introgressão mitocondrial em TcI.
- Avaliar a riqueza alélica dos isolados de TcI mediante a análise de loci de microsatélites.
- Avaliar o potencial de didelfídeos como bioacumulador da diversidade de TcI.

4 RESULTADOS

Os resultados obtidos serão apresentados no formato de artigos:

Artigo 1. Multilocus sequence typing: genetic diversity in *Trypanosoma cruzi* I (TcI) isolates from Brazilian didelphids. *Parasites & Vectors* (2018) 11:107: doi.org/10.1186/s13071-018-2696-9.

Artigo 2. Dissecting the phyloepidemiology of *Trypanosoma cruzi* I (TcI) in Brazil by the use of high-resolution genetic markers. *PLoS Neglected Disease* (2018) 21;12(5):e0006466: doi: 10.1371/journal.pntd.0006466.

Artigo 1. Multilocus sequence typing: genetic diversity in *Trypanosoma cruzi* I (TcI) isolates from Brazilian didelphids.

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Parasites & Vectors (2018) 11:107:
doi.org/10.1186/s13071-018-2696-9.

Neste trabalho, foi avaliada a diversidade genética em isolados de TcI através da análise de quatro genes constitutivos. Os isolados foram obtidos de hospedeiros didelfídeos de quatro biomas brasileiros.

Os resultados mostraram:

- Alta diversidade genética intra DTU observada na análise com MLST.
- A ausência de estritas associações com áreas geográficas ou surtos de doença de Chagas. No entanto, foi observado um grupo integrado com isolados exclusivamente do bioma Caatinga.
- A presença de intercâmbio genético, evidenciada pela incongruência entre as árvores geradas com os genes individuais. Além disso, haplótipos para cada gene indicaram a presença de intercâmbio genético em dois fragmentos de genes.

Roman et al. Parasites & Vectors (2018) 11:107
https://doi.org/10.1186/s13071-018-2696-9

Parasites & Vectors

RESEARCH Open Access

Multilocus sequence typing: genetic diversity in *Trypanosoma cruzi* I (TcI) isolates from Brazilian didelphids

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Abstract

Background: *Trypanosoma cruzi* is a protozoan parasite characterized by extensive genetic heterogeneity. There are currently six recognised, genetically distinct, monophyletic clades designated discrete typing units (DTUs). TcI has the broadest geographical range and most genetic diversity evidenced by a wide range of genetic markers applied to isolates spanning a vast geographical range across Latin America. However, little is known of the diversity of TcI that exists within sylvatic mammals across the geographical expanse of Brazil.

Results: Twenty-nine sylvatic TcI isolates spanning multiple ecologically diverse biomes across Brazil were analyzed by the application of multilocus sequence typing (MLST) using four nuclear housekeeping genes. Results revealed extensive genetic diversity and also incongruence among individual gene trees. There was no association of intralineage genotype with geography or with any particular biome, with the exception of isolates from Caatinga that formed a single cluster. However, haplotypic analyses of *METIII* and *LYT1* constitutive markers provided evidence of recombination events in two isolates derived from *Didelphis marsupialis* and *D. albiventris*, respectively. For diversity studies all possible combinations of markers were assessed with the objective of selecting the combination of gene targets that are most resolutive using the minimum number of genes. A panel of just three gene fragments (*DHFR-TS*, *LYT1* and *METIII*) discriminated 26 out of 35 genotypes.

Conclusions: These findings showed geographical association of genotypes clustering in Caatinga but more characteristically TcI genotypes widely distributed without specific association to geographical areas or biomes. Importantly, we detected the signature of recombination events at the nuclear level evidenced by haplotypic analysis and incongruence.

Keywords: *Trypanosoma cruzi*, Chagas disease, Multilocus sequence typing, MLST, Recombination, *T. cruzi* I, Genetic diversity

Background

The protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas disease, is a vector-borne zoonosis transmitted by hematophagous triatomine bugs (Hemiptera: Reduviidae: Triatominae). They are maintained in the sylvatic environment by a wide range of mammalian hosts species and endemic from southern USA to southern Argentina [1, 2]. The primary route of infection in humans is contact with infected triatomine bug faeces that are deposited on the skin during the blood meal. Infection occurs when parasites enter mammal hosts through skin lesions, the insect bite wound or directly through the mucosa. Domiciliated infestation of triatomine bugs has not been reported from the Amazon basin but enzootic transmission from non-domiciliated adult triatomines is known to occur [3]. Moreover sporadic human infection by *T. cruzi* is re-emerging as a food-borne disease in areas that were not previously endemic for Chagas disease. In the Amazon, oral infection is associated primarily with the ingestion of infected açaí juice and bacaba juice [4–6]. Also, in Venezuela, an outbreak of over 100 cases of acute Chagas disease was caused by the ingestion of fresh guava juice in one

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school in Cacaras [7] due to poor hygiene measures in the preparation of the fruit juice.

A salient feature of *T. cruzi* is extensive genetic heterogeneity [8–11]. The species is currently subdivided into six genetically discrete typing units (DTUs), TcI to TcVI [12, 13], and an additional clade associated with bats (TcBat) has been proposed [14]. TcI and TcII are ancient lineages that diverged from a common ancestor approximately 1–3 million years ago [10]. TcV and TcVI clearly have a relatively recent hybrid origin derived from TcII and TcIII [15]. According to some authors, TcIII and TcIV also originated from a more ancient hybridization of TcI and TcII [16, 17], although others claim that it is not the case [10, 18].

TcI is widespread from the southern USA to northern Argentina and Chile and infects many different mammal host including humans, domestic and sylvatic species, transmitted by triatomine bug vectors. TcI is the most frequently sampled DTU in wild transmission cycles, although it was also observed in domestic cycles [19, 20] and is the dominant DTU in terms of Chagas disease transmission in endemic regions north of the Amazon Basin [21]. In Brazil, TcI represents 58% of recovered sylvatic *T. cruzi* isolates [8]. Moreover, in Brazil, the distribution of DTUs does not appear to have an association with particular biomes or geographical areas [8]. Some species including bats and, in particular, marsupials (*Didelphis* spp.) are considered to be ancient reservoir hosts of *T. cruzi* [22]. Didelphids are nomadic sylvatic/synanthropic species and widely distributed throughout Brazil's biomes, inhabiting both terrestrial and arboreal niches [8]. They are omnivorous and highly adaptable, capable of colonizing environments degraded by humans and are classically associated with the *T. cruzi* I genotype [23]. However, they are also able to harbor other *T. cruzi* DTUs. Hence, *Didelphis* spp. are exposed to *T. cruzi* infection across different transmission cycles and act as bioaccumulators of TcI intralineage genotypes [8].

The genetic diversity present in TcI has been assessed by a plethora of molecular methods including random amplification of polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MLEE), internal transcribed spacer (ITS), and polymorphisms in minicircles and in the miniexon gene [23–28]. Several studies based on the microsatellite motif of spliced ladder intergenic regions (SL-IR) indicate the existence of five groups within TcI (Ia-Ie) and potential associations to anthropogenic or wild environments [16, 28–32]. However, a review of the SL-IR classification of five subgroups of TcI showed that there was no conclusive evidence of genetic structuring between domestic and wild isolates, but rather an association with geographical distribution [33, 34]. Ramirez et al. [35], using nuclear gene targets and MLST, demonstrated the existence of two TcI groups in Colombian isolates: one associated with the wild transmission cycle (TcI SILV) and the other with domestic transmission (TcI DOM). The latter has previously been classified as TcIa and TcI VEN_{Dom} [28, 36]. Ramirez & Hernandez [37] provided evidence for a possible subdivision of TcI (into DOM and SILV), although they also stated that the substancial diversity present in TcI did not allow conclusive identification of TcIDom as a robust near-clade.

MLST involves the sequencing of constitutive gene fragments. Initially applied to bacteria and yeast, and subsequently adapted and applied to diploid organisms, including *Trypanosoma cruzi* [38, 39] and *Leishmania* spp. [40]. The major advantage of MLST analysis is that sequence data are unequivocal with sufficient resolution for epidemiological diversity and population studies [38]. Furthermore, results are objective and easily accessible for some pathogens via online database repositories such as MLST.net [38]. Previously, and specific to *T cruzi*, Yeo et al. [38] applied MLST analysis using nine constitutive genes to evaluate the diversity across different DTU's. Additionally, Lauthier et al. [39] assessed diversity on *T. cruzi*, using ten markers. Moreover, Messenger et al. [41] developed an MLST maxicircle typing scheme using ten gene targets applied to TcI isolates from disparate locations revealing extensive mitochondrial introgression and heteroplasmy. Furthermore, to analyze the variability of TcI in Colombia, Ramirez et al. [35], applied MLST analysis to 13 constitutive genes.

Brazil is a vast country, containing an enormous diversity of habitats and high biodiversity. Yet, little is known of the intra-DTU diversity of TcI in mammals, and more specifically in *Didelphis* spp., in Brazil. We investigate the potential for TcI subpopulation associations in the context of species of the marsupial genus *Didelphis* and their role in transmission cycles and as bioaccumulators of *T. cruzi*. We apply MLST analysis of four housekeeping genes, to assess the genetic heterogeneity of TcI isolates obtained from *Didelphis* spp. that were captured in four different Brazilian biomes spanning a vast geographical range.

Methods

Parasite isolates

A total of twenty nine TcI isolates, available in the Coleção de Trypanosomas de mamiferos silvestres, domesticos e vetores (ColTryp) at the Laboratório de Biologia de Tripanosomatídeos do Instituto Oswaldo Cruz/FIOCRUZ, were sampled from *Didelphis marsupialis*, *D. albiventris* and *D. aurita*, captured in four Brazilian biomes: Atlantic Forest, Amazon, Caatinga and Cerrado (Fig. 1 and Table 1). TcI isolates that had previously been confirmed as TcI using Mini-Exon polymerase chain

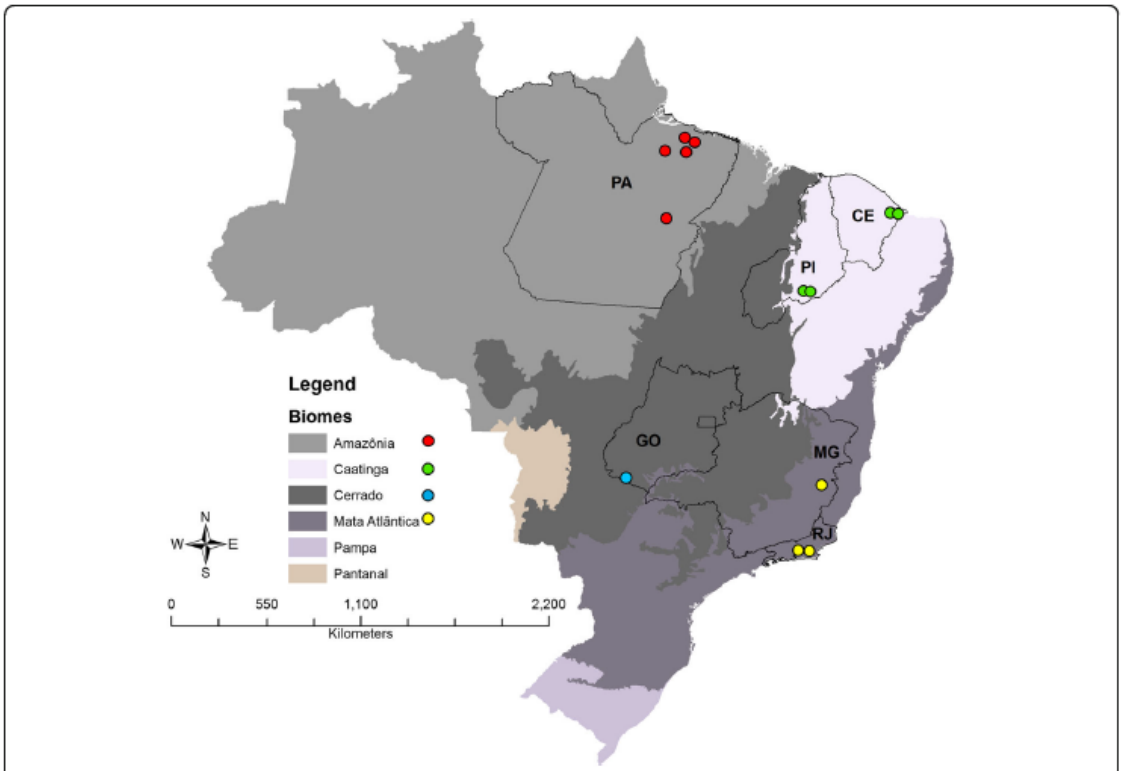

Fig. 1 Map of the spatial distribution of the *T. cruzi* I isolates. Circles with colors represent the municipalities in each biome where samples were obtained: Amazon (red); Caatinga (green); Atlantic Forest (yellow); Cerrado (blue)

reaction (PCR) [42] by ColTryp, were genotyped by MLST with appropriate reference sequences.

Details of the origin and geographical distribution of the isolates are given in Table 1 and Fig. 1, respectively.

MLST: Choice of loci

Four nuclear TcI gene fragments were selected for MLST analysis. The choice of targets was based on a previous work conducted by Yeo et al. [38] that showed the intralineage discriminatory capacity of 9 nuclear genes applied to *T. cruzi*. The four genes selected were: *DHFR-TS* (dihydrofolate reductase-thymidylate synthase); *RB19* (RNA-binding protein-19), *METIII* (metacyclin-III), and *LYT1* (lytic pathway protein).

Molecular methods

PCRs were performed using different reaction conditions following Yeo et al. [38] with some modifications. For *DHFR-TS*, initial denaturation was at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 1 min and 72 °C for 2 min. The cycle conditions for amplification of *RB19*, *METIII* and *LYT1* genes were: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, with an annealing temperature of 53 °C (*RB19*), 51 °C (*METIII*) or 57 °C (*LYT1*) for 30 s, and an extension of 72 °C for 45 s. All reactions had a final additional extension of 72 °C for 10 min. Each 25 μl total reaction volume contained: 50 ng genomic DNA, 0.2 μM of each primer, 2 mM of each dNTPs, 50 mM $MgCl_2$ solution and 1 U *Taq* (BIO-21086, Bioline, London, UK). The products were visualized on agarose (2%) stained with ethidium bromide. PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, Little, Chalfont, Buckinghamshire, UK). Bi-directional sequencing was performed using PCR primers and Big Dye Terminator Cycle Sequencing v.3.1 (Applied Biosystems, Foster city, CA, USA) in an ABI PRISM 3730 DNA Sequencer (Applied Biosystems) using standard protocols. Bio Edit v.7.0.4.1 [43] and DNASTAR Lasergene SeqMan v.7.0.0 programs were used to align and edit DNA sequences. Heterozygous positions were identified manually by the presence of two coincident peaks at the same locus ("split peaks"), verified in both forward and reverse directions and scored according to one-letter International Union of Pure and Applied Chemistry (IUPAC) nomenclature. All

Table 1 MLST: Characteristics of TcI isolates from *Didelphis* spp. from four Brazilian biomes

ID	Host	Biome	Municipality/ State	Year of collection	GenBank accession number			
					DHFR-TS	*RB19*	*LYT1*	*METIII*
D7	*D. aurita*	Atlantic Forest	Silva Jardim/ Rio de Janeiro	1996	MG228296	MG228324	MG228352	MG868974
G05	*D. marsupialis*	Atlantic Forest	Silva Jardim/ Rio de Janeiro	2003	MG228297	MG228325	MG228353	MG868975
G15	*D. marsupialis*	Atlantic Forest	Silva Jardim/ Rio de Janeiro	2003	MG228298	MG228326	MG228354	MG868976
G45	*D. marsupialis*	Atlantic Forest	Guapimirim/ Rio de Janeiro	2000	MG228300	MG228328	MG228356	MG868978
G41	*D. marsupialis*	Atlantic Forest	Silva Jardim/ Rio de Janeiro	2003	MG228299	MG228327	MG228355	MG868977
762	*D. aurita*	Atlantic Forest	Silva Jardim/ Rio de Janeiro	unknown	MG228290	MG228318	MG228346	MG868968
5574	*D. aurita*	Atlantic Forest	Capitão Andrade/ Minas Gerais	2003	MG228284	MG228312	MG228340	MG868962
12640	*D. marsupialis*	Amazonia	Abaetetuba/ Pará	2008	MG228280	MG228308	MG228336	MG868958
10272	*D. marsupialis*	Amazonia	Cachoeira do Arari/ Pará	2006	MG228276	MG228304	MG228332	MG868954
12625	*D. marsupialis*	Amazonia	Abaetetuba/ Pará	2008	MG228279	MG228307	MG228335	MG868957
10290	*D. marsupialis*	Amazonia	Cachoeira do Arari/ Pará	2006	MG228277	MG228305	MG228333	MG868955
6737	*D. marsupialis*	Amazonia	Itupiranga/ Pará	2004	MG228286	MG228314	MG228342	MG868964
6716	*D. marsupialis*	Amazonia	Itupiranga/ Pará	2004	MG228285	MG228313	MG228341	MG868963
12667	*D. marsupialis*	Amazonia	Curralinho/ Pará	2009	MG228281	MG228309	MG228337	MG868959
12668	*D. marsupialis*	Amazonia	Curralinho/ Para	2009	MG228282	MG228310	MG228338	MG868960
6824	*D. albiventris*	Caatinga	Jaguaruana/ Ceará	2004	MG228289	MG228317	MG228345	MG868967
M3	*D. albiventris*	Caatinga	Coronel José Dias/ Piauí	1998	MG228302	MG228330	MG228358	MG868980
8648	*D. albiventris*	Caatinga	Jaguaruana/ Ceará	2005	MG228293	MG228321	MG228349	MG868971
8622	*D. albiventris*	Caatinga	Jaguaruana/ Ceará	2005	MG228292	MG228320	MG228348	MG868970
11639	*D. albiventris*	Caatinga	Russas/ Ceará	2008	MG228278	MG228306	MG228334	MG868956
3510	*D. albiventris*	Caatinga	Jaguaruana/ Ceará	2001	MG228283	MG228311	MG228339	MG868961
6812	*D. albiventris*	Caatinga	Jaguaruana/ Ceará	2004	MG228287	MG228315	MG228343	MG868965
6813	*D. albiventris*	Caatinga	Jaguaruana/ Ceará	2004	MG228288	MG228316	MG228344	MG868966
M1	*D. albiventris*	Caatinga	Coronel José Dias/ Piauí	1998	MG228301	MG228329	MG228357	MG868979
10171	*D. albiventris*	Caatinga	São Raimundo Nonato/ Piauí	2006	MG228275	MG228303	MG228331	MG868953
9149	*D. albiventris*	Cerrado	Aporé/ Goiás	2006	MG228294	MG228322	MG228350	MG868972
9425	*D. albiventris*	Cerrado	Aporé/ Goiás	2006	MG228295	MG228323	MG228351	MG868973
8552	*D. albiventris*	Cerrado	Aporé/ Goiás	2005	MG228291	MG228319	MG228347	MG868969

edited sequences were deposited in the GenBank database under the accession numbers MG228275–MG228358 and MG868953–MG868980 (Table 1).

Data analyses for MLST

Gene sequences were analyzed to investigate intralineage diversity, evidence of recombination via phylogenetic incongruence at the level of individual diplotypes and haplotypes, and also to determine the best combination and minimum combination of loci that enable identification of the maximum number of diploid sequence types (DSTs). MLSTest software [44] was initially applied to calculate the typing efficiencies (TE) and discriminatory power (DP) for each target. TE is defined as the number of identified genotypes divided by the number of polymorphic sites within the target locus [45]. DP is defined as the probability that two strains are distinguishable when chosen at random from a population of unrelated strains [39].

Trypanosoma cruzi is a minimally diploid organism and as such heterozygosity renders MLST analysis more difficult than in haploid organisms. As mentioned above, heterozygosity was inferred from electropherograms by a double peak (two bases) at the same variable bi-allelic site [46]. One consequence of multiple bi-allelic sites is the presence of ambiguous allelic phases within loci and also ambiguous combinations of alleles across separate loci. However, it is possible for diploid sequence data (without phase resolution) to be concatenated across multiple loci [38, 45] and subsequently subjected to distance-based phylogenetic methods for the study of lineage assignment and of recombination. Here we apply an average state methodology described by Diosque et al. [45]. In more detail, the genetic distance between

T and Y (heterozygosity composed of T and C) is considered as the mean distance between the T and the possible resolutions of Y (distance T-T = 0 and distance T-C = 1, average distance = 0.5, see Diosque et al. [45] and MLSTest software [44] for further details.

Incongruence between phylogenetic trees derived from individual gene targets was analyzed via incongruence length difference (ILD) tests, in MLSTest software [44]. ILD tests evaluate differences between expected and observed incongruences between loci in the context of random unstructured homoplasy. All combinations from 2 to 4 fragments were analyzed using the optimization algorithm scheme in MLSTest which identifies the minimum combination of loci producing the maximum number of DSTs.

Phylogenetic analyses, for individual and concatenated genes sequences were performed using neighbor-joining (NJ) trees, implemented in MLSTest v.1.0 [44]. NJ trees were constructed using uncorrected p-distances, considering heterozygous sites as average states. One thousand bootstrap replications were used to evaluate branch support.

To infer haplotypes for each gene, diploid sequence data were analized using PHASE software v.2.1 [47]. This program is based on a modified Markov chain Monte Carlo (MCMC) algorithm which identified and assembles all unambiguous haplotypes. Bayesian phylogenetic analysis was subsequently performed (MrBayes), implemented through TOPALi v.2.5 [48], using the best-fit model selected, Hasegawa-Kishino-Yano plus gamma distributed rate variation among sites (HKY + G), based on the Bayesian information criterion. Five MCMC runs were carried out in parallel for one million generations with sampling every 100 generations and 25% burn in.

Reference sequences were obtained from LSHTM collaborators, from Yeo et al. [38] comprising TcI [C8 cl1, SAXP18 cl1, 9210601P cl1, PI (CJ007), PII (CJ005)] and also Tu18cl2 (TcII) and GenBank sequence (X10/1, CP015651.1). In addition to phylogenetic incongruence between single gene trees, analysis of allelic recombination to detect allelic gene mosaics at the level of individual genes was undertaken. Isolates with unambiguous phases were applied through the software package RDP3 (recombination detection program) [49], incorporating the following methods: RDP [50], Bootscanning [51], GENECONV [52], Maximum Chi Square method [53, 54], the Chimaera method [53], the Sister Scanning Method [55], the 3SEQ method [56].

Results

Genetic diversity and discriminatory power of MLST by diploid sequence typing

We observed a significant genetic diversity in the context of single nucleotide polymorphisms (SNPs), as well as differences and variations in discriminatory powers (DP) of the four constitutive gene fragments under study (Table 2).

For individual genes, the number of polymorphic sites ranged from 4 (*DHFR-TS*) to 14 (*LYT1*) and the associated number of alleles ranged from 4 (*DHFR-TS*) to 14 (*LYT1* and *RB19*). The most resolutive marker, i.e. distinguishing most genotypes, was *RB19* (TE = 2), which also possessed the highest discriminatory power (DP = 0.92). *LYT1* and *DHFR-TS* were the least resolutive genes (TE = 1), with *DHFR-TS* also possessing the lowest DP value of 0.31. The DP of 4 concatenated targets by MLST was 0.993, discriminating 33 out 35 isolates.

Single locus phylogenies and MLST

Phylogenetic trees were generated for each marker (Additional file 1: Figure S1 and Additional file 2: Figure S2) to assess diversity and incongruence in topology. In more detail, *DHFR-TS* isolates formed a single cluster, indicative of limited variation, a finding in agreement with TE and DP figures for this target. All isolates grouped with reference sequences, with the exception of one isolate (8622) from Caatinga (Additional file 1: Figure S1). *RB19* revealed the presence of several clusters, although with relative low bootstrap support. Two clusters displayed bootstrap support > 50%: one containing 3 isolates from the Cerrado biome (9425, 8552, 9149), and the other cluster including 3 isolates from Amazonia (12667, 10272, 12625), one from the Atlantic Forest (G41) and 2 reference sequences (B187, X10). *LYT1* produced a single cluster containing all 10 isolates from Caatinga (bootstrap value = 61%) (Additional file 2: Figure S2). Here again, 8552 and 9149 samples from Cerrado grouped together (bootstrap value = 64%). Within *LYT1*, reference sequences X10 (Bolivian *T. infestans*), C8 (Bolivian *T. infestans*) and SAXP18 (Peruvian *Didelphis marsupials*) clustered with moderate and high support (bootstrap values of 87 and 98%, respectively). The *METIII* tree (Additional file 1: Figure S1) supported a single cluster (bootstrap support > 58%) and grouped the sample 12668 from the Amazon region and 6812 from Caatinga with the reference sequences 9210160 (*Didelphis marsupials*, US) and X10, respectively.

Trees generated with *LYT1* and *RB19* (Additional file 2: Figure S2) and *METIII* (Additional file 1: Figure S1) genes, also exhibited topological inconsistencies. For example, within *LYT1* the subgroup containing all isolates from the Caatinga biome was not observed in other trees. The Atlantic Forest isolate G41 was similarly positioned in both *LYT1* and *METIII* topologies within Amazonian, Cerrado and Atlantic Forest isolates. In contrast, G41 was positioned in a well-defined cluster for *RB19* which contained isolates solely from the Amazon region (bootstrap value of 53%) (Additional file 2: Figure S2).

Table 2 Measures of genetic diversity for 4 MLST housekeeping genes

Gene	No. of alleles	No. of polymorphisms	Typing efficiency (TE)	Discriminatory power (DP)
DHFR-TS	4	4	1	0.31
MET III	11	10	1.1	0.828
RB19	14	7	2	0.92
LYT1	14	14	1	0.89

Intra DTU I diversity

The resolutive power for all four genes was assessed by concatenation to produce a single phylogeny. Generally, clusters consisted of a mixture of isolates from geographically disparate regions with the exception of the isolates originating from the Caatinga biome. In more detail, five clusters with moderate bootstrap support were observed (Fig. 2).

Cluster a (bootstrap value of 56%) exclusively included isolates from the Caatinga biome, as observed previously with *LYT1*. Cluster b (bootstrap value of 62%) included isolates from very distant regions, including the Atlantic Forest (G41 - Rio de Janeiro) and Amazonia (12667 - Curralinho), indicating the wide distribution of this TcI genotype. Cluster c (bootstrap value of 51%) grouped Atlantic Forest isolates (Rio de Janeiro) and a single isolate from Cerrado (Goias) further evidencing the wide distribution of the TcI genotypes (Fig. 1). Cluster d (bootstrap value of 60%) (Fig. 2) was comprised of remaining isolates from the Cerrado biome. Lastly, cluster e (bootstrap value of 57%) included the reference sequences X10, C8 and SAXP, as previously observed in the *LYT1* phylogeny.

Applying measures of incongruence across all combinations reveal the four fragment datasets are significantly incongruent ($P < 0.05$). However, on excluding

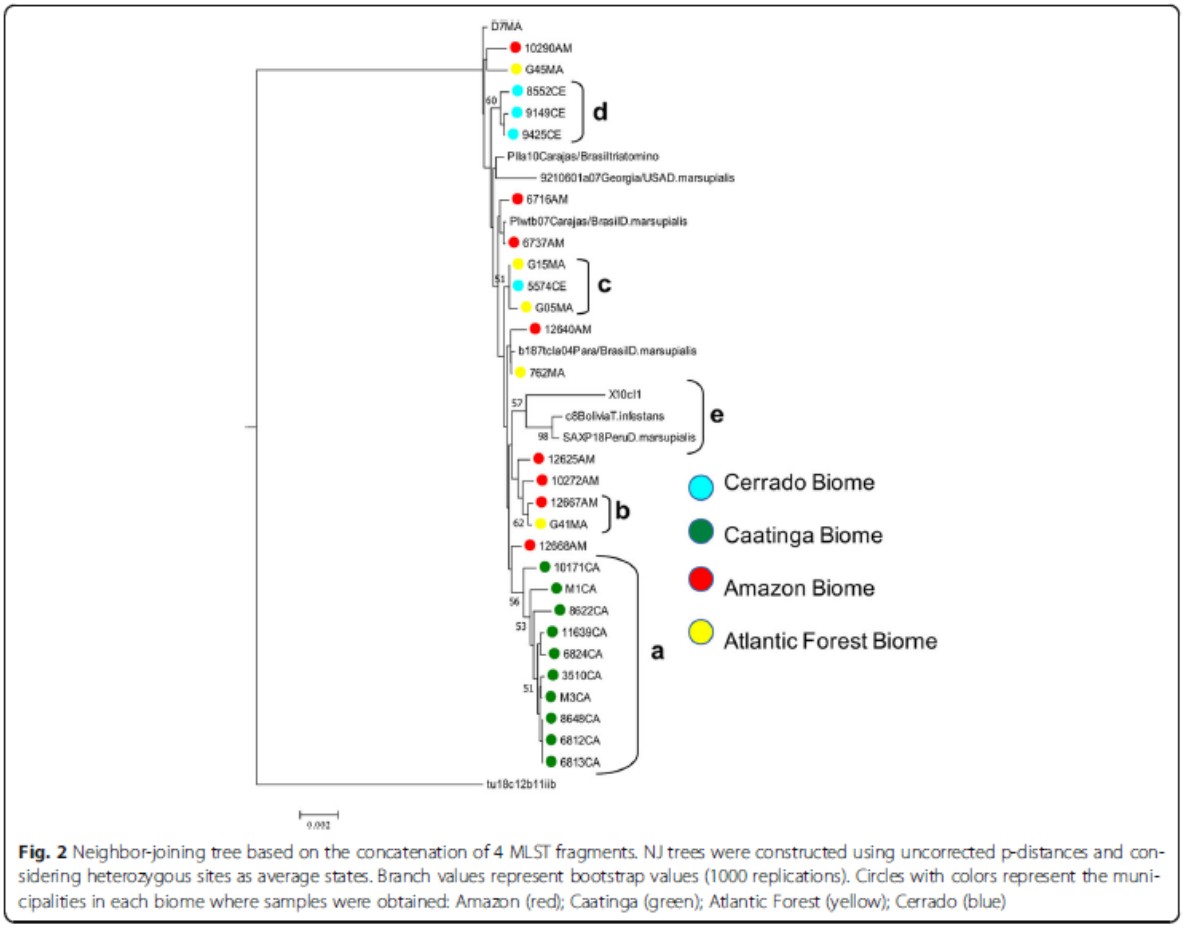

Fig. 2 Neighbor-joining tree based on the concatenation of 4 MLST fragments. NJ trees were constructed using uncorrected p-distances and considering heterozygous sites as average states. Branch values represent bootstrap values (1000 replications). Circles with colors represent the municipalities in each biome where samples were obtained: Amazon (red); Caatinga (green); Atlantic Forest (yellow); Cerrado (blue)

RB19 the *P*-value for ILD was not significant (BIONJ-ILD = 0.4) indicating that *DHFR-TS*, *LYT1* and *MET III* are broadly congruent (Fig. 3).

Intralineage recombination

To further assess evidence for recombination, the allelic origins for each target were investigated. Haplotypes and associated phylogenies were generated for each of the three gene fragments (*LYT1*, *METIII* and *RB19*). Based on these haplotypes we observed evidence of genetic recombination among isolates in two genetic targets.

The haplotypic trees for *METIII* revealed the putative homozygous donors genotypes, situated in different clusters and their corresponding heterozygous profiles (Fig. 4). Specifically, heterozygous isolate 12640 contained alleles that are consistent with putative donor haplotypes from homozygous isolates 12625 and 12668. Putative donor homozygous SNP profiles and the corresponding heterozygous profiles are presented in the Additional file 3: Table S1.

Secondly *LYT1* demonstrated heterozygous haplotypes for isolate 9149 and D7, which clustered with its putative homozygous donors 8552 and 9425, and 6737 and 6716, respectively (Fig. 5). Putative donor-homozygous SNP profiles and the corresponding heterozygous profiles are presented in the Additional file 4: Table S2. Although this gives evidence of recombination at the level of allelic inheritance, for example through the segregation of alleles, we did not observe evidence of allelic recombination (mosaic alleles) through the RDP approach.

Discussion

In this study, we evaluated the genetic diversity present in Brazilian TcI isolates, obtained from *Didelphis* spp. using an MLST approach. Our results confirmed the existence of significant genetic diversity within TcI in Brazilian didelphids [29, 32, 36]. Although we observed some evidence of geographical association in the Caatinga biome forming a single cluster (*LYT1* target) there was a general lack of population structure in association to any particular biome or habitat. Moreover, isolates originated from *Didelphis* spp. from geographically disparate areas and biomes possessed identical or similar genotypes.

MLST applied to 35 sequences (including reference sequences) indicated that the concatenation of the four gene fragments was highly discriminatory, identifying 33 out of 35 possible DSTs; however, a reduced panel of markers that included *DHFR-TS*, *METIII* and *LYT1* can discriminate 26 of 35 DSTs (Fig. 3). *LYT1* was the most polymorphic of the four fragments analyzed, in agreement with Ramirez et al. [36] and Yeo et al. [38]. However, a caveat to routine use of this target is the difficulty

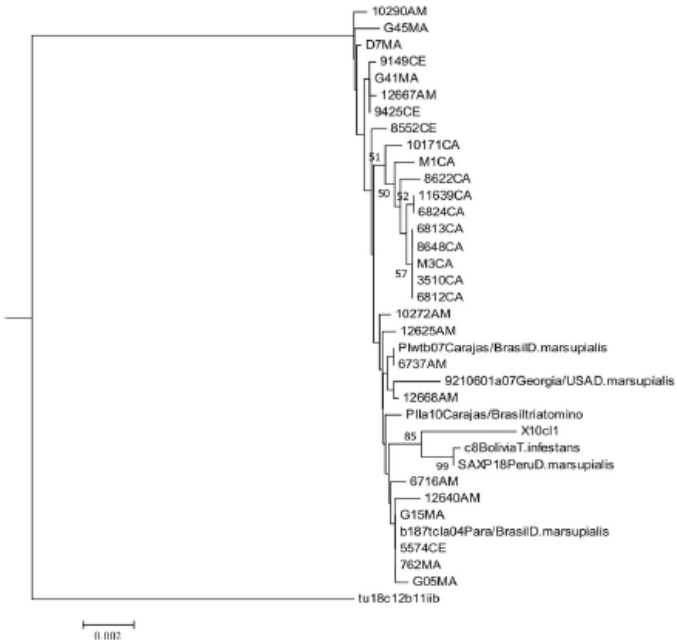

Fig. 3 Neighbor-joining phylogenetic reconstruction using the three nuclear genetic markers (*DHFR-TS*, *LYT1* and *METIII*) concatenated. The tree was constructed using uncorrected p-distances and considering heterozygous sites as average states

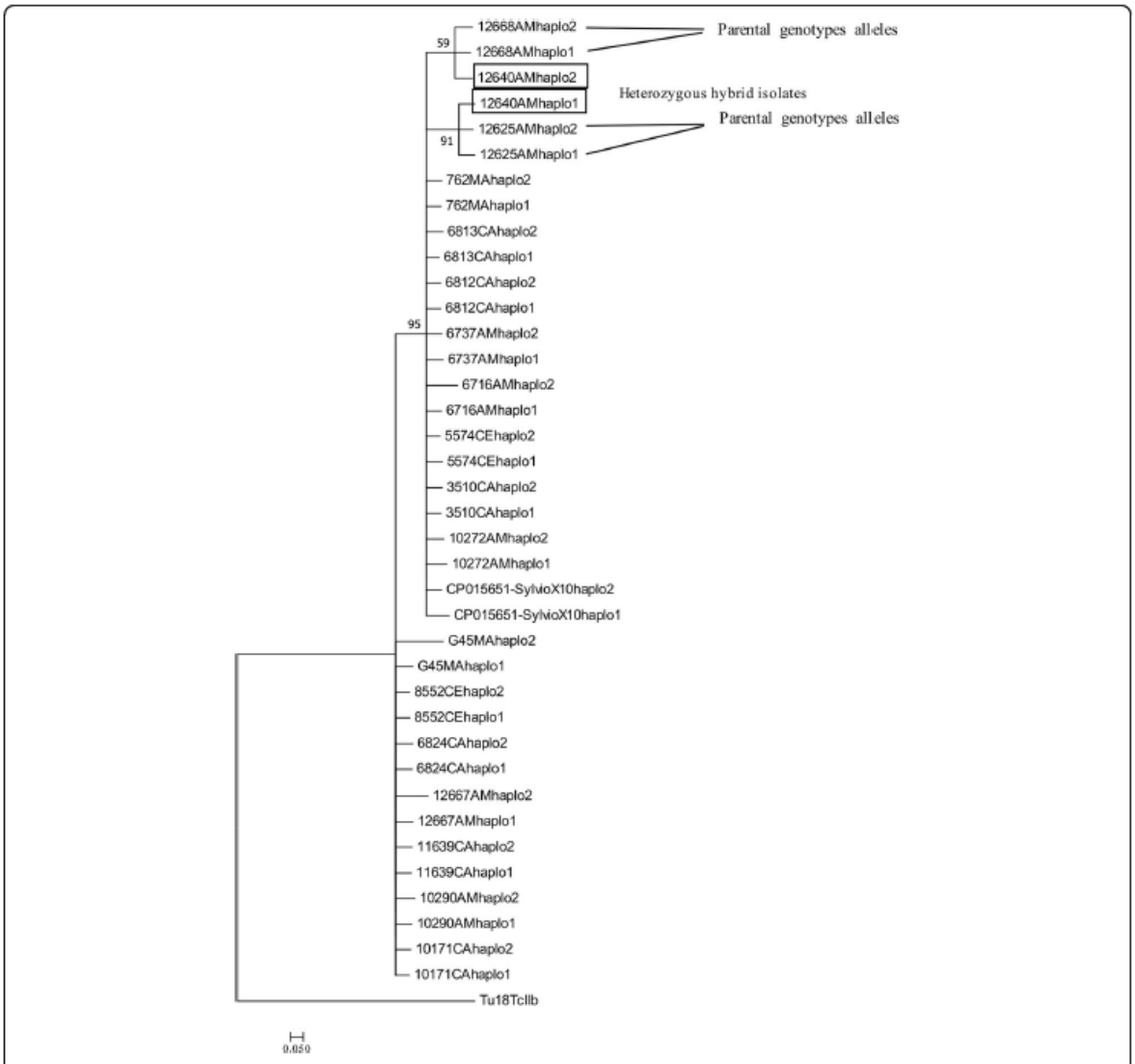

Fig. 4 Haplotype neighbor-joining tree with *MET III* locus. The tree based on haplotypes with the program PHASE v.2.1. Black circles indicate heterozygous hybrid isolates (12640) and black line potential parental genotypes alleles (12625–12668)

in optimizing reaction conditions to produce consistent quality sequence data, also observed by Yeo et al. [38].

RB19, with 7 polymorphic sites, possessed the highest typing efficiency (TE = 2), indicating that it is an excellent candidate for TcI diversity studies. Conversely, *DHFR-TS* was the least polymorphic. Yeo et al. [38] applied this marker for intralineage diversity studies finding it useful for discriminating DTUs TcVI and TcV. Despite the low number of SNPs we observed that the DP increased (0.25–0.31) with the inclusion of the *DHFR-TS*. However, in the context of a TcI specific cohort we suggest alternative, more discriminatory markers to *DHFR-TS*, be considered.

We also observed incongruences between phylogenetic trees, constructed with individual gene fragments, one explanation for which would be genetic recombination. Our data showed that isolates appeared in different clusters in the individual trees (Additional file 1: Figure S1 and Additional file 2: Figure S2). The existence of incongruences between constitutive gene trees has previously been observed in different lineages [19, 40, 45, 57] and it is considered a marker of populations that have undergone genetic recombination. The evidence for recombination is further supported by haplotypic phylogenies that infer allelic inheritance from homozygous donor genotypes. Importantly, for *MET III* and *LYT1*, heterozygous

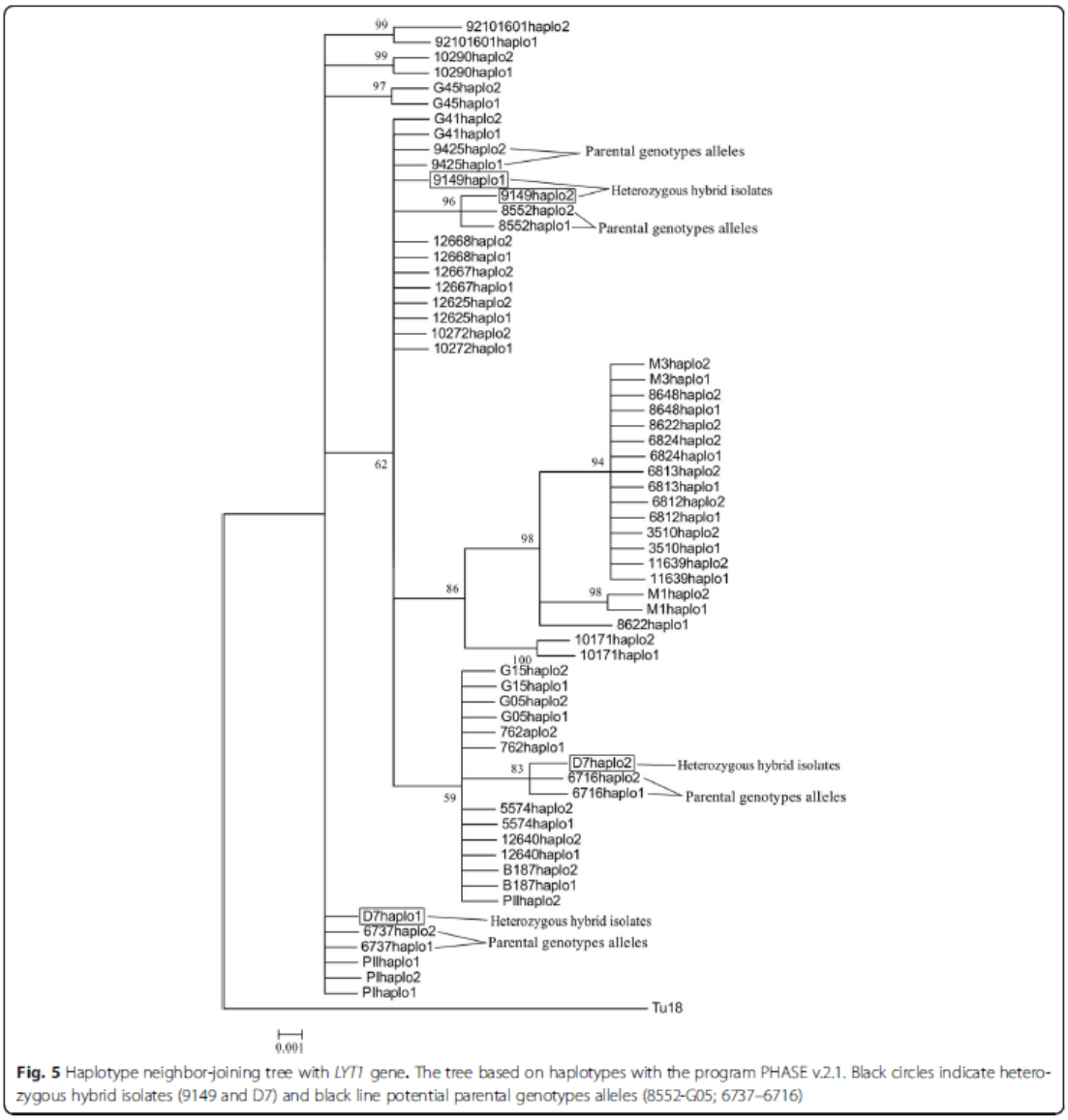

Fig. 5 Haplotype neighbor-joining tree with *LYT1* gene. The tree based on haplotypes with the program PHASE v.2.1. Black circles indicate heterozygous hybrid isolates (9149 and D7) and black line potential parental genotypes alleles (8552-G05; 6737–6716)

isolates were observed with their corresponding homozygous SNP donor genotypes (Additional file 3: Table S1 and Additional file 4: Table S2). We also examined haplotype sequences using various recombination detection algorithms (through RDP3), applied to individual alleles; we found no evidence of mosaics. As already observed by Yeo et al. [38], this result is not unexpected as the allelic recombination (mosaics) may be a rare event in comparison to inheritance by segregation of allelic haplotypes from and homozygous parental donors [38, 46]. TcV and TcVI, known recombinants of TcII and TcIII, were originally characterized by SNP distribution, in this way. Recombination in Tcl has been proven experimentally in a landmark publication [58], and in natural populations [20, 35, 41]. The frequency of recombination is unknown and may be common [41, 59] or rare [60, 61]. To obtain more refined results in the future, it would be important to include a much larger cohort of isolates with further cloning for downstream recombination analysis.

Concatenation of the four genes resulted in clustering all Caatinga isolates and excluded isolates from the other biomes, suggesting a geographical association at some level. It is worth noting that the area in question is rather extended, e.g. the municipalities of Jaguaruana and Sao Reimundo Nonato are separated by a distance of nearly 900 km (much more than the typical hosts displacement) and numerous barriers to hosts movement, including cities and roads [62]. The presence of similar genotypes over a wide area is in agreement with previous studies [63].

We generally observed similar genotypes spanning vast geographical distances, which is likely indicative of host/vector dispersion or clonal propagation over time against a background of intermittent recombination. As a further case in point, genetically similar genotypes were also present in both Atlantic Forest and Amazonia (cluster b, Fig. 2) forming a discrete subcluster, despite the corresponding localities being separated by a huge geographical distance. Amazonian isolates 12625 (Abaetetuba) and 10272 (Cachoeira do Arari) were clustered together despite their localities being separated by Bahia de Marajo, providing further evidence that similar genotypes span substantial distances across geographical barriers (Fig. 2). From our data, it is clear that different genotypes circulate sympatrically and infect multiple *Didelphis* spp. Of note, some of the localities, such as Abaetetuba and Curralinho in the state of Para, correspond to outbreak areas of Chagas disease [64]. Noticeably, Abaetetuba (Amazonian 12640, 12625) has had several outbreaks of Chagas disease reported [65]. Moreover, Llewellyn et al. [36] and also Ramirez et al. [35] identified specific genotypes involved in domestic and sylvatic transmission cycles in Venezuela (by microsatellites) and Colombia (by MLST), respectively. However, to investigate this possibility in Brazil a larger cohort of isolates originated from vectors, mammals, sylvatic and domestic isolates would be required.

We also observed local genotypic diversity as all isolates from the Cerrado biome ($n = 4$) were obtained from the same municipality (Apore), yet, only three of them grouped in the same cluster indicating localized diversity (Fig. 2). This phenomenon was observed by Lima et al. [63] who also detected extensive genetic diversity, in mainly sylvatic hosts, by the application of microsatellite analysis to isolates from the Cerrado biome (Tocantins), in which case these isolates clustered with isolates from the Atlantic Forest. Hence, the recurring theme from TcI in didelphids, presented here and also from also other TcI studies in Brazil, is one of extensive diversity but also with genetically similar isolates being present in disparate regions.

The presence of multiclonality in single hosts is known to occur [60, 66] and is a potential confounder for the genetic analysis from DNA from uncloned isolates. However, evidence from this particular cohort suggests that multiclonality can be ruled out as an explanation of the observed results. Specifically, as stated above, RDP analysis testing for recombination at the level of alleles (intra-allelic recombination) did not detect allelic mosaics in the clonal controls or the field isolates, which would likely be observed in the presence of mixed clones in individual isolates. Moreover, the evidence for recombination is derived from discrete allelic contributions from potential donor homozygous genotypes (and not allelic mosaics), further supporting the case for genetically discrete isolates over that of admixtures. Finally, these data show that similar genotypes span huge geographical distances. This is also suggestive of genetically discrete isolates and not admixtures of different clones. Together these results provide robust evidence for genetic diversity between discrete isolates within TcI.

The main epidemiological implication of our findings, is that there is no observed association between the distribution of subpopulations of TcI and the appearance of outbreaks of CD. Moreover, in Brazil, TcI has been classically associated with the sylvatic transmission cycle. This is, in part, due to its high prevalence in mammals and its high dispersion [42]. However, TcI has also been detected in human cases [67], in which it originated from sylvatic mammals including *Didelphis* spp. These marsupials are found in all forest strata in all biomes of Brazil. Additionally, they are known to dwell habitats in the proximity of human activity. These facts, together with their ability to harbour diverse TcI genotypes as seen in this study, clearly imply that understanding the role of the didelphids is of central importance to a more thorough elucidation of the TcI transmission cycles in Brazil.

Conclusions

We conducted a MLST study using four nuclear genes applied to a panel of TcI isolates obtained from three didelphid hosts and spanning four ecologically disparate Brazilian biomes. Our results revealed considerable intra DTU genetic diversity using a sensitive panel of MLST markers and demonstrated a lack of clear associations of TcI genotypes to geographical location or transmission cycle. The one exception are isolates from Caatinga which clustered together. These data suggest that multiple TcI genotypes circulate sympatrically in mammalian host species, transmission cycles and probably insect vectors. We also infered the presence of intralineage genetic recombination by SNP distribution patterns and phylogenies at two loci (*METIII* and *LTY1)* in two isolates derived from *D. marsupialis* and *D. albiventris*, thus substantiating the important role of didelphids in TcI transmission in Brazil.

Additional files

Additional file 1 Figure S1 Phylogenetic incongruence between individual nuclear markers applied to 35 TcI Brazilian isolates. a NJ phylogenetic reconstruction using *DHFR-TS*. b NJ phylogenetic reconstruction using *METIII*. (PDF 122 kb)

Additional file 2 Figure S2 Phylogenetic incongruence between individual nuclear markers applied to 35 TcI Brazilian isolates. a NJ phylogenetic reconstruction using *LYT1*. b NJ phylogenetic reconstruction using *RB19*. (PDF 122 kb)

Additional file 3 Table S1 SNP data showing putative donor and recipient isolates for *METIII*. Sequences containing heterozygous SNPs (R) and putative homozygous donor (D) genotypes. (XLSX 12 kb)

Additional file 4 Table S2 SNP data showing putative donor and recipient isolates for *LYT1*. Sequences containing heterozygous SNPs (R) and putative homozygous donor (D) genotypes. (XLSX 8 kb)

Abbreviations

aCD: acute Chagas disease; ColTryp: Coleção de Trypanosomas de mamiferos silvestres, domesticos e vetores; *DHFR-TS*: Dihydrofolate reductase-thymidylate synthase; DP: Discrimination power; DST: Diploid sequence types; DTU: Discrete typing units; ILD: Incongruence length difference; ITS: Internal transcribed spacer; *LYT1*: Lytic pathway protein; MCMC: Markov chain -Monte Carlo; *METIII*: Metacyclin-III; MLEE: Multilocus enzyme electrophoresis; MLST: Multilocus sequence typing; NJ: Neighbor-joining; PCR: Polymerase chain reaction; RAPD: Random amplification of polymorphic DNA; *RB19*: RNA-binding protein-19; RDP: Recombination detection program; SL-IR: Spliced lader intergenic region; SNP: Single nucleotide polymorphism; TcI: *Trypanosoma cruzi* I; TE: Typing efficiency

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Availability of data and materials

The newly generated sequences were submitted to the GenBank database under the accession numbers MG228275–MG228302 (*DHFR-TS*); MG228303–MG228330 (*RB19*); MG228331-MG228358 (*LYT1*) and MG868953-MG868980 (*METIII*).

Authors' contributions

MY designed the experiments. FR performed the experiments and wrote the first version of manuscript. AMI, AMJ and FR analyzed the results. FR, AMI, AMJ and MY contributed to the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable. No special permission was required for the present study. We used DNA extracted from the cultures obtained from animals collected during previous field expeditions conducted by our group.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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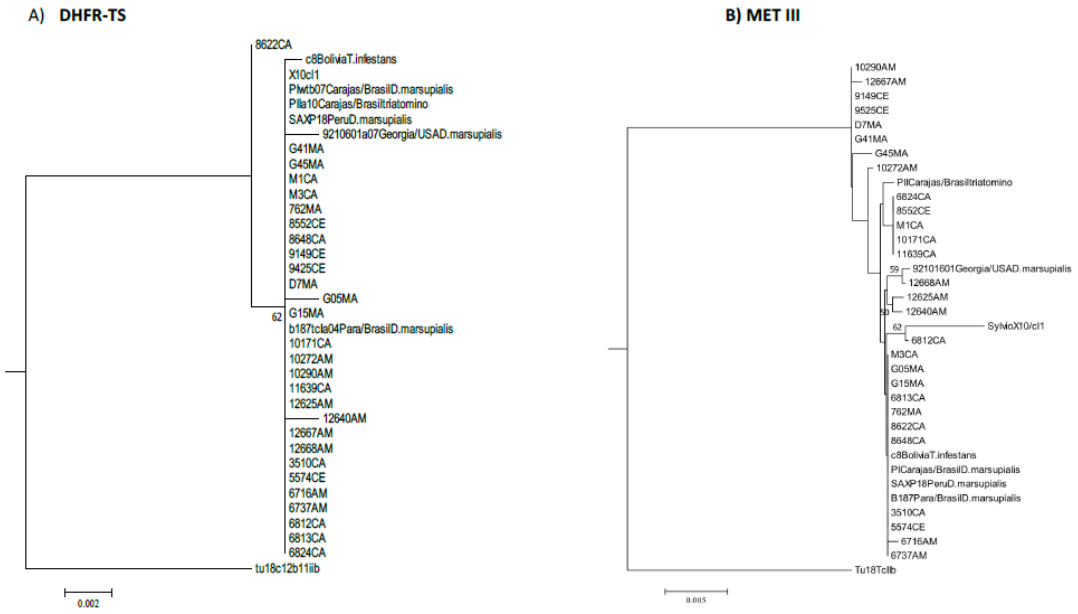
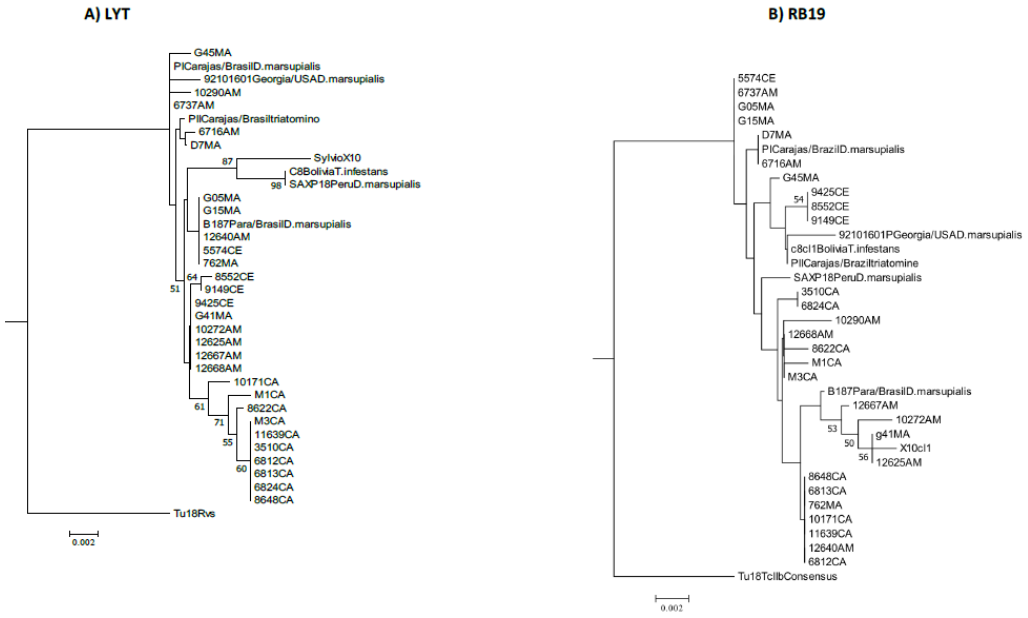
Additional file 1: Figure S1: Phylogeny incongruence between nuclear markers applied to 35 TcI Brazilian isolates. A. NJ phylogenetic reconstruction using *DHFR-TS* gene B. NJ phylogenetic reconstruction using *METIII* gene.



Additional file 2: Figure S2: Phylogeny incongruence between nuclear markers applied to 35 TcI Brazilian isolates. A. NJ phylogenetic reconstruction using *LYT* gene B. NJ phylogenetic reconstruction using *RB19* gene



Additional file 3: Table S1. SNP data showing putative donor and recipient isolates for *METIII* . Sequences containing heterozygous SNP's (R) and putative homozygous donors (D)

	Strain	1	2	3	4	5	6	7
	c8 Bolivia T. infestans	G	T	T	T	T	C	C
	PI Carajas/Brasil D. marsupialis	G	T	T	T	T	C	C
	PII Carajas/Brasil triatomino	G	K	T	T	Y	C	C
	SAXP18 Peru D. marsupialis	G	T	T	T	T	C	C
	9210601 Georgia/USA D. marsupialis	G	K	T	T	T	C	T
	b187 tcI Para/Brasil D. marsupialis	G	T	T	T	T	C	C
	10171 CA	G	G	T	T	T	C	C
	10272 AM	A	T	T	T	T	C	C
	10290 AM	A	G	T	T	T	C	C
	11639 CA	G	G	T	T	T	C	C
D	12625 AM	G	T	T	T	T	C	C
R	12640 AM	G	T	T	T	T	C	C/T
	12667 AM	A	G	T	W	T	C	C
R	12668 AM	G	T	T	T	T	C	T
	3510 CA	G	T	T	T	T	C	C
	5574 CE	G	T	T	T	T	C	C
	6716 AM	G	T	T	T	T	Y	C
	6737 AM	G	T	T	T	T	C	C
	6812 CA	G	T	T	T	T	C	C
	6813 CA	G	T	T	T	T	C	C
	6824 CA	G	G	T	T	T	C	C
	762 MA	G	T	T	T	T	C	C
	8552 CE	G	G	T	T	T	C	C
	8622 CA	G	T	T	T	T	C	C
	8648 CA	G	T	T	T	T	C	C
	9149 CE	A	G	T	T	T	C	C
	9425 CE	A	G	T	T	T	C	C
	D7 MA	A	G	T	T	T	C	C
	G05 MA	G	T	T	T	T	C	C
	G15 MA	G	T	T	T	T	C	C
	G41 MA	A	G	T	T	T	C	C
	G45 MA	A	G	Y	T	T	C	Y
	M1 CA	G	G	T	T	T	C	C
	M3 CA	G	T	T	T	T	C	C

Additional file 4: Table S2. SNP data showing putative donor and recipient isolates for *LYT1* . Sequences containing heterozygous SNP's (R) and putative homozygous donors (D). The first four sequences are reference sequences.

	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	PI Carajas/Brasil D. marsupialis	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	G
	PII Carajas/Brasil triatomino	C	G	A	A	C	C	A	A	A	T	G	Y	A	C	C	A	R
	92101601 Georgia/USA D. marsupialis	C	G	T	A	C	C	A	A	A	T	R	C	A	C	C	A	G
	B187 Para/Brasil D. marsupialis	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	A
	10171 CA	T	G	A	T	C	C	A	A	A	T	G	C	A	C	C	A	A
	10272 AM	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	A
	10290 AM	C	G	A	A	C	C	A	A	A	T	G	C	A	T	C	A	G
	11639 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A
	12625 AM	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	A
	12640 AM	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	A
	12667 AM	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	A
	12668 AM	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	A
	3510 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A
	5574 CE	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	A
	6716 AM	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	G
	6737 AM	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	G
	6812 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A
	6813 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A
	6824 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A
	762 MA	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	A
D	8552 CE	C	G	A	A	C	C	A	A	A	C	G	C	A	C	C	A	A
	8622 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	Y	A	R
	8648 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A
R	9149 CE	C	G	A	A	C	C	A	A	A	T/C	G	C	A	C	C	A	A
D	9425 CE	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	A
	D7 MA	C	G	A	A	C	C	A	A	A	T	G	Y	A	C	C	A	G
	G05 MA	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	A
	G15 MA	C	G	A	A	C	C	A	A	A	T	G	T	A	C	C	A	A
	G41 MA	C	G	A	A	C	C	A	A	A	T	G	C	A	C	C	A	A
	G45 MA	C	A	A	A	C	C	A	A	A	T	G	C	A	C	C	A	G
	M1 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	C	G	A
	M3 CA	T	G	A	A	T	C	A	A	A	T	G	C	A	C	T	A	A

Artigo 2. Dissecting the phyloepidemiology of *Trypanosoma cruzi* I (TcI) in Brazil by the use of high-resolution genetic markers.

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O estudo da diversidade genética de TcI foi aprofundado neste trabalho através da avaliação de marcadores com taxa de evolução rápida (genes de microssatélites e gene do maxicírculo), além dos genes de evolução lenta como genes constitutivos. As análises foram aplicadas a isolados de TcI obtidos de diferentes hospedeiros e vetores de 5 biomas brasileiros. Neste trabalho foi observada alta diversidade genética de TcI e uma ausência de associações estritas de *clusters* com específicos hospedeiros-vetores ou biomas. No entanto, algum grau de associação de um cluster de MLMT estava presente em isolados do bioma Amazônia. Nenhuma outra relação evidente entre clusters e biomas foi identificada; no entanto, os testes estatísticos são consistentes com a possibilidade de alguma forma de associação. Além disso, foi observada a presença de eventos de introgressão mitocondrial em isolados TcI e evidências de intercâmbio genético intra-DTU.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Dissecting the phyloepidemiology of *Trypanosoma cruzi* I (TcI) in Brazil by the use of high resolution genetic markers

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Data Availability Statement: Data are all contained within the paper and/or Supporting Information. Sequence data are available with the following accession numbers in GenBank (https://www.ncbi.nlm.nih.gov/genbank/): MF781085-MF781124; MF615620 - MF615679; MF615680 - MF615739; MF615740 - MF615799; MF615800 - MF615859; MF615860 - MF615919; MF615920 - MF615979.

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Abstract

Background

Trypanosoma cruzi, the causal agent of Chagas disease, is monophyletic but genetically heterogeneous. It is currently represented by six genetic lineages (Discrete Typing Units, DTUs) designated TcI-TcVI. TcI is the most geographically widespread and genetically heterogeneous lineage, this as is evidenced by a wide range of genetic markers applied to isolates spanning a vast geographic range in Latin America.

Methodology/Principal findings

In total, 78 TcI isolated from hosts and vectors distributed in 5 different biomes of Brazil, were analyzed using 6 nuclear housekeeping genes, 25 microsatellite loci and one mitochondrial marker. Nuclear markers reveal substantial genetic diversity, significant gene flow between biomes, incongruence in phylogenies, and haplotypic analysis indicative of intra-DTU genetic exchange. Phylogenetic reconstructions based on mitochondrial and nuclear loci were incongruent, and consistent with introgression. Structure analysis of microsatellite data reveals that, amongst biomes, the Amazon is the most genetically diverse and experiences the lowest level of gene flow. Investigation of population structure based on the host species/genus, indicated that *Didelphis marsupialis* might play a role as the main disperser of TcI.

Conclusions/Significance

The present work considers a large TcI sample from different hosts and vectors spanning multiple ecologically diverse biomes in Brazil. Importantly, we combine fast and slow evolving markers to contribute to the epizootiological understanding of TcI in five distinct Brazilian biomes. This constitutes the first instance in which MLST analysis was combined with the use of MLMT and maxicircle markers to evaluate the genetic diversity of TcI isolates in

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Brazil. Our results demonstrate the existence of substantial genetic diversity and the occurrence of introgression events. We provide evidence of genetic exchange in TcI isolates from Brazil and of the relative isolation of TcI in the Amazon biome. We observe the absence of strict associations with TcI genotypes to geographic areas and/or host species.

Author summary

T. cruzi is a zoonotic protozoan parasite infecting mammals and widely dispersed throughout endemic Latin America. It is known to possess considerable genetic diversity, comprising six discrete genetic lineages designated Discrete Typing Units (DTUs) TcI-TcVI. TcI is the most genetically diverse DTU and the most frequently sampled lineage in Brazil. We use a combination of high resolution molecular techniques to analyze the genetic diversity of Brazilian TcI isolates obtained from a wide geographical area encompassing five distinct biomes isolated from different mammal hosts and insect vectors. The results reveal significant genetic diversity and no clear association of genotypes with areas or host/vector species. Evidence from incongruent phylogenetic topologies based on nuclear and mitochondrial markers are indicative of genetic exchange and/or introgression events. The relevance of these findings in the context of population structure, ecology and epizootiology is discussed.

Introduction

Trypanosoma cruzi, a protozoan parasite (Kinetoplastidea: Trypanosomatidae), is known to possess a complex epidemiology and is widely distributed from the southern states of the United States of America to the Argentinian Patagonia. *T. cruzi* is a pervasive zoonosis capable of affecting more than 150 domestic and wild mammal species, distributed across 8 orders. *T. cruzi* infection in humans, may result in Chagas disease [1–3].Transmission to humans is mainly vectorial in endemic areas and over 100 species of hematophagous triatomine insects can harbor the parasites [4, 5]. Moreover, migration of individuals from highly endemic regions to the United States and Europe has resulted in significant public health concerns in recipient countries [6]. Domestic transmission of Chagas disease (CD) in Brazil by *Triatoma infestans* has been successfully interrupted [7]. However, human infection by *T. cruzi* is re-emerging as a food-borne disease in previously non-endemic areas [8–10]. Annual outbreaks have occurred, particulary in the northern Brazilian Amazon region during the past decade. Here, some local products derived from fruit juice have been contaminated with infected feces of triatomine bugs of different genera [9, 11, 12].

T. cruzi is characterized by a remarkable genetic heterogeneity [13, 14] and is currently comprised of six lineages or discrete typing units (DTUs), designated TcI to TcVI [15, 16]. In addition, recent evidence also supports the existence of a seventh lineage (TcBat) associated to bats [17]. The most genetically distant DTUs are TcI and TcII [18]. The evolutionary origins of TcIII and TcIV was initially proposed to be the result of an old hybridization between TcI and TcII [19], however more recent evidence shows that TcIII and TcIV have no hybrid origin, but rather are a monophyletic group with TcI that diverged from TcII [20, 21]. TcV and TcVI are known hybrid lineages which share haplotypes with TcII and TcIII [22, 23]. Whether given subpopulations of the parasite are associated with particular vector or host species or with distinct human disease characteristics is still unresolved.

TcI is the most frequently isolated DTU in the sylvatic environment, infecting diverse host and vector species across the Americas with an ancestral parental origin estimated at ~0.5–0.9 MYA [24, 23]. In Brazil, it is also the most widely distributed DTU, in terms of geography and diversity of host and vector species. Furthermore, it is, by far, the most genetically diverse DTU [25–30]. Llewellyn et al. [31] applied Multilocus Microsatellite Typing (MLMT) to the study of TcI population substructure in samples that originated from eight countries, isolated from 18 host and vector species, across 48 tandem repeats [32]. Results revealed extensive intra DTU diversity and spatial structuring of specific genotypes associated with acute oral outbreaks or vectorial infections in Venezuela. In addition, remarkable genetic diversity, through multiclonality, was observed when a single *Didelphis* reservoir host of TcI was studied [33].

Attempts to subdivide TcI strains into epidemiologically relevant groups are ongoing [34]. Herrera et al. [28] and Cura et al. [35] described five haplotypes associated with transmission cycles in Colombia, Chile and Bolivia. Ramirez et al. [36], used MLST to identify TcI genotypes specifically associated with human infection (TcI_{DOM}) and others associated with peridomestic/sylvatic areas. MLST exploits nucleotide diversity present in four to ten single-copy housekeeping genes and has previously been applied to the study of *T. cruzi* using different marker combinations for lineage assignment and intraspecific characterization [18, 37]. Evidence for genetic exchange in TcI has been reported, for example, in strains isolated from *Didelphis marsupialis* and *Rhodnius prolixus* in the Amazon Basin [38] and in a domestic/peridomestic TcI population in Ecuador [39]. Experimental generation of intra-lineage hybrids suggest that TcI also displays a potential for genetic exchange [40].

Mitochondrial DNA in *T. cruzi* has a unique structure and function consisting of approximately 20–50 maxicircles (~20kb) and thousands of smaller minicircles (~1.4kb) [38]. Maxicircle DNA is uniparentally inherited and represents a useful taxonomic marker as it is highly mutable in comparison to nuclear DNA. Messenger et al. [41] developed a high resolution maxicircle multilocus sequence typing (mtMLST) scheme to describe intra-DTU diversity in TcI, revealing multiple mitochondrial introgression events and heteroplasmy within South American TcI. Introgression had already been detected in North America [21, 22] and in Brazil [42] and also Bolivia [43].

Together these studies illustrate several remarkable characteristics of TcI, namely the immense geographic distribution, diversity of host and vector species, extensive genetic diversity, and the capacity for genetic exchange. However, little is known about TcI in Brazil and extraordinarily there is only one relevant Brazil centric publication specifically addressing diversity of TcI [42]. Unlike Colombia and Venezuela, in Brazil there is no evidence of population substructure in the context of geographical distribution of intra DTU genotypes, distribution of host/vector species, or genotypes associated with acute outbreaks of CD in Brazil. In the present work, we comprehensively analyze a large cohort of Brazilian TcI isolates from five ecologically disparate biomes. Through the use of high resolution nuclear markers (MLST and MLMT) and a maxicircle region (*COII*), we investigate the phyloepizootiology of TcI from different Brazilian biomes. The study described herein was conducted with the following major hypothesis: DTU I of *T. cruzi* in Brazilian isolates displays extensive heterogeneity with no particular association of subpopulations to geographic areas, or host/vector species.

Materials and methods

Parasite isolates

A total of 78 TcI isolates were supplied by Coleção de *Trypanosoma* sp de Mamíferos Silvestres, Domésticos e Vetores COLTRYP/FIOCRUZ. deposited by several researchers and maintained in liquid nitrogen. DNA was extracted immediately following initial isolation in NNN medium

and one round of expansion in LIT. The isolates had previously been confirmed as TcI using Mini-Exon PCR [44].

In this work, TcI isolates were characterized using three high resolution methods comprising MLST, MLMT and maxicircle sequencing (COII) using appropriate reference isolates. Full isolate details are shown in S1 Table and include characterization methods applied to each sample, isolate localities and collection dates. To increase the robustness of the results, microsatellite information from 50 additional isolates, published by Lima et al. [42], was included in our MLMT analyses.

Isolates originated from vector and mammalian reservoir hosts across five Brazilian biomes; namely Atlantic Forest, Amazon, Caatinga, Cerrado, and Pantanal (Fig 1 and S1 Table).

The Cerrado biome is primarily open scrubland (savannah) covering approximately 2 million km^2 of Central Brazil, comprising 23% of the total land surface area [45]. Scrubland is interspersed with gallery forests and is seasonally dry but with permanent swamplands dominated by *Mauritia flexuosa* palm trees [46].

The Pantanal biome is a large seasonal floodplain covering approximately 140000 km^2 at the core of South America [47]. It is a biodiversity hotspot and freshwater ecosystem of global significance containing diverse mammal species and habitats. Climate instability results in periodic floods and droughts, affecting the population number and behavior of some species [48].

The **Amazon** biome is in the largest hydrographic basin of the world, comprising 44% of the South American subcontinent. The biome is a complex mosaic of very diverse ecosystems, dominated by tropical rain forests, with semi-arid regions, and a variety of man-made landscapes. The Amazon biome contains the greatest biological diversity (in absolute terms) on the planet [49].

The Atlantic Forest biome extends from the south of Pernambuco to the south of Rio Grande do Sul, and it is characterized by humid tropical forest. This biome is extensively impacted by human activities. It originally encompassed 12 percent of the national territory but only 1 to 5 percent (less than 100,000 km^2) is intact today [50]. Containing more than 8,000 endemic species, the Atlantic Forest is recognized as one of the world's most significant biodiversity hotspots [51].

The Caatinga biome in northeast Brazil, comprises a semi-arid ecological landscape with only 1% of its territory currently conserved, it is threatened by agriculture and cattle ranching [52]. This biome is characterized by clay and sandy soils with open plains supporting flora that is typical of semi-arid regions [53].

Multilocus sequence typing (MLST)

MLST Loci. Ten housekeeping gene fragments were initially considered with the objective of detecting intra-lineage variation within TcI. Fragments were selected from Yeo et al. [37] and Diosque et al. [18] using the following rationale. Firstly, those known to be phylogenetically informative for TcI based on typing efficiency and discriminatory power [18, 37]; secondly, reliable PCR amplification, and thirdly consistent sequencing quality in both 5' and 3' directions. Two genes previously described by Yeo et al. [37]: mitochondrial peroxidase (*TcMPX)* and RNA-binding protein-19 (*RB19*) and eight housekeeping genes from Lauthier et al. [54]: glutathione peroxidase (*GPX*), 3-hydroxy-3-methylglutaril-CoA reductase (*HMCoAR*), pyruvate dehydrogenase component E1 subunit alfa (*PDH*), small *GTP*-binding protein Rab7 (*GTP*), Rho-like *GTP*-binding protein (*RHO1*), glucose-6-phosphate isomerase (*GPI*), superoxide dismutase B (*SODB*) and leucine aminopeptidase *(LAP*) were chosen for initial evaluation. Post screening, four of the ten targets (*TcMPX*, *SODB*, *GPX* and *GPI*) were

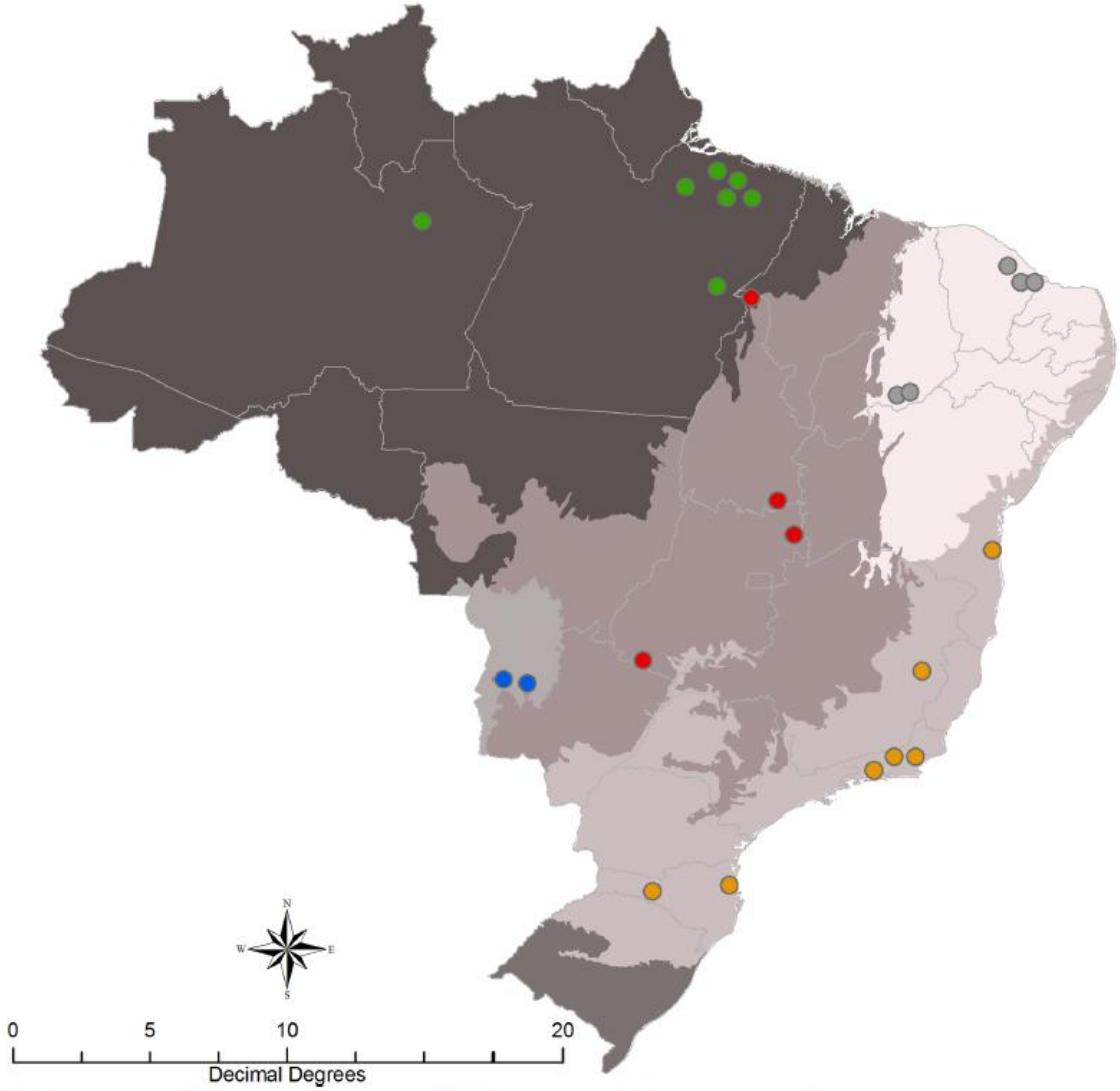

Fig 1. Map of the spatial distribution of TcI isolates used in the study. Colours represent the different biomes where samples were obtained: Green: Amazon; Orange: Atlantic Forest; Red: Cerrado. Blue: Pantanal. Grey: Caatinga.

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discarded due to non-specific PCR amplification products and/or inconsistent sequence quality. The ratio of non-synonymous to synonymous amino acid changes (dN/dS) was calculated according to the Nei-Gojobori method [55] to infer relative selection pressures, where genes possessing a dN/dS ratio <1 meet the criteria for stabilizing selection for the conservation of metabolic function. Taylor & Fisher [56] recommend incorporating some loci with a dN/dS ratio of >1 in order to obtain sufficient sequence diversity. Chromosome location and other

characteristics for each locus are shown in S2 Table. Additionally, we employed the FEL (Fixed Effects Likelihood) analyses [57], through the HiPhy application [58] via the Datamonkey web server [59] to infer selection pressure. Briefly, FEL analyses yields nonsynoymous (dN) and synonymous (dS) substitution rates on a per-site basis (assuming a significance level of 10% to all comparisons) for a given coding alignment and corresponding phylogeny through a maximum-likelihood approach.

MLST sequencing. PCR reactions were carried out in 20 uL reaction volumes containing 20 ng of DNA; 0.2 uM of each primer (S2 Table), 1U of Taq polymerase (Bioline, London, UK), 5X reaction buffer (Bioline, London, UK), 0.2 mM of each dNTPs (New England Biolabs, Hitchin, UK) and 1.5 mM MgCl2 solution (Bioline, London, UK). Amplification conditions for all targets were: 5 min at 94˚C followed by 35 cycles of 94˚C for 1 min; 55˚C for 1 min, and 72˚C for 1 min, with a final extension at 72˚C for 5 mins. Products were visualized on 2% agarose gels, stained with ethidium bromide, followed by electrophoresis. Bi-directional sequencing was performed with Big Dye Terminator Cycle Sequencing V3.1 (Applied Biosystems, Foster City, CA) and ABI PRISM 3730 DNA Sequencing (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Sequences were aligned with BioEdit v7.1 [60] and DNASTAR Lasergene SeqMan v. 7.0 [61]. The presence of two coincident peaks at the same locus was verified in forward and reverse sequences and scored using IUPAC nomenclature.

MLST data analysis. Different approaches were used to analyze MLST data. Firstly, for each locus, typing efficiency (TE) and discriminatory power (DP) were assessed (MLSTest software, http://ipe.unsa.edu.ar/software) [62] to determine the resolution of individual markers. Following this, both diplotypic and haplotypic phylogenies were constructed. Diplotypic phylogenetic trees were initially constructed to investigate isolate diversity and enable concatenation across loci, as described by Yeo et al. [37] and Tavanti et al. [63], which renders diplotypic data suitable for use in distance based dendrograms. Briefly, individual loci between isolates can be considered either homozygous or heterozygous, assuming that *T. cruzi* is minimally diploid. For example, a homozygous locus scored as A (adenine) was modified by duplication to AA, and a heterozygous locus, for example Y (C or T, in accordance with IUPAC nomenclature), scored as CT, effectively creating a difference matrix across the panel. Phylogenies were also constructed from heterozygous SNPs were considered as average states. In more detail, the genetic distance between T and Y (heterozygosity composed of T and C) is considered as the mean distance between the T and the possible resolutions of Y (distance T-T = 0 and distance T-C = 1, average distance = 0.5 [18, 62].

Suitability of targets for inclusion into an MLST scheme was assessed via Incongruence Length Difference (ILD) tests, implemented through MLSTest 1.0 software using 1000 permutations [62]. This evaluates differences between expected and observed incongruences between loci in the context of random unstructured homoplasy [38]. Topological incongruence (TI), through MLSTest, was applied to the tree derived from concatenated loci to evaluated the number of fragment trees that are topologically incompatible. [64].

In a parallel approach, to investigate genetic exchange, gene haplotypes were inferred using PHASE v. 2.1 [65]. Genetic exchange within individual haplotypic gene phylogenies was examined by assessing allelic contributions to heterozygous isolates from putative homozygous donor genotypes with unambiguous phase. Genetic exchange within haplotypes was also examined in isolates with unambiguous phase, through RDP3 [66], in an attempt to detect allelic mosaics. RDP3 software implements an extensive array of methods for detecting and visualizing genetic exchange.

Lastly, MLST data were concatenated, testing all fragment combinations, using MLSTest to define the minimum combination of loci that resolves the maximum number of diploid

sequence types (DSTs). Individual and concatenated phylogenies were generated initially with MrBayes, implemented through TOPALI v. 2.5 [67], using the best-fitting model, according to the Bayesian Information Criterion BIC. Two independent analyses were performed for 1.000.000 generations, sampling every 100 simulations (25% burn-in). Secondly, Neighbor Joining (NJ) method with uncorrected p-distances and ranch support was calculated by bootstrap set at 1,000 replications.

Maxicircle (*COII*) data analysis

The TcI *COII* locus was amplified and sequenced according to Messenger et al. [41]. Nucleotide sequences per gene fragment are available at GenBank under accession numbers: MF781085-MF781124. Phylogenies were constructed implementing the substitution model based on the Akaike Information Criterion (AIC) in MEGA 6 [68]. To compare nuclear and mitochondrial topologies, Maximum-Likelihood (ML) phylogenies were constructed (T92+I model, Tamura 3-parameter) which assumes that a fraction of sites is evolutionarily invariable [68]. TcIII (CM17) and TcIV (Saimiri3 cl1, X10/610 cl5, ERA cl2 and 10R26) strains were included as outgroups (accession numbers: JQ581330.1, JQ581331.1, JQ581329.1, JQ581328.1 and JQ581327.1, respectively) [41].

MLMT sequencing and data analysis

Twenty-five microsatellite loci were amplified as previously described by Llewellyn et al. [31] with some modifications (S3 Table). Markers were distributed across 11 chromosomes, including six groups of physically linked loci [69]. The following reaction conditions were implemented across all loci: a denaturation step of 4 mins at 95˚C, 30 amplification cycles 95˚C (20 s), 57˚C (20 s), 72˚C (20 s) with a final 20 mins elongation step at 72˚C. Reactions were performed in a final volume of 10 μL containing, 1X ThermoPol Reaction Buffer (New England Biolabs (NEB, UK), 4 mM MgCl2, 34 μM dNTPs; 0.75 mM of each primer, 1 unit of Taq polymerase (NEB, UK) and 1 ng of genomic DNA. Five fluorescent dyes were used to label forward primers, 6-FAM & TET (Proligo, Germany), NED, PET & VIC (Applied Biosystems, UK). Allele sizes were determined using an automated capillary sequencer (ABI 3730, Applied Biosystems, UK), in conjunction with a fluorescently tagged size standard (GeneScan– 500 LIZ, Applied Biosystems, UK), and manually checked for errors in GeneMapper software v3.7 (Applied Biosystems, UK).

Microsatellite data were assessed in accordance with Lewellyn et al. [31]. Individual-level clustering defined by Neighbour-Joining (NJ) phylogenies (D_{AS}: 1 –proportion of shared alleles at all loci/n) between microsatellite genotypes was calculated in MICROSAT v. 1.5 [70] under the infinite-alleles model (IAM). To accommodate multi-allelic genotypes ($\geq$3 alleles per locus), a script was developed in Microsoft Visual Basic to generate random multiple diploid re-samplings of each Multilocus profile. A final pair-wise distance matrix was derived from the mean across multiple re-sampled datasets and used to construct a NJ phylogenetic tree in PHYLIP v3.67 [71]. Majority rule consensus analysis of 10,000 bootstrap trees was performed in PHYLIP v 3.6 by combining 100 bootstraps generated in MICROSAT v. 1.5 [70], each drawn from 100 randomly re-sampled datasets.

Population assignment with a prior assumption of subdivision by collection sites was estimated with the Bayesian clustering program Structure v. 2.3 [72]. We assumed the *admixture model* due to the lack of information regarding ancestry, with correlated allele frequencies (i.e. frequencies in different populations are similar as a consequence of migration or shared ancestry) [72]. Simulations were set at 10^6 Markov Chain Monte Carlo (MCMC) interactions, with 2.5 x 10^5 iterations as burn-in. Ten independent runs were performed for each value of K (that

correspond to the number of groups, 2–10), as suggested by Pritchard et al. [72]. The most likely K value was estimated with the ΔK method [73].

An alternative approach to summarize genetic polymorphism was performed using a non-parametric approach, free from Hardy-Weinberg assumptions. Briefly, a *K*-means clustering algorithm, executed in ADEGENET [74] was used to identify the optimal number of 'true' populations, with reference to the BIC, which reaches a minimum when approaching the best support for assignment of isolates to the appropriate number of clusters. The relationship between clusters and the strains contained within them was evaluated using a discriminant analysis of principal components (DAPC), as described in Jombert et al. [74].

A single randomly sampled diploid dataset, generated using a custom Microsoft Visual Basic script to re-sample random multiple diploid combinations of each Multilocus profile, was used for all subsequent analyses, as described in Jombert et al [75]. Population genetic statistics were calculated considering strains assigned to their DAPC-derived population clusters. DTU-level genetic diversity was evaluated using sample size corrected allelic richness (Ar) in FSTAT v 2.9 [76]. Intra-population sub-clustering was calculated as mean pairwise D_{AS} values and associated standard deviations in MICROSAT v1.5 [70]. Sample size corrected private (population-specific) allele frequency per locus (PA/L) was calculated in HP-Rare [77]. Mean F_{IS}, a measure of the distribution of heterozygosity within and between individuals, was calculated per population in FSTAT 2.9. F_{IS} varies between -1 (all loci are heterozygous for the same alleles) and +1 (all loci are homozygous for different alleles). DTU-level heterozygosity indices were calculated in ARLEQUIN v3.11 [78] and associated significance levels for p-values derived after performing a sequential Bonferroni correction to minimize the likelihood of Type 1 errors [79]. Population subdivision was estimated using pairwise $F_{ST,}$ linearized with Slatkin's correction, in ARLEQUIN v 3.11. Statistical significance was assessed via 10.000 random permutations of alleles between populations. Three different strategies were performed to group the samples and calculate pairwise F_{ST} values: i) using isolate collection locations to investigate local diversity, ii) to assess levels of gene flow between the five ecologically disparate biomes and, iii) investigate the role of host/vector specificity in the context of host movement and the distribution of TcI genotypes. Within-population subdivision was evaluated in ARLEQUIN v 3.11 [74] using a hierarchal analysis of molecular variance (AMOVA). A Mantel test for the effect of isolation by distance within populations (pairwise genetic vs. geographic distance) was implemented in GENAIEX 6.5 using 10,000 random permutations [80]. The association between host/vectors and genotypic clusters based on DAPC were calculated using contingency tables along with a Chi-squared test.

Nucleotide sequence and read data accession numbers

Nucleotide sequences per gene fragment are available from GenBank under the accession numbers: MF615620-MF615679; MF615680-MF615739; MF615740-MF615799; MF615800-MF615859; MF615860-MF615919; MF615920-MF615979.

Results

In our cohort of 78 isolates, 60 isolates were successfully characterized using all six MLST markers, 62 isolates by maxcicircle gene sequencing and 42 using MLMT markers. In particular, only twenty two isolates could be analyzed using all three methods. Furthermore, 50 more isolates were reassessed with MLMT, totaling 92 isolates considered for microsatellite analysis. S1 Table provides details of the typing methodologies applied to each particular isolate.

Six MLST markers were sequenced in 78 *T. cruzi* isolates, of which 60 consistently produced amplicons and sequences of acceptable quality. Concatenated gene fragments comprised a

Table 1. Properties of six *T. cruzi* MLST loci.

Targets	N° of Polymorphic Sites	N° of Genotypes	Typing Efficiency	Discriminatory Power	Ratio Of Nonsynonymous To Synonymous Changes
CoAR	9	9	1	0.529	0.16
GTP	7	10	1.42	0.646	0.032
LAP	1	3	3	0.383	0.30
PDH	5	10	2	0.549	0.073
RB19	4	9	2.25	0.829	0.014
RHO1	10	19	1.9	0.935	0.957

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total of 2571 bp for each isolate. No single gene was able to differentiate all 60 isolates on the basis of TE and DP. Table 1 describes the level of diversity seen in each gene fragment; the number of polymorphic sites ranged from 10 (*RHO1*) to 1 (*LAP*). Typing efficiency (number of ST/number of polymorphisms) was variable among loci and the gene fragment distinguishing the highest number of genotypes per polymorphic site was *RB19* (TE = 2.25). In contrast, *CoAR* showed the lowest efficiency (TE = 1). *RHO1* demonstrated the highest DP (0.935) for our cohort; and *LAP*, the lowest (DP = 0.383).

All fragments met the criterion for stabilizing selection (dN/dS<1) for conservation of metabolic function. FEL analyses detected signs of purifying (negative) selection in 10 sites across four gene fragments (*LAP* - 24th site; *RB19* - 35th and 63th sites; *RHO1* - 76th site; and *GTP* - 13th, 15th, 42th, 98th, 119th, 136th positions; $p < 0.1$).

A comparison of diplotypic phylogenies of individual gene trees revealed differences in topology and clustering between gene fragments (S1–S9 Figs). However, similarities did exist, most notably, the highly diverse loci *RB19* and *RHO1* possessed similar topologies (S8 Fig). Following concatenation of all six markers, DP increased to 0.997, differentiating 55 genotypes from 60 isolates.

The minimum number of loci needed to derive the maximum DP was assessed for all combination of fragments (2 loci to 6 loci) through MLSTest. A combination of 5 fragments: *CoAR-GTP-LAP-RB19-RHO1* (S10 Fig) also yielded a high DP (0.995), discriminating 53 genotypes (2 genotypes less than yielded by the use of all 6 MLST markers).

MLST intraspecific diversity

Individual gene fragment trees revealed multiples polytomies in all six phylogenetic trees (S1–S9 Figs). Substantial congruences between the phylogenetic trees generated with SNP duplication (with Bayes) and Average State (with NJ) were observed (S1–S6 Figs), The two fragments with the most pronounced inconsistencies between Bayes and NJ were *PDH* and *RHO1* (S4 and S6 Figs).

S7–S9 Figs, show the comparison between the six gene trees. *RB19* and *RHO1* each produced a cluster corresponding to isolates from Atlantic Forest, Cerrado and Pantanal, which are mostly congruent. However, no two gene fragments showed completely identical topologies. The remaining loci (*CoAR*, *LAP*, *PDH* and *GTP*), which had lower TE and DP values, generally yielded trees that were less congruent. None of the gene fragments showed 100% congruence between their clusters.

Topological incongruence analyses revealed a mean of 2.86 incongruences per branch and 25% of branches with at least n-1 incongruent fragments. These correspond to moderate levels of incongruence (S11 Fig), where moderate incongruence was defined as being between 20 and 40% [64]. The ILD tests of discrepancies were no higher than expected, indicating that the combination of six gene fragments produces reliable branches (ILD = 0.05).

To evaluate intra-DTU diversity of TcI, phylogenies were inferred from the concatenated alignment of six gene fragments (Fig 2). Both, NJ and Bayesian methods produced similar results, although NJ analysis showed lower bootstrap values. Clusters with >50% support in both analyses are indicated. The presence of several sub-clusters was observed, revealing considerable intraspecific diversity within TcI and also similar genotypes circulating sympatrically over large geographical areas.

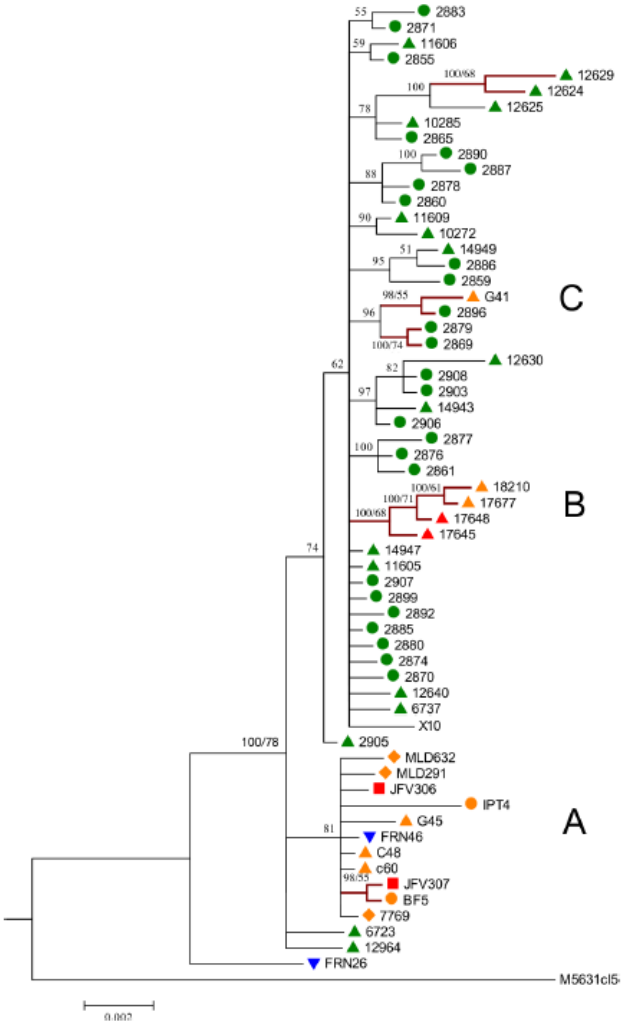

Fig 2. Bayesian inference based on the concatenation of 6 gene fragments from 63 TcI isolates. The highlighted clusters indicate congruence between SNPs duplication (Bayesian analysis) and Average State (NJ). Colors represent the biome of collection: green, Amazon; orange, Atlantic Forest; red, Cerrado; blue, Pantanal. Bullets correspond to mammal host or insect vector species: triangle, *Didelphis* spp.; square, *Chiroptera* spp.; circle, triatomine; diamond, primate; inverted triangle, rodent.

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Specific phyloepidemiological observations are as follows. Cluster A grouped isolates originating from very distant localities including the Atlantic Forest, Cerrado and Pantanal biomes (BPP = 81% and bootstrap <50%). Of particular note, cluster A (Fig 2) lacked genotypes present in the Amazon, in congruence with results from maxicircle phylogenies (below). Also, similar genotypes were isolated from different species. For example, isolates from *Didelphis* spp, primates, chiroptera, one rodent, and triatomine bugs grouped within cluster A (Fig 2).

Amazonian isolates were genetically diverse and were mostly contained within a single clade (Fig 2). Cluster B (Fig 2) contained genotypes from the Atlantic Forest and Cerrado biomes, comprising an enormous geographical distance (~1.130 km). Interestingly, cluster C comprised isolates from distant biomes, Amazon and Atlantic Forest. Likewise, isolates from Abaetetuba (11609) and Cachoeira do Arari (10272), separated by vast geographical distances (~78.38 km), were grouped in the same cluster. Of note, a single isolate FRN26, *Oecomys mamorae* from the Pantanal, was genetically dissimilar from all other TcI strains and placed in different topological positions in the context of MLST and maxicircle phylogenies.

Although isolates were collected in different years and localities (S1 Table), no clear clustering by collection date or biome was apparent; however, statistical tests were not able to rule out the existence of some association. Details of these tests are described below.

Haplotype analyses

Haplotype analysis, applied to nuclear loci, was used to generate phylogenies and investigate the allelic origins of heterozygous isolates from homozygous putative donor genotypes (Fig 3 and S12–S16 Figs). Here isolates with haplotypes present in two different genetically clusters that also contained respective homozygous donor isolate genotypes were considered potential hybrids.

Three genetic loci (*GTP*, *PDH* and *RB19*) revealed heterozygous isolates and allelic profiles that could be derived from homozygous genotypes (Fig 3, S12 and S13 Figs, respectively). In more detail, Fig 3 shows the *GTP* locus and alleles from homozygous donor isolates: 10285, haplotypes 1 and 2, in one clade; and 14943, haplotypes 1 and 2, in another. Within *GTP*, two isolates contain heterozygous allelic profiles, 12630 and 12624, corresponding to one allele from each homozygous donor. For *PDH*, five isolates contain heterozygous allelic profiles: 2892, 2896, 10285, 17645 and G41 (S12 Fig). The potential parental alleles for *PDH* were: 2879 and 2869, in one clade, and 2880, in another clade (S12 Fig). Similarly, for *RB19*, eight isolates showing potential genetic exchange were identified. The most plausible parental alleles for each putative hybrid are shown in S13 Fig, while the SNP profile for putative homozygous donors and the corresponding heterozygous profiles are shown in the S4–S6 Tables. Putative recombinants were different in *PDH*, *GTP*, and *RB19*; possibly indicating that there have been multiple genetic exchange events over time. Although we detect the signature of genetic exchange through heterozygous genotypes and their associated homozygous "donor" isolates, we observe no evidence of genetic exchange at the level of individual alleles, since allelic mosaics were not detected using RDP3 software.

Mitochondrial analysis

Sixty two *COII* sequences produced a 449 bp alignment, 10 unique haplotypes and 64 polymorphic sites. Maximum-Likelihood trees (Fig 4) revealed two major clades and almost complete congruence with cluster A derived from concatenated MLST (S17 Fig). This cluster contains strains from the Atlantic Forest, Cerrado and Pantanal with the notable exclusion of Amazonian isolates (bootstrap = 100%). Interestingly, isolate FRN26 from the Pantanal, and isolate G41 from the Atlantic Forest formed a strongly supported sub-clade (bootstrap = 100%).

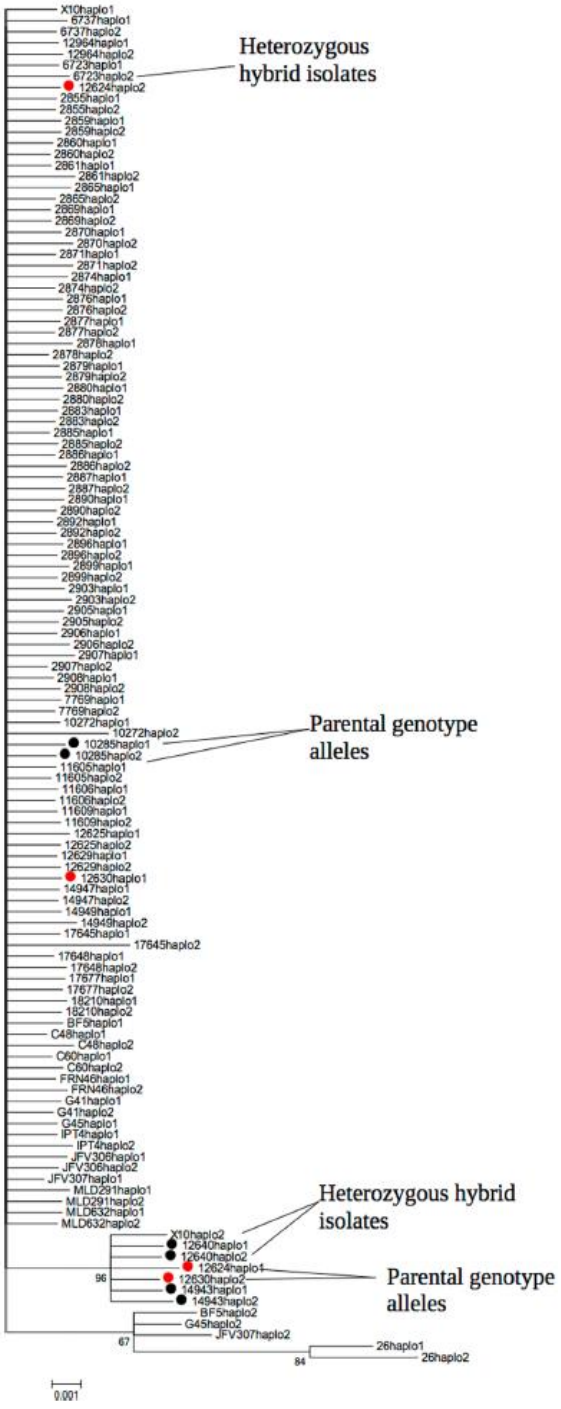
Heterozygous hybrid isolates
Parental genotype alleles
Heterozygous hybrid isolates
Parental genotype alleles
96
67
84
0.001

Fig 3. Haplotypic Bayesian Tree with *GTP* locus. Haplotypes inferred by PHASE V2.1. Red labels indicate heterozygous hybrid isolates (12630–12624) and black potential parental alleles (10285–14943).

https://doi.org/10.1371/journal.pntd.0006466.g003

In contrast, nuclear phylogenies grouped G41 with Amazonian isolates. Also of note, isolates within sub-clusters were highly homogeneous. Analyses with MLST and maxicircle were congruent in relation to the isolates of Amazon, in which they formed a separate group that included a cluster with isolates from Cerrado and Atlantic Forest (S17 Fig).

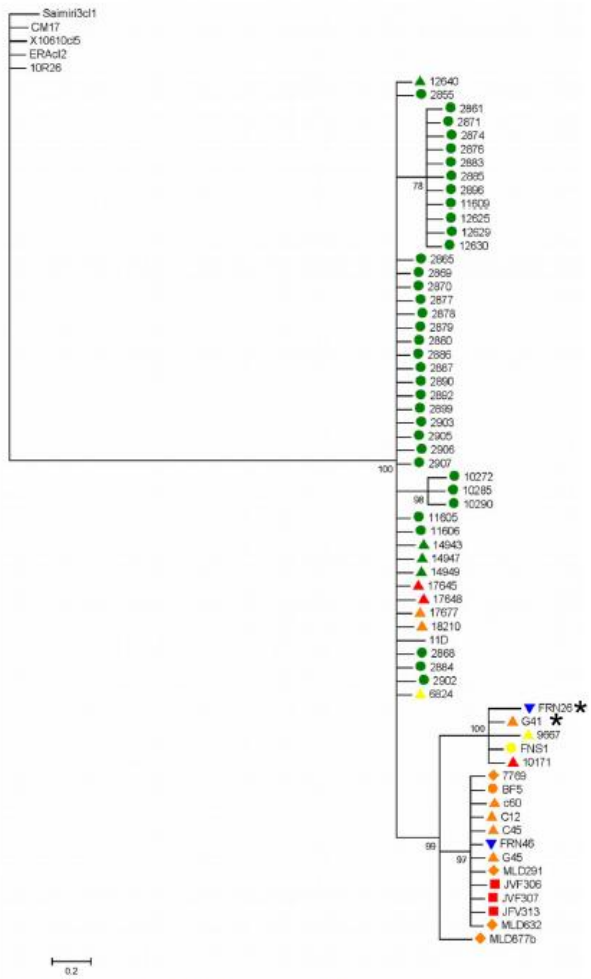

Fig 4. Maximum-Likelihood tree constructed from maxicircle sequences for 62 TcI Brazilian isolates. Colors represent the biomes where samples were obtained: Green, Amazon; orange, Atlantic Forest; red, Cerrado; blue, Pantanal; gray, reference sequences from Caatinga. Symbols correspond to strain host/vector: Triangle, *Didelphis* spp.; square, *Chiroptera* spp.; circle, triatomine; diamond, primate; inverted triangle, rodent.

https://doi.org/10.1371/journal.pntd.0006466.g004

The presence of genetically identical mitochondrial sequences despite a mutation rate one order of magnitude greater than that of nuclear genes provides support for the occurrence of multiple mitochondrial introgression events (Fig 4 and S17 Fig). Additionally, these sequences correspond to geographically dispersed isolates, obtained from different biomes and hosts and vectors, further supporting the case for introgression.

Microsatellite analysis

In total, 4595 alleles were identified, corresponding to 92 unique multilocus genotypes. Multiple (≥3) alleles were observed at 1.87% of markers. This is most likely attributable to aneuploidy in a small proportion of the loci (S7 Table). Bayesian clustering applied to 92 strains revealed the existence of four discrete phylogenetic groups without apparent association to the biome of origin (Fig 5). For example, isolates from Atlantic Forest clustered across three groups (Fig 5, yellow, green and pink label), in which specimens from the state of Rio de Janeiro are genetically similar to those from Posse, Goias (Cerrado biome) and specimens from the states of Minas Gerais and Santa Catarina clustered together with samples from Pantanal. Moreover, TcI specimens of the state of Bahia are genetically more similar to samples from the states of Piauí (Caatinga), Pará and Amazonas (Amazon) than to other samples from the Atlantic Forest biome. It is worth mentioning that, in general, samples from Cerrado, Caatinga and Amazon biomes were grouped together in two different groups (Fig 5, red and pink coloured groups).

The DAPC analysis with the 92 strains yields five genetic clusters, evidenced by a slight 'elbow' in the distribution of the BIC values across optimal cluster numbers at K = 5, once 22 principal components (PCs) were retained and analyzed (representing 80% of the total variation) (S18 Fig). DAPC-derived populations were broadly congruent with patterns of nuclear clustering identified by NJ and Bayesian clustering analysis. The five DAPC clusters, showed in S1 Table, corresponded to: Population 1 that includes Caatinga (n = 13) and Cerrado (n = 4); population 2, Atlantic Forest (n = 4), Pantanal (n = 10) and Cerrado (n = 1);

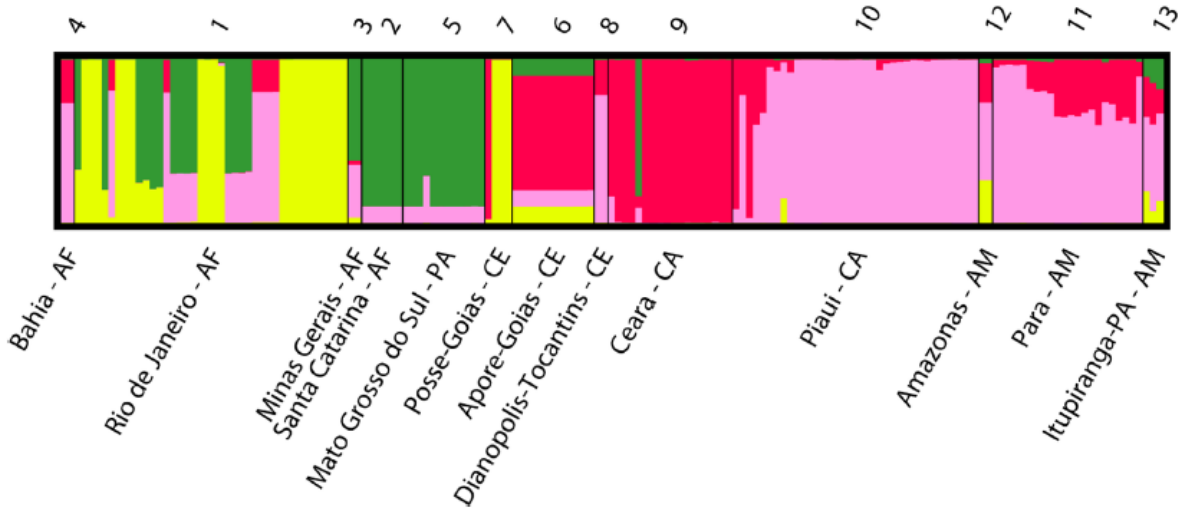

Fig 5. Population structure based on Bayesian clustering (K = 4) for 92 TcI isolates from five Brazilian biomes, genotyped for 25 microsatellite loci. Each number (above) represents the collection sites (below). Colours in vertical bars represent the proportion of the individual's genome derived from four assumed clusters.

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Population 3, Amazon (n = 30), Atlantic Forest (n = 6), Pantanal (n = 1) and Caatinga (n = 1); population 4, Atlantic Forest (n = 14) and Cerrado (n = 3) and population 5, the remaining parasites principally from opossums and primates in the Atlantic Forest (n = 14) and bats and an opossum in Cerrado (n = 3). Similarly, the NJ tree (Fig 6) reveals that parasites from the Atlantic Forest, Cerrado and Pantanal were generally admixed together. We observe no strict specific association between biomes, species or collection years and the clusters based on DAPC; however, the chi square contingency test ($p<0.05$) can not completely exclude an association between these clusters and host/vector species, collection biome or dates.

Cluster A, derived from MLST data, was congruent with one MLMT cluster, the equivalent maxicircle cluster (S17 and S19 Figs paired trees). The isolate G41 (Atlantic Forest), grouped with isolates from Amazonia for MLST but was grouped with Atlantic Forest isolates with MLMT analysis. Similarly, topological positions for FRN26/26 were different for MLST and maxcircle trees (S17 Fig paired trees)

Population genetic parameters were calculated for strains grouped *a priori* according to their biome of origin, as well as *a posteriori* DAPC cluster assignments (Table 2 and S8 Table). Consistent results are observed when strains are grouped according to DAPC-assigned clusters. Table 2 shows high levels of genetic heterogeneity in Amazon (DAPC population 3), as well as excess homozygosity, high numbers of private alleles per locus and a low standard deviation associated with D_{AS} value. *T. cruzi* strains from Atlantic Forest, Cerrado and Caatinga displayed similar, but lower levels of diversity, with comparatively lower numbers of private alleles per locus.

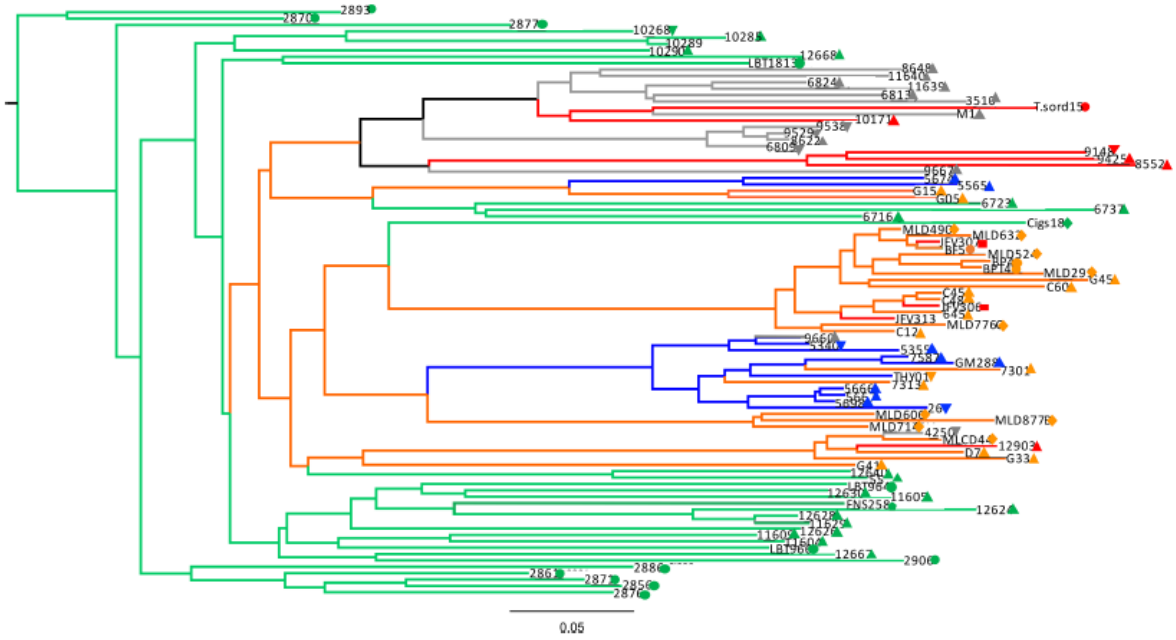

Fig 6. Unrooted neighbour-joining tree based on D_{AS} values between multilocus genotypes generated for 92 sylvatic Brazilian TcI strains. D_{AS} values were calculated as the mean across 1000 random diploid re-samplings of the data set. Branch colors represent the biomes where samples were collected: Green, Amazon; orange, Atlantic Forest; red, Cerrado; Bblue, Pantanal; gray, Caatinga. Symbols correspond to host/vector species: Triangle, *Didelphis* spp.; square, *Chiroptera* spp.; circle, Triatomine; diamond, Primate; inverted triangle, rodent; pentagon, dog; hexagon, Raccoon.

https://doi.org/10.1371/journal.pntd.0006466.g006

Table 2. Population genetic parameters for *a priori* sylvatic populations of TcI in Brazil.

Populations	G/N	PL	PA/L±SE	Ar±SE	D_{AS} ± SD	Ho	He	% PL He	% PL Hd	F_{IS}±SE
Atlantic Forest	26/26	23	0.44 ± 0.12	3.61 ± 0.35	0.388 ± 0.188	0.37	0.46	26.1	43.5	0.212 ± 0.08
Amazon	32/32	25	0.97 ± 0.17	4.24 ± 0.38	0.457 ± 0.0846	0.42	0.51	0	36	0.194 ± 0.04
Pantanal	11/11	17	0.27 ± 0.14	2.46 ± 0.36	0.198 ± 0.157	0.35	0.31	23.1	5.9	-0.004± 0.16
Cerrado	09/09	19	0.36 ± 0.16	3.72 ± 0.74	0.482 ± 0.166	0.39	0.53	0	26.3	0.279 ± 0.12
Caatinga	14/14	20	0.13 ± 0.07	3.14 ± 0.46	0.341 ± 0.118	0.34	0.40	0	5	0.206 ± 0.09

N: number of isolates in population; G: number of multilocus genotypes (MLGs) per population based on microsatellite data of 25 loci analyzed; PL: number of polymorphic loci out of 25 loci analysed; PA/L: mean number of private alleles per locus ± SE, calculated in HP-Rare (Kalinowski, 2005); Ar: allelic richness as a mean over loci ± SE, calculated in FSTAT 2.9.3.2 (Goudet, 1995); D_{AS}: mean pairwise allele sharing ± SD, calculated in MICROSAT v1.5d (Minch, 1997); Ho: mean observed heterozygosity across all loci, calculated in Arlequin v3.11 (Excoffier, 2005); He: mean expected heterozygosity across all loci, calculated in Arlequin v3.11 (Excoffier, 2005); %PL He: proportion of polymorphic loci showing a significant excess in heterozygosity after a sequential Bonferroni correction (Rice, 1989), calculated in Arlequin v3.11 (Excoffier, 2005); %PL Hd: proportion of polymorphic loci showing a significant deficit in heterozygosity after a sequential Bonferroni correction (Rice, 1989), calculated in Arlequin v3.11 (Excoffier, 2005); F_{IS}: mean fixation index ± SE, calculated in FSTAT 2.9.3.2 (Goudet, 1995).

https://doi.org/10.1371/journal.pntd.0006466.t002

Three diverse populations (Atlantic Forest, Cerrado and Caatinga) were characterized by elevated standard deviations associated with D_{AS} values and positive F_{IS} values (Table 2). A hierarchical AMOVA demonstrated 83.1% of total genetic variation was present within populations, compared to 16.9%, among populations ($p<0.0001$ for both).

The observed subdivision between a priori populations suggests the existence of gene flow between *T. cruzi* from the Atlantic Forest and those of the Cerrado biome ($F_{ST} = 0.067$) (Table 3). Gene flow was also inferred to have occurred between *T. cruzi* populations of Caatinga and Cerrado ($F_{ST} = 0.0982$) (Table 3). The admixed character of these isolates was also supported by Bayesian assignment. More geographically-distant TcI populations display similar levels of subdivision, as observed between Caatinga and Pantanal, Caatinga and Atlantic Forest, Cerrado and Pantanal and Pantanal and the Atlantic Forest. *T. cruzi* isolates from the Amazon biome exhibited lower F_{ST} values than populations of all other biomes (Table 3). This observation is also supported by F_{ST} values calculated for the a posteriori populations (S9 Table).

At a local-level structure analysis (i.e. when samples were grouped using the collection site as prior information; Table 4), it is clear that some isolates from Atlantic Forest grouped with others from Cerrado and Pantanal due to the genetic similarity between samples of Rio de Janeiro and Possas, Goias ($F_{ST} = 0.04$), of Bahia and Tocantins ($F_{ST} = 0.13$), and of Santa Catarina and Mato Grosso do Sul ($F_{ST} = 0.09$). Similarly, samples from Cerrado (Piaui) and Amazon (Para) showed low levels of structure ($F_{ST} = 0.09$). The investigation of parasite population structure based on host taxonomy suggests that *Didelphis marsupialis* might play a role as the

Table 3. Pairwise F_{ST} values in a five-way comparison between *a priori* populations.

	Atlantic Forest	Amazon	Pantanal	Cerrado	Caatinga
Atlantic Forest	*				
Amazon	0.146 **	*			
Pantanal	0.227 **	0.172 **	*		
Cerrado	0.067(p = 0.0156 ± 0.0037)	0.114 **	0.267 **	*	
Caatinga	0.216 **	0.142 **	0.347 **	0.0982 **	*

* 0.000

**P<0.001

https://doi.org/10.1371/journal.pntd.0006466.t003

Table 4. Pairwise F_{ST} values for microsatellite data grouped according to the collection site.

	RJ	SC	MG	BA	MS	APGO	POGO	TO	CE	PI	PA	ITPA
RJ												
SC	0.26											
MG	0.29	0.56										
BA	0.24	0.57	0.56									
MS	0.24	0.09	0.47	0.48								
APGO	0.35	0.50	0.45	0.51	0.48							
POGO	0.04	0.39	0.46	0.33	0.35	0.43						
TO	0.28	0.58	0.52	0.13	0.50	0.49	0.41					
CE	0.22	0.39	0.42	0.31	0.34	0.34	0.22	0.39				
PI	0.18	0.28	0.32	0.27	0.23	0.35	0.23	0.31	0.20			
PA	0.16	0.25	0.27	0.25	0.22	0.30	0.20	0.26	0.16	0.09		
ITPA	0.17	0.37	0.31	0.33	0.33	0.39	0.23	0.37	0.23	0.19	0.13	
AM	0.22	0.45	0.5	0.41	0.44	0.45	0.31	0.37	0.23	0.19	0.13	0.30

False Discovery Rate (FDR) = 0,016. Yellow cells denote 0,016<p<0,05, blue cells denote 0,001<p<0,016 and, grey cells denote p<0,001

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main disperser of TcI (S10 Table), since its overall pairwise F_{ST} values were lower than the others ($F_{ST} \leq 0.2$; median = 0.15).

Finally, to determine the extent of spatial genetic structure, a Mantel test was conducted, demonstrating significant parasite isolation by distance across the sampled geographical range ($RXY = 0.384$; $P = 0.01$). (S20 Fig).

Discussion

In this study we explored the genetic diversity of Brazilian TcI isolates, obtained from different vectors and mammal hosts, spanning 5 different ecological biomes. To this end, we analyzed data from six protein coding genes (MLST), 25 microsatellite loci and one mitochondrial locus. We observed substantial genetic diversity with no strict association of clusters with particular host/vector species or biomes. However, some degree of association to a cluster from MLMT was present in isolates from Amazon. No other noticeable relation between clusters and biomes was identified; nevertheless, statistical tests are consistent with the possibility of some form of association. In addition, we observed mitochondrial introgression events and evidence of intra-DTU genetic exchange. Previous works [29, 41, 81, 82] have studied the genetic diversity within DTU I using different methods encompassing nuclear and mitochondrial markers. However, this constitutes the first instance in which MLST analyses in combination with two other high resolution genetic markers (microsatellite and maxicircle sequencing) have been used to evaluate intra TcI diversity of isolates from Brazil.

The criteria for justifying the selection of MLST markers used were based broadly on Diosque et al. [18], and fragments were assessed, in order of importance, in terms of intra-DTU diversity (TE), genotype discrimination (DP) and statistical support in phylogenetic trees. MLST analysis using all six concatenated gene fragments discriminated 55 genotypes out of 60 isolates. The use of a combination of five fragments of concatenated genes (*CoAr*, *GTP*, *LAP*, *RHO1* and *RB19*) also proved to be a viable alternative to the six genes, discriminating 53 of a possible 60 isolates. However, in light of the slightly reduced discriminatory power, we recommend the use of all six gene fragments.

There was significant variation in TE and DP among tested loci (Table 1). The most variable loci were *RHO1* and *GTP* which possessed 17 and 13 polymorphic sites, respectively. This is in

line with the observations made previously by Diosque et al. [18] and Ramirez et al. [36]. The *LAP* locus contained the least number of SNPs (4 polymorphic sites), in accordance with Ramirez et al. [36]. Some previous works have assessed *RB19*, *GPI*, *LAP* and *TR* and considered them non-informative when applied to typing schemes for cohorts spanning all six DTUs [18, 19, 36]. However, in the context of Brazilian isolates, *RB19* proved to be a highly informative marker for investigating intra-TcI diversity (TE = 1.9). Variation in TE is likely the result of selective pressures on individual loci, genetic drift or differences in mutation rates. Additionally, non-synonymous SNPs in MLST fragments, contributing to amino acid alterations, have previously been reported in *T. cruzi* [36, 37]. Here all gene fragments met the criteria for stabilizing selection (<1) for conservation of metabolic function. FEL analyses provided evidence that 10 sites in four of the six gene fragments are under purifying selection.

Bayesian and NJ analyses of the trees generated with each individual gene showed the presence of polytomies (S1–S6 Figs). One possible explanation for the existence of polytomies is the relatively small number of informative polymorphisms in the markers analyzed. This type of structure (Genetic Structure Type 2) was previously observed by Tomasini et al [64] when studying *A. fumigatus* through the application of MLST.

We generated phylogenetic trees with NJ to assess the robustness of our findings, since Bayesian analyses with SNP duplication can lead to artificially high bootstrap values [64]. Indeed, the support values were higher in the Bayesian analysis with SNP duplication; nevertheless, the clusters in the concatenated tree were mainly consistent between both analyses. Concatenation across loci by MLST has been successfully applied to many prokaryotic and eukaryotic organisms [22, 61, 63, 83, 84–86]. Nevertheless, when using this methodology, high levels of inbreeding or genetic exchange at particular loci may confound true phylogenetic relationships; therefore, in the presence of these effects, results must be interpreted cautiously. In this study, concatenation of six genes resulted in two main groups: the first included all isolates from Amazon and some representatives from Cerrado and Atlantic Forest (Fig 2, clusters B and C), and the second group included the remaining isolates from Atlantic Forest, Cerrado and part of Pantanal (Fig 2, cluster A). The epizootiological significance of these findings are discussed below.

Nuclear genes, phylogenies and genetic exchange

The BPP values supporting those clusters that show incongruences varied widely between individual gene phylogenies. Similar patterns of incongruence have been previously observed in nuclear genes [37, 87]. Such incongruence, where isolates differ in topological positions, are a classical marker in populations that have undergone genetic exchange. To investigate further, haplotypic phylogenies were constructed for each genetic locus in order to define heterozygous isolates and their potential homozygous allelic donors. (Fig 3, S12–S16 Figs) The results indicate potential allelic recombinants in 3 of the 6 loci. Putative recombinant isolates possessed heterozygous allelic profiles, each present in two different homozygous putative donor isolates, situated in different phylogenetic clusters. Potential allelic recombinant isolates across 3 genes is suggestive of multiple genetic exchange events. PHASE is a Bayesian method for the reconstruction of haplotypes. It is generally considered one of the most accurate haplotype reconstruction methodologies. However, there are potential confounders, for example, population size and frequency of recombination have the potential to skew outcomes. Furthermore, one must be cautious when using PHASE to infer frequency of genetic exchange, as this is one of the assumptions of the method. Nevertheless, the presence of recombinants and potential "donor" genotypes inferred in three independent nuclear markers is confirmed by heterozygous and homozygous SNPs derived from nuclear sequences (S4–S6 Tables). Together, these

observations constitute evidence for the presence of genetic exchange at the nuclear level. Population structure of *T. cruzi* is frequently regarded as clonal [88]. This model does not exclude genetic exchange, but considers it to be infrequent [89]. However, exchange across DTUs has been demonstrated using MLST [18, 36]; and intra-TcI genetic exchange in a single isolate has been observed in a cohort of Colombian samples [36]. Similarly, Messenger et al. [41] and Ramirez et al [82] observed multiple incongruence and introgression events within TcI on the basis of MLMT, MLST and maxicircle phylogenies, concluding that genetic exchange within DTU I is frequent. Genetic exchange is inferred in the current data set, however the frequency of genetic exchange is presently unknown and a topic of enthusiastic debate.

Mitochondrial analysis

In comparison with nuclear genes, remarkably low levels of intra DTU *COII* diversity were observed. Paradoxically, the mutation rate of mitochondrial genes is generally considered one order of magnitude higher than that of nuclear genes [90]. The spectrum of reduced diversity observed in maxicircle clades is consistent with introgression events as also reported in different TcI populations in South America [36, 41]. Two major clades were observed, the first consisting of all samples from the Amazon biome, together with a few samples from other biomes. The second clade grouped all of the remaining samples. This pattern was congruent across both nuclear and mitochondrial loci, and is indicative of genetically discrete populations. MLMT analysis (below) suggests limited gene flow (F_{ST}) between the Amazon biome and other studied areas. Although nuclear and mitochondrial phylogenies shared some topological characteristics, there were substantial incongruences between them. For example, isolate G41 clustered with Amazonian isolates at the nuclear level, but associated in mitochondrial phylogenies with isolates of non-Amazonian origin (S17 Fig). In the context of introgression, the discordance between nuclear and mitochondrial phylogenies is indicative of a prolonged and continuous association between populations from very distant localities [41]. This is consistent with the suggestion that genetic exchange in *T. cruzi* involves the independent exchange of kinetoplasts and nuclear genetic material [41]. Reciprocal nuclear genetic exchange among parasite strains undergoing mitochondrial introgression has not yet been detected, which may support an asymmetric, cryptic hybridization mechanism, or perhaps more likely, reflect the minor amount of nuclear genetic information sampled [81]. However, without the resolution of whole nuclear genome sequences, it is only possible to define the contributions of elements of meiosis, mitochondrial introgression and/or parasexual fusion [15, 82, 91]. The results presented here, include isolates from geographically distant sites (approximately 1790 km) and imply multiple introgression events occurring between different clades encompassing a large geographical area.

Microsatellite analysis

MLMT, the most sensitive method for assessing diversity, identified 4 groups when collection sites were used to group TcI specimens (Fig 5) or 5 clusters when no prior clustering was imposed. Three groups draw attention, one with isolates originating from Caatinga (gray branch), another from Pantanal (blue branch) and a third, consisting of an admixture of Atlantic Forest and Cerrado (Fig 6, orange and red branch). The third group contained genotypes that occurred in primates, bats, *Didelphis* and *Rhodnius* spp., in agreement with mitochondrial phylogenetic topology. There was a tendency for TcI isolates to cluster with other locally obtained isolates, which may reflect a sampling bias or clonal expansion. However, when samples were grouped according to their collection sites (Table 4), the analysis revealed specific examples of similar genotypes present across nearby states. Examples include Santa Catarina (Atlantic Forest) and Mato Grosso do Sul (Pantanal), Bahia (Atlantic Forest) and

Tocantins (Cerrado), and Piaui (Caatinga) and Pará (Amazon). In stark contrast, Amazon demonstrated significant intraspecific heterogeneity (Table 2 and Table 3) and clustering indices suggest that parasites from Amazon (DAPC population 3) have undergone long-term, undisturbed, sylvatic diversification. The relative lack of human impact, particularly in some municipalities in the state of Para, may account for allelic richness evolving over time in a biome with an abundance of host species. [39]. Interestingly, D_{AS} values from three diverse populations (Atlantic Forest, Cerrado and Caatinga) suggest the presence of intra-population sub-clusters, which is likely a consequence of the fragmentation due to intense human activity in these areas. Significant gene flow is observed over vast distances, for example between Cerrado and Atlantic Forest (Fig 1 and Table 3). The most parsimonious explanation is host movement, particularly aerial dispersion with bats, as exemplified over large distances in African clades of *Trypanosoma* sp. [92]. In South America, bats are known to harbor diverse trypanosome genotypes [92, 93], but their role in biogeography and dispersion is not fully understood. Unfortunately, TcI samples from Chiroptera species were collected from a single location (in Cerrado). A much more comprehensive effort to study Tc1 isolates in Chiroptera would be of interest to adequately address the nature of their role in dispersal in Brazil. Notwithstanding, we observed that *D. marsupialis* acts as a disperser of TcI genotypes across different biomes [94], this is evidenced by generally low F_{ST} values in pairwise comparisons with samples obtained from other hosts ($F_{ST} \leq 0.2$, S10 Table). Isolates from Atlantic Forest, Amazon and Cerrado showed significantly low heterozygosity levels, which may be due to gene conversion or under sampling used in the study. In this case, processes such as inbreeding are expected to shape the genetic background of populations [94]. Indeed, isolates from the Amazon biome presented low gene flow and moderate levels of inbreeding ($F_{IS} = 0.194 \pm 0.04$), relatively to other biomes, indicating a degree of genetic isolation. (Table 2).

Epizootiology of Brazilian TcI

Our analyses of three classes of genetic markers revealed broadly similar patterns of intra-DTU diversity in Brazil. MLST and maxicircle marker analysis yielded two principal phylogenetic groups. One included all isolates from the Amazon region, with representatives from Cerrado and Atlantic Forest (Fig 2, clusters B and C). The second group included all other isolates from Atlantic Forest, Cerrado and part of Pantanal (Fig 2, cluster A). MLMT analysis comprising fast evolving markers, as expected, revealed the most diversity, five discrete populations and variable amounts of gene flow and fragmentation indicators. Among all biomes it is evident that Amazon harbors the most extensive diversity and comparatively low gene flow. High diversity and low fragmentation indicate a biome exposed to less ecological pressure and undisturbed sylvatic diversification. Generally, there was no clear evidence of specific host/vector associations. In particular, similar genotypes were represented in different vector/host species. For example, genotypes represented in cluster A (Fig 2) consisted of closely related genotypes observed in a diversity of hosts species including didelphids, rodents, chiroptera, primates and triatomines scattered across diverse municipalities within the Atlantic Forest, Cerrado and Pantanal biomes. Additionally, this cluster included hosts whose habitat is principally arboreal, with *Didelphis* spp occupying all strata. The presence of *Didelphis* spp. in all clades and low associated F_{ST} values (S10 Table) is compatible with the hypothesis that they are bioaccumulators of multiple genotypes [83, 94], they are highly permissive to infection and are known to move between all ecological strata from terrestrial to arboreal. The genealogical relationship of isolates in cluster A in MLST was preserved across MLMT and mitochondrial analyses (S17 and S19 Figs).

Evidence from all markers reveals that similar genotypes are found across vast geographical distances, over ecological barriers, diverse habitats, and different hosts species. Noticeably, isolates G41 (Atlantic Forest) and 2896, from Belem in the Amazon biome (Figs 1 and 2), have identical genotypes. Other examples include isolates 10272 and 11609, which possess identical genotypes despite being separated by the *Marajo bay* (a distance of 4500 km); and isolates from Belem (2855) and Abaetetuba (11606), which are genetically homogenous despite vast geographic separation. Human activity is likely to have an impact on the dispersal of genotypes. A case in point is provided by Combu and Murucutu, which are two island localities situated in the municipality of Belem (Amazon) that are sparsely occupied by humans and used primarily for açaí production [53]. They form a robust enzootic transmission cycle, and remote human infections are acquired by unwitting transport of infected triatomines in açai baskets [53]. Comparatively high indicators of gene flow between other biomes inferred by MLMT analysis are also compatible with the influence of human activity that may facilitate gene flow. Lima and collaborators [42] using MLMT, applied to Brazilian TcI, observed that isolates from Atlantic Forest and the Amazon formed distinct and separate clusters. Their proposition was that geographic distance separating biomes was the likely explanation for topological features. However, in this work, through the application of MLST, MLMT and maxicircle analysis, we find not only localized diversity but also genetic homogeneity over large distances. In summary, this study included a large number of samples and revealed extensive intra DTU diversity, an absence of strict associations to host/vector species, and similar genotypes circulating over vast areas. We provide evidence of genetic exchange based on phylogenetic incongruence among loci, haplotypic analysis of nuclear markers and also mitochondrial introgression. It is likely that gene flow between biomes is influenced by the movement of mammals and also facilitated by human activity.

Supporting information

S1 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *COAR* gene fragment.
(PDF)

S2 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *GTP* gene fragment.
(PDF)

S3 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *LAP* gene fragment.
(PDF)

S4 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *PDH* gene fragment.
(PDF)

S5 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *RB19* gene fragment.
(PDF)

S6 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *RHO1* gene fragment.
(PDF)

S7 Fig. Trees generated with individual fragments using Bayesian analysis. (A) *CoAR*, (B) *GTP*.
(PDF)

S8 Fig. Trees generated with individual fragments using Bayesian analysis. (A) *RHO1*, (B) *RB19*.
(PDF)

S9 Fig. Trees generated with individual fragments using Bayesian analysis. (A) *PDH* and (B) *LAP*.
(PDF)

S10 Fig. MLST: Reduced 5 loci combination scheme.
(PDF)

S11 Fig. Topological incongruence based on the six concatenate gene markers.
(PDF)

S12 Fig. Haplotypic Bayesian Tree with *PDH* locus.
(PDF)

S13 Fig. Haplotypic Bayesian Tree based on *RB19* locus.
(PDF)

S14 Fig. Haplotypic Bayesian Tree based on *CoAR* locus.
(PDF)

S15 Fig. Haplotypic Bayesian Tree based on *LAP* locus.
(PDF)

S16 Fig. Haplotypic Bayesian Tree based on *RHO1* locus.
(PDF)

S17 Fig. Comparison between (A) MLST and maxicircle trees (B).
(PDF)

S18 Fig. Nuclear genetic clustering among 92 Brazilian sylvatic TcI strains.
(PDF)

S19 Fig. Comparation between (A) MLST and (B) MLMT trees.
(PDF)

S20 Fig. Nuclear spatial genetic analysis of *T. cruzi* I isolates from five Brazilian biomes.
(PDF)

S1 Table. *Trypanosoma cruzi* I isolates used in the study.
(PDF)

S2 Table. MLST gene targets.
(PDF)

S3 Table. Panel of microsatellite loci and primers.
(PDF)

S4 Table. SNP data of isolates for *RB19*.
(PDF)

S5 Table. SNP data of isolates for *PDH*.
(PDF)

S6 Table. SNP data of isolates for *GTP*.
(PDF)

S7 Table. Complete dataset of 25 microsatellite markers.
(PDF)

S8 Table. Population genetic parameters for a posteriori sylvatic population of TcI in Brazil.
(PDF)

S9 Table. F_{ST} values in a five- way comparison between a posteriori population.
(PDF)

S10 Table. Pairwise F_{ST} values for microsatellite data grouped according to the parasites' hosts.
(PDF)

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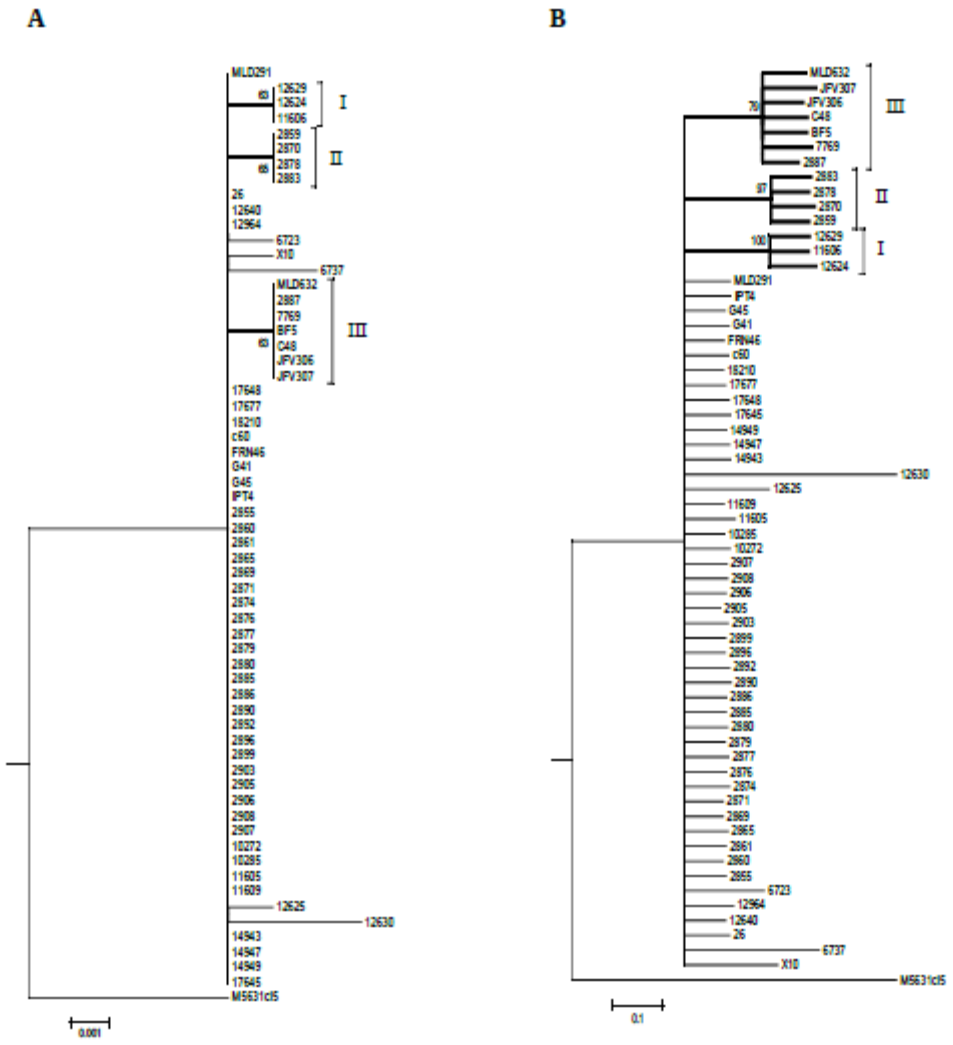
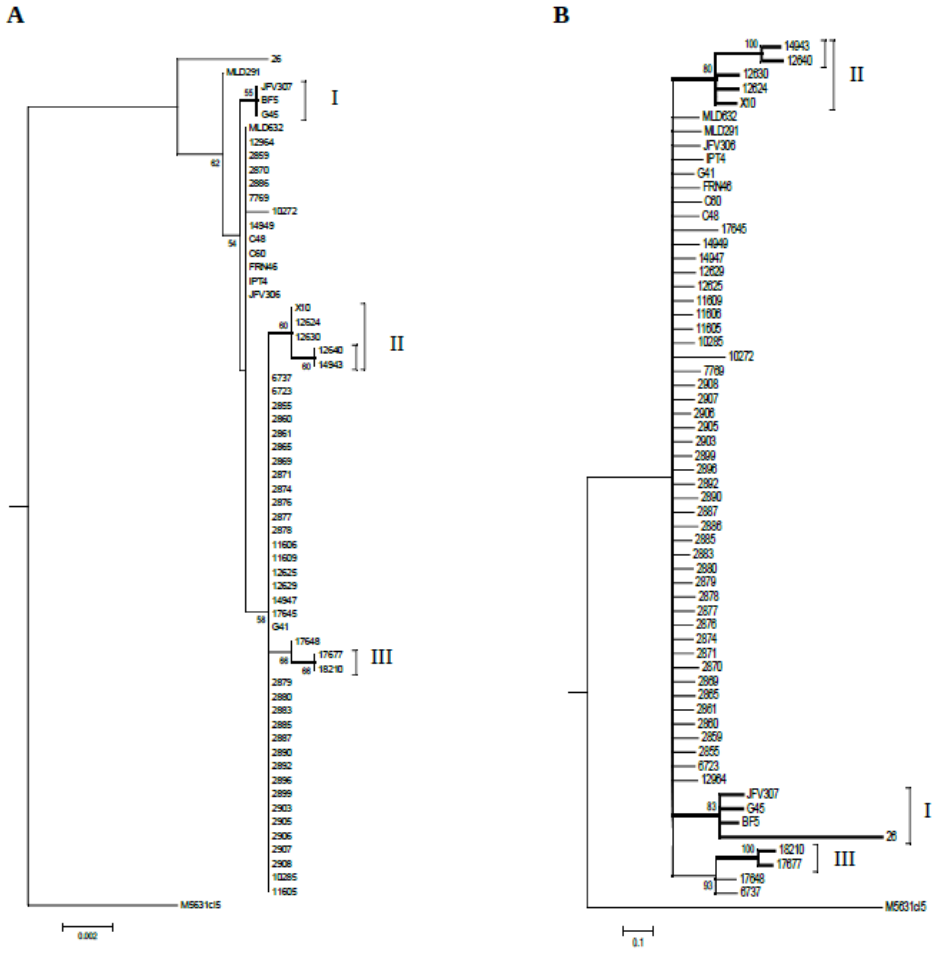
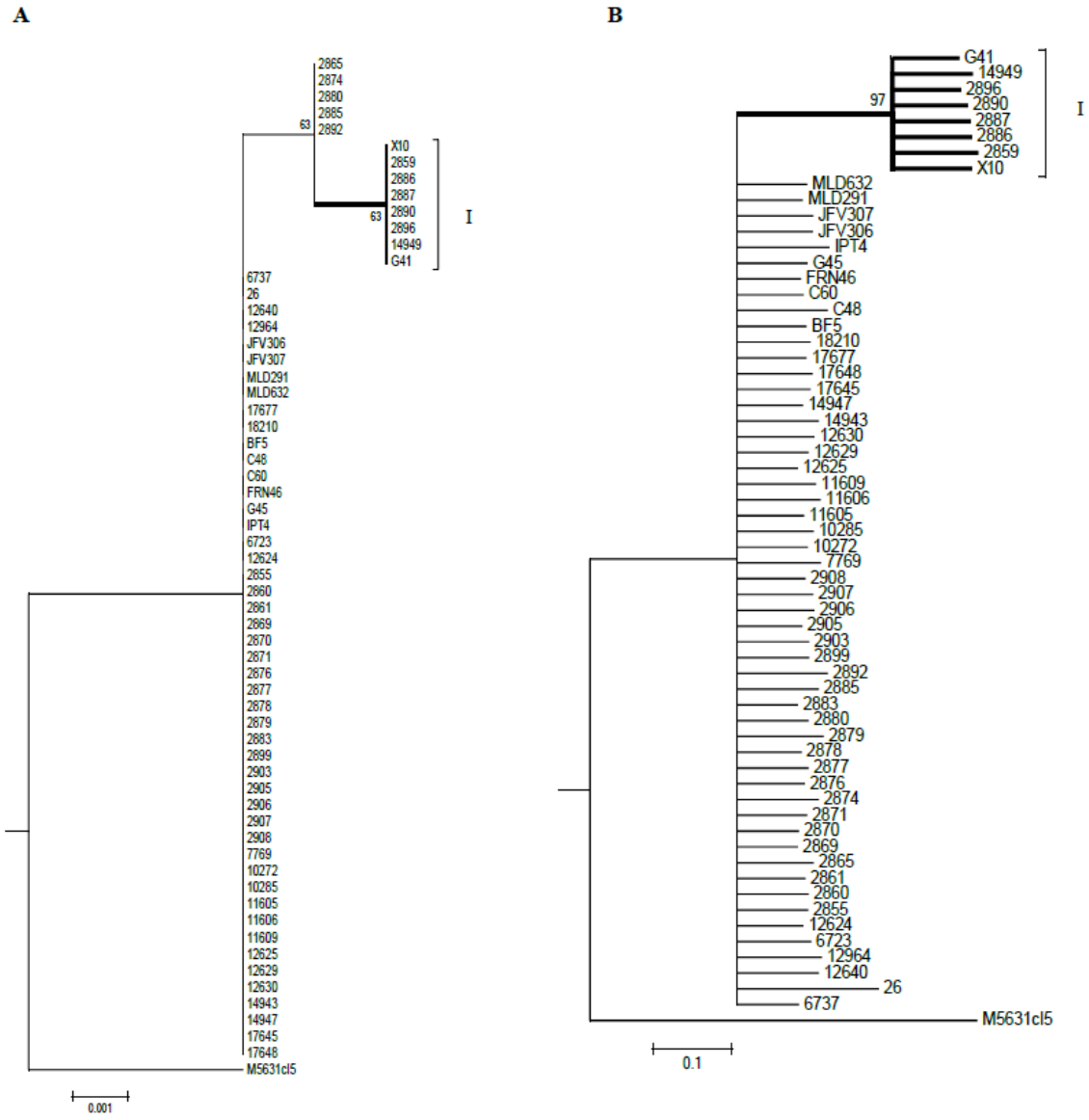


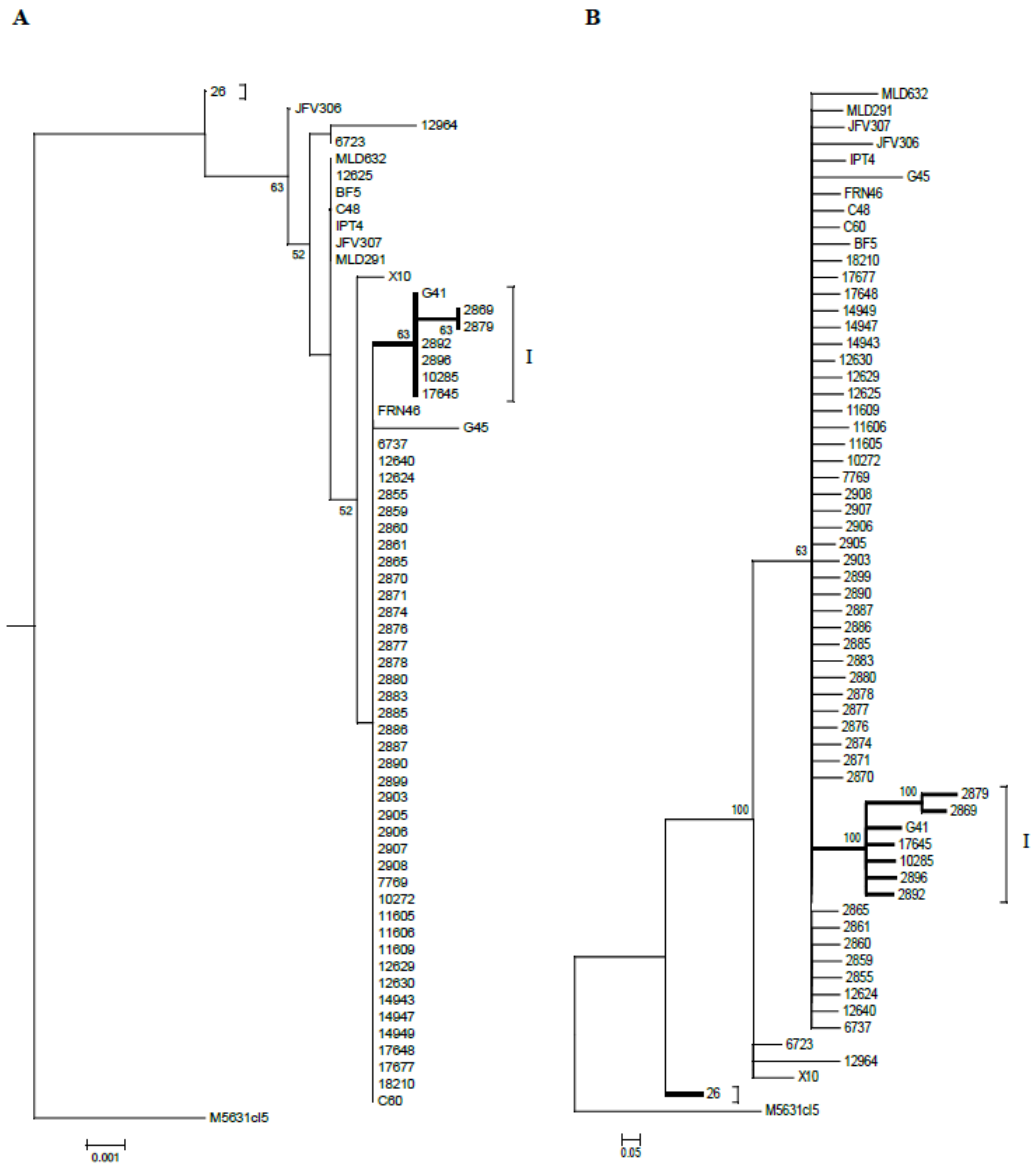
S1 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *COAR* gene fragment.
Roman numerals identify clusters that were consistent across both analyses.



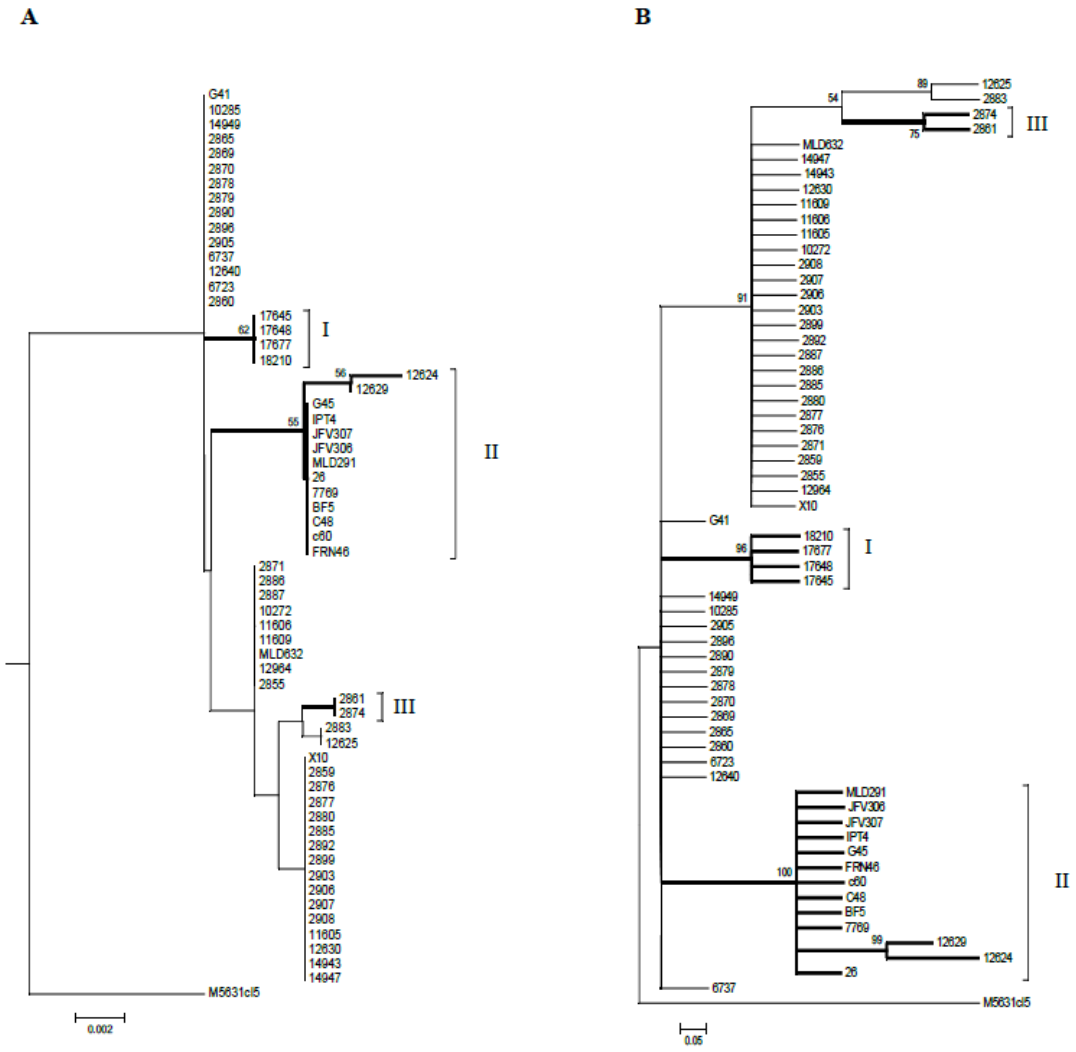
S2 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *GTP* gene fragment. Roman numerals identify clusters that were consistent across both analyses.



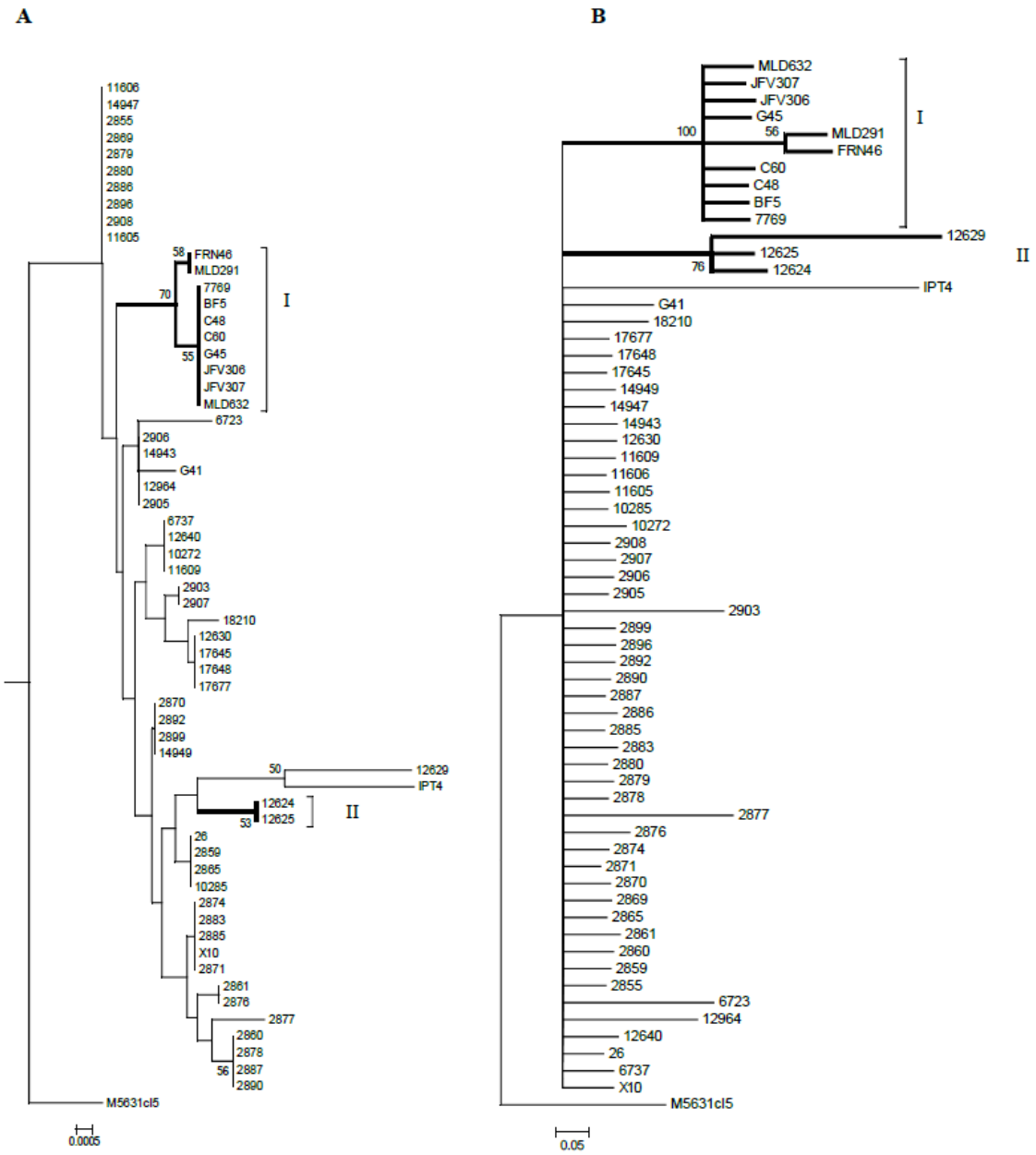
S3 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *LAP* gene fragment. Roman numerals identify clusters that were consistent across both analyses.



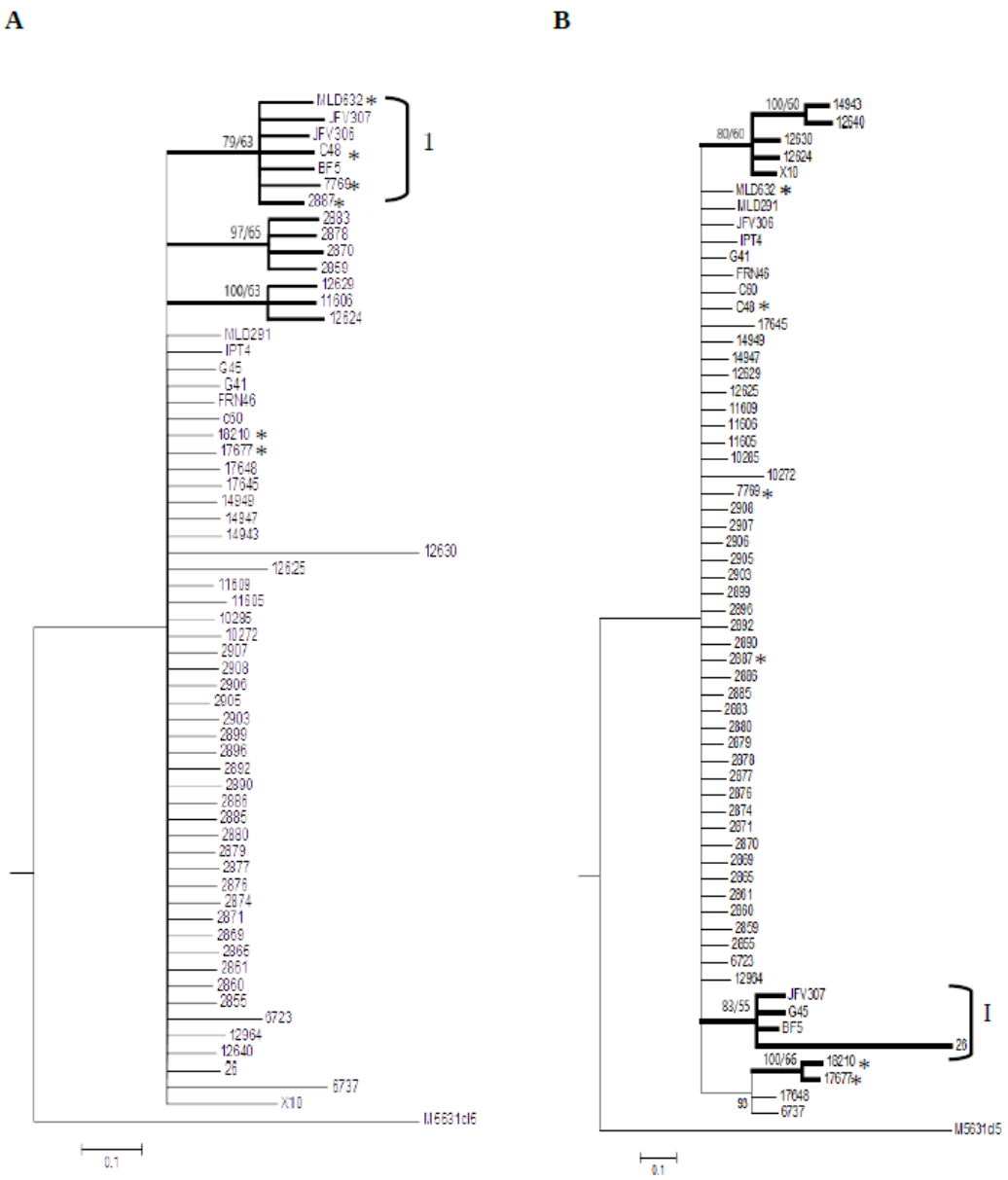
S4 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *PDH* gene fragment. Roman numerals identify clusters that were consistent across both analyses.



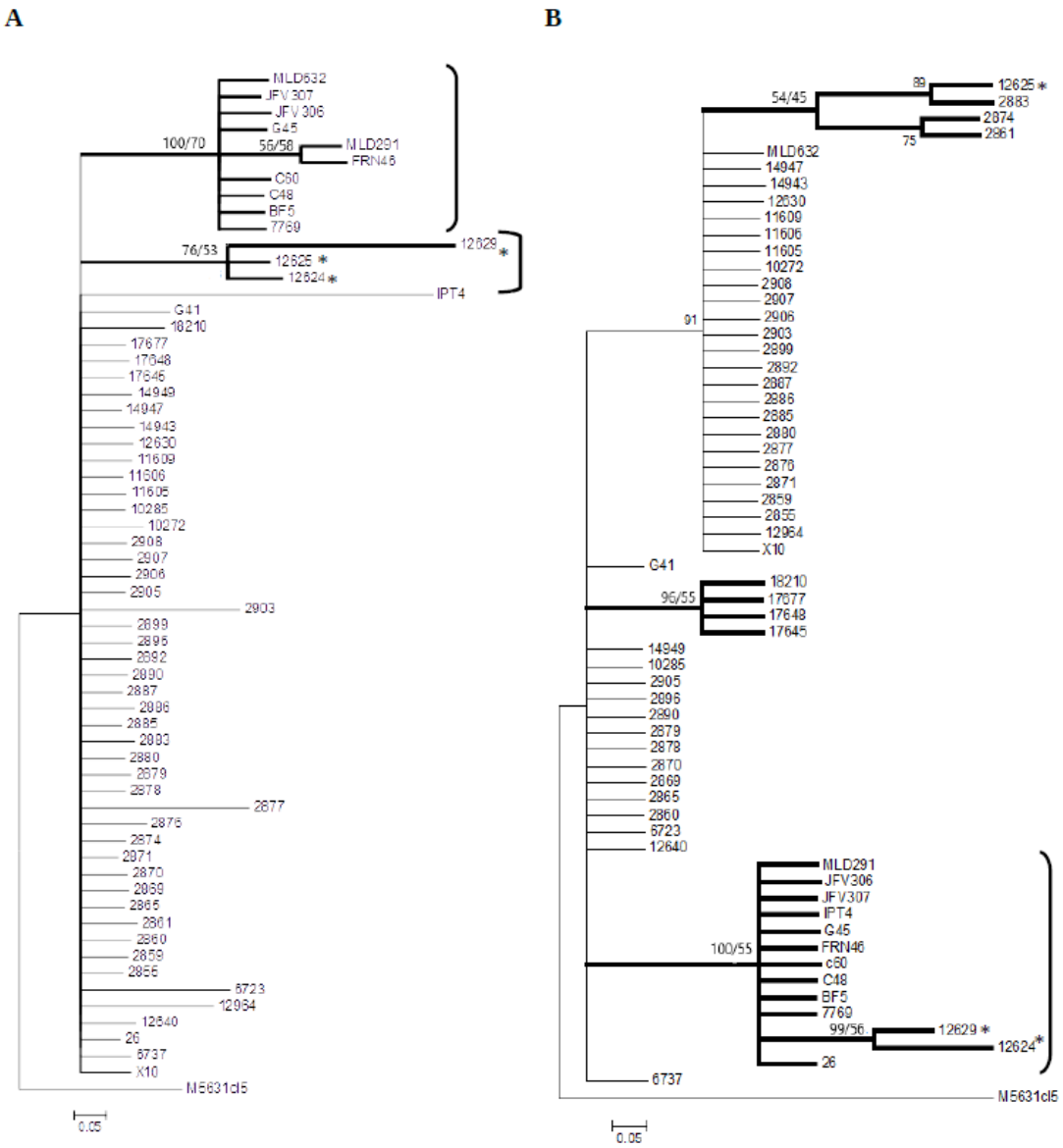
S5 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *RB19*gene fragment. Roman numerals identify clusters that were consistent across both analyses.



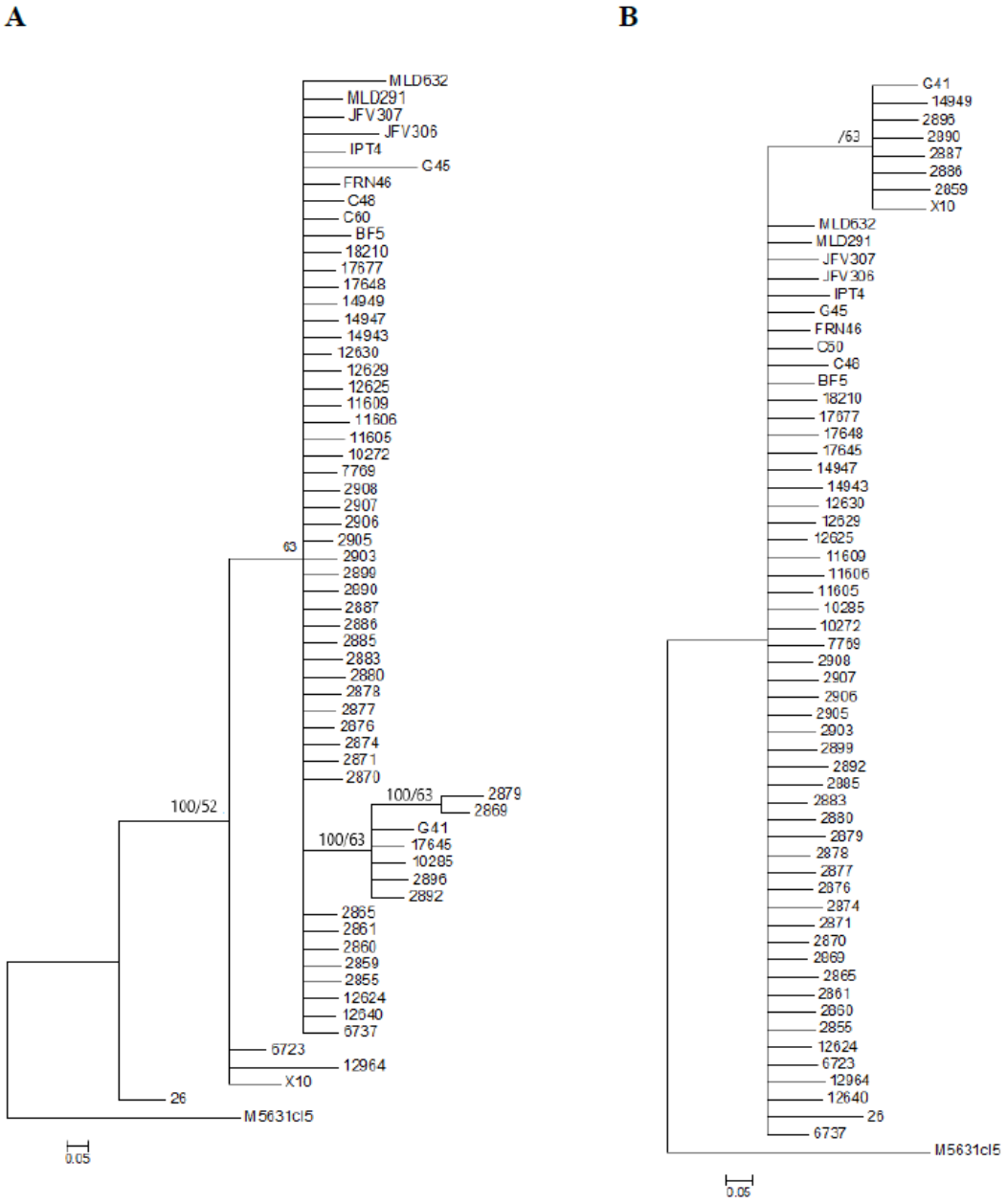
S6 Fig. Neighbor Joining tree (A) and Bayesian tree (B) based in *RHO1* gene fragment. Roman numerals identify clusters that were consistent across both analyses.



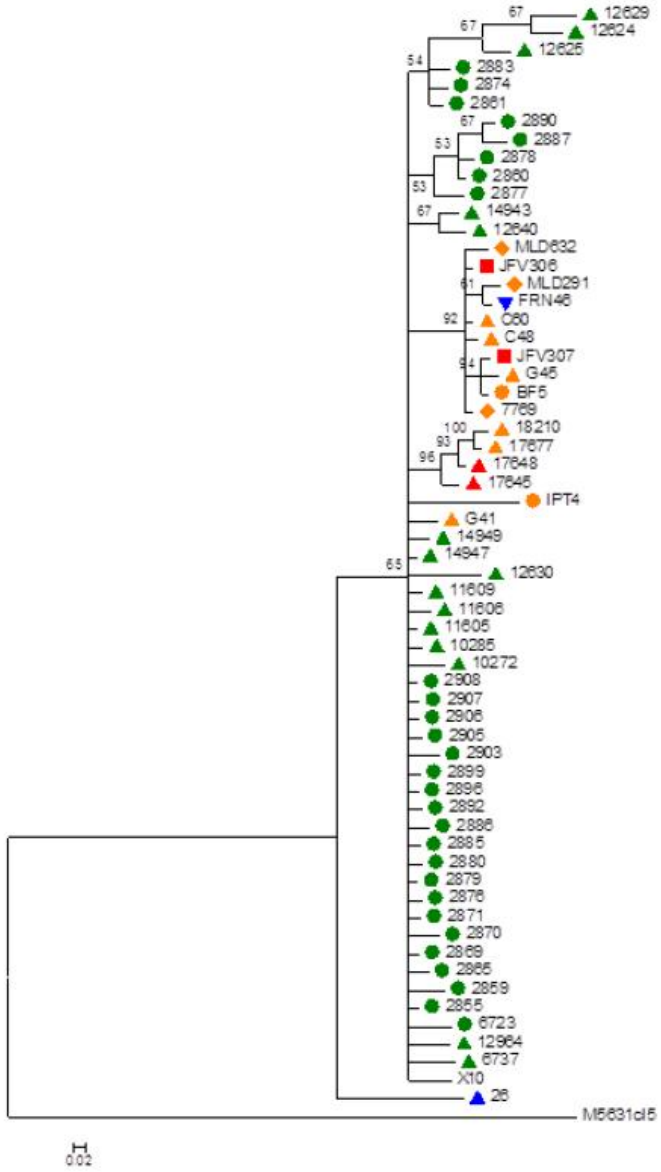
S7 Fig. Trees generated with individual fragments using Bayesian analysis. (A) *CoAR*, (B) *GTP*. Highlighted clusters in each tree indicate agreement between Bayesian analyses and NJ, for which both bootstrap values are shown. Roman numerals identify partial congruence across both markers and asterisks signal outstanding incongruences.



S8 Fig. Trees generated with individual fragments using Bayesian analysis. (A) *RHO1*, (B) *RB19*. Highlighted clusters in each tree indicate agreement between Bayesian analyses and NJ, for which both bootstrap values are shown. Roman numerals identify partial congruence across both markers and asterisks signal outstanding incongruences.

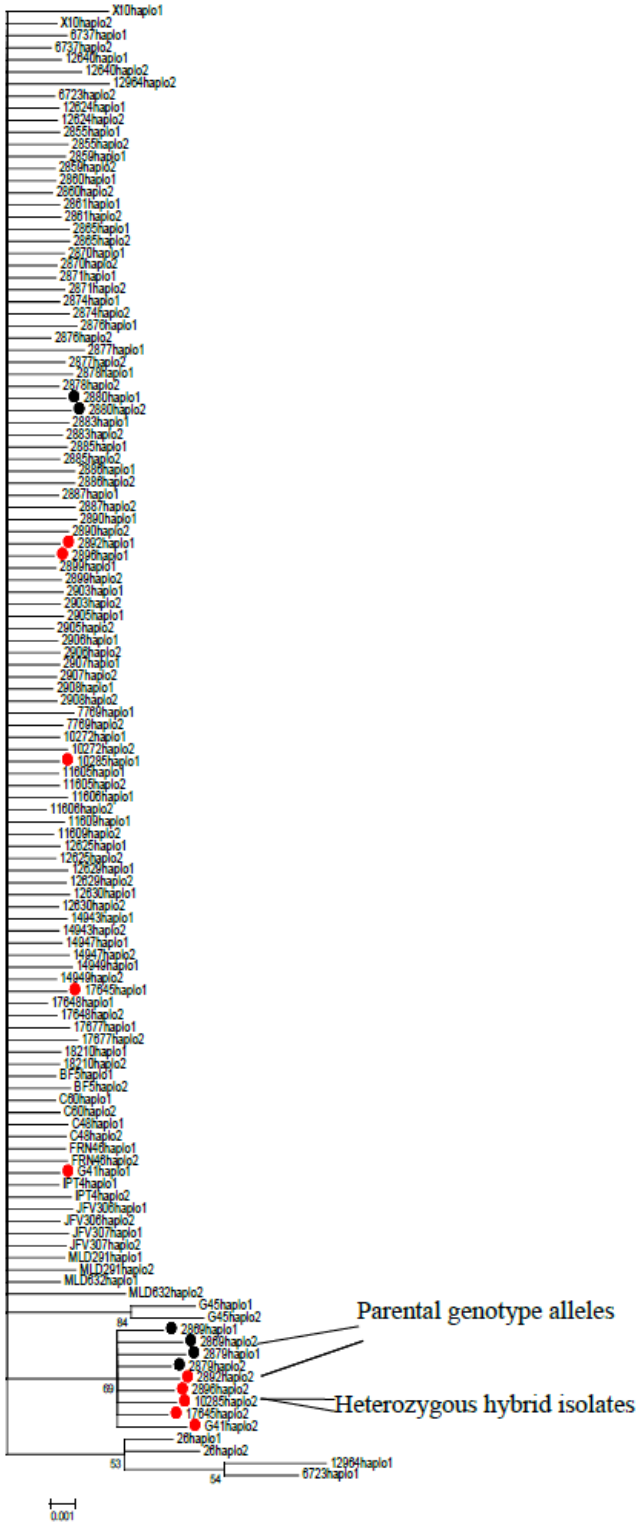


S9 Fig. Trees generated with individual fragments using Bayesian analysis. (A) *PDH* and (B) *LAP*. Bootstrap values (>50%) for Bayesian analysis and NJ are shown. Substantial incongruence were observed.

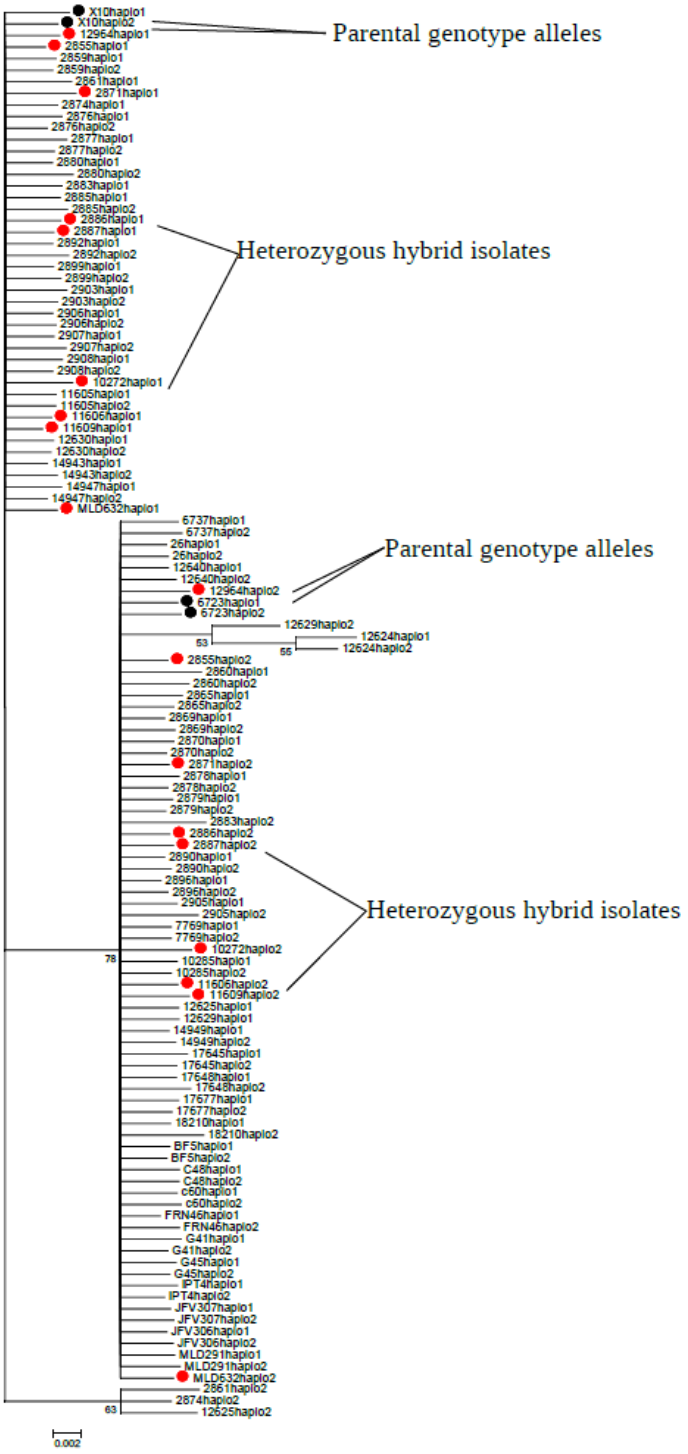


S10 Fig. MLST: Reduced 5 loci combination scheme *(CoAR-GTP-LAP-RHO1-Rb19)*

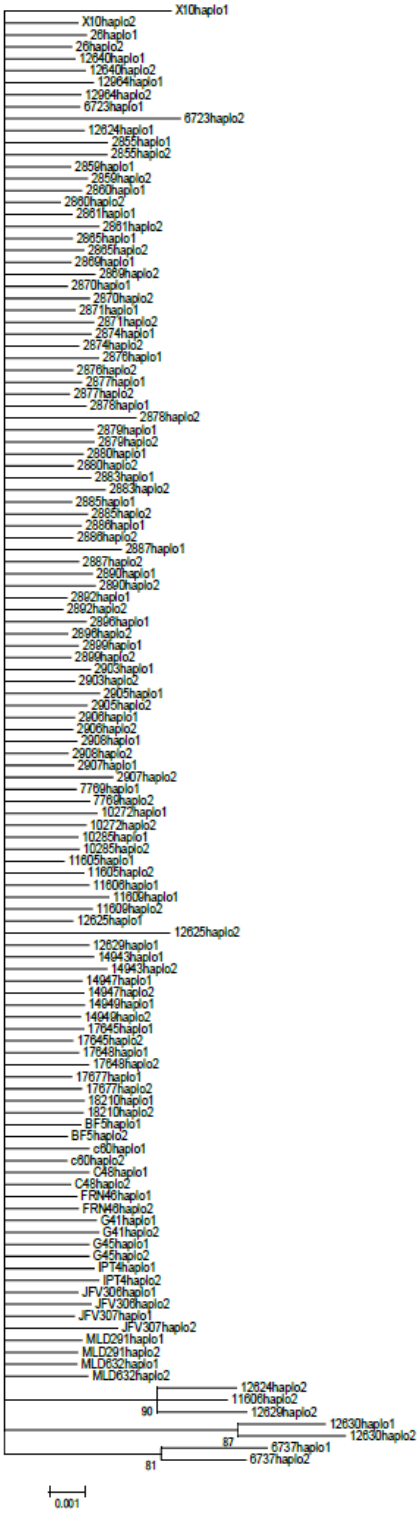
S11 Fig. Topological incongruence based on the six concatenate gene markers. Number represent the fragments topologically incompatible in each branch.



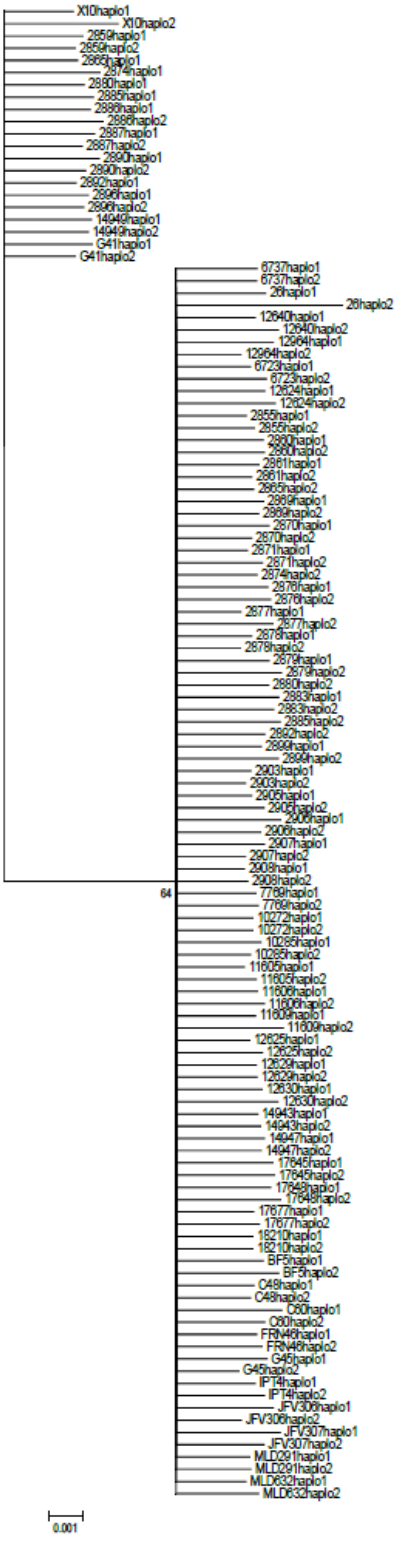
S12 Fig. Haplotypic Bayesian Tree with *PDH* locus. Haplotypes inferred by PHASE V2.1. Red circles indicate heterozygous hybrid isolates, black circles correspond to potential parental alleles.



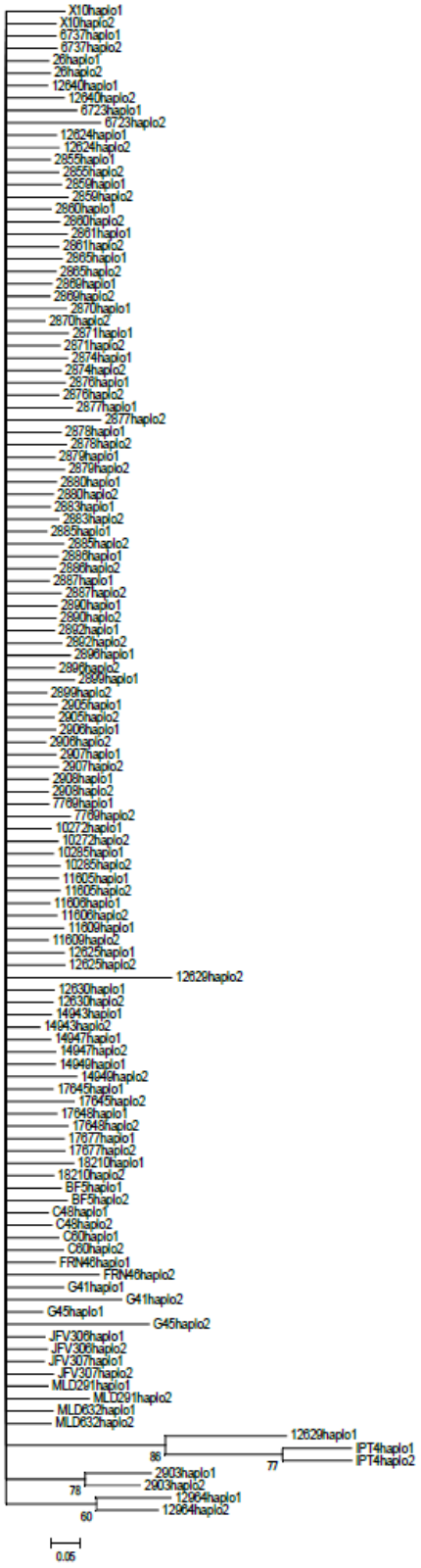
S13 Fig. Haplotypic Bayesian tree with *RB19* locus. Haplotypes inferred by PHASE V2.1. Red circle indicate heterozygous hybrid isolates and black circle correspond to potential parental alleles.



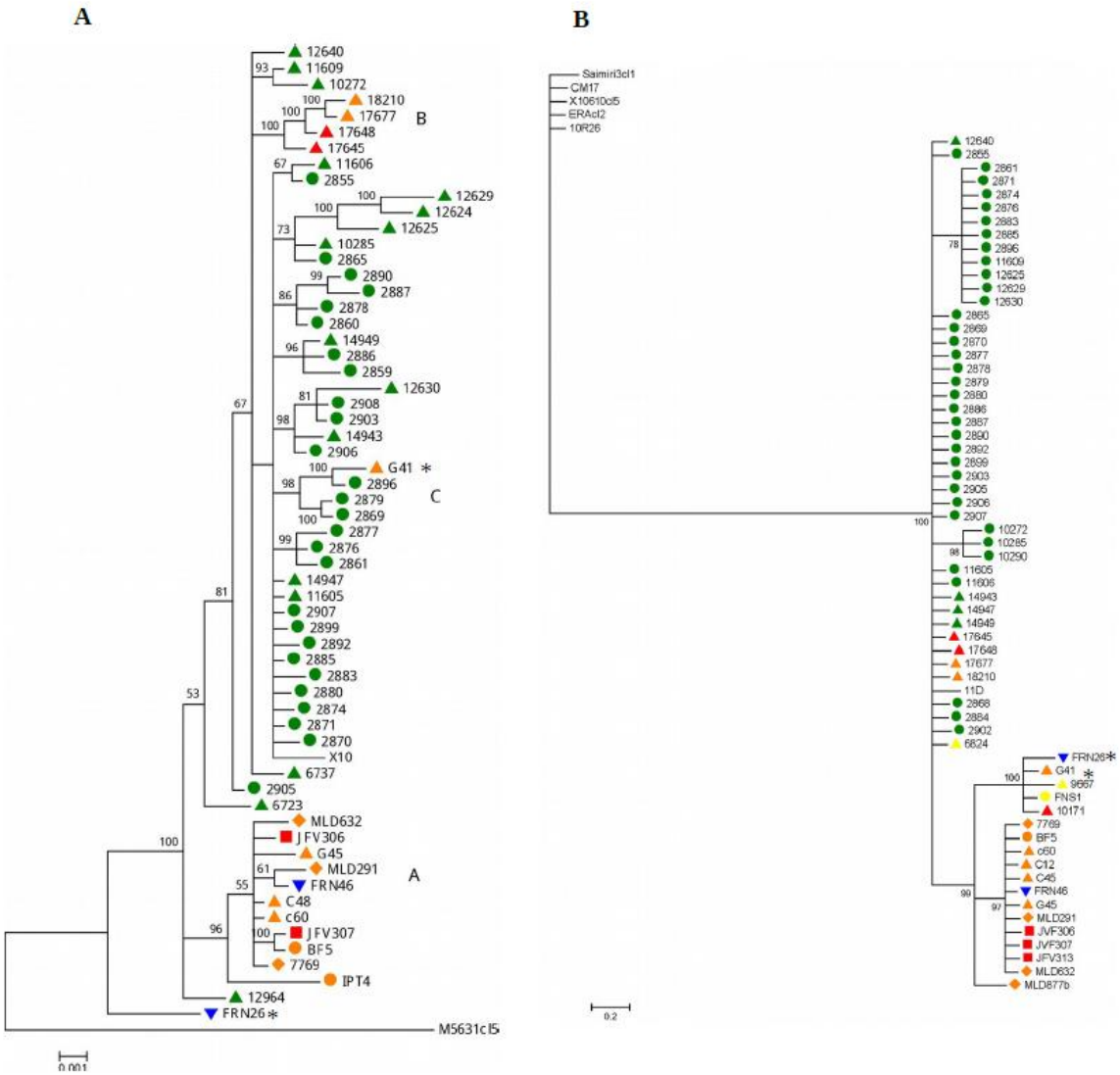
S14 Fig. Haplotypic BayesianTree *COAR* locus. Haplotypes inferred by PHASE V2.1. Heterozygous hybrid isolates were not observed.



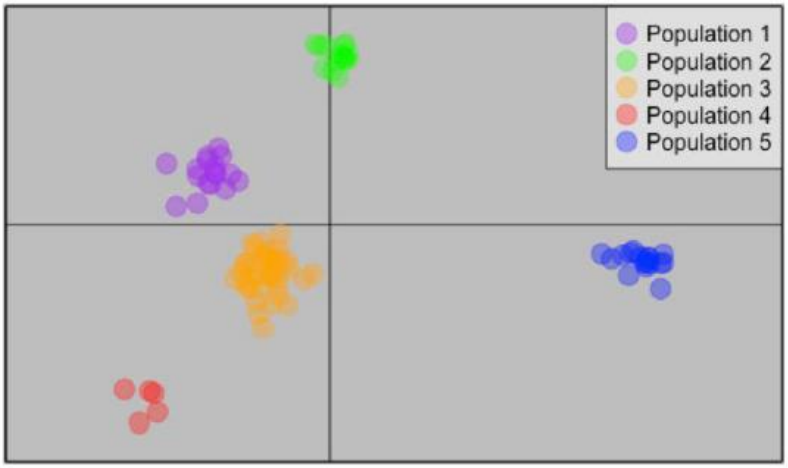
S15 Fig. Haplotypic Bayesian Tree, *LAP* locus. Haplotypes inferred by PHASE V2.1. Heterozygous hybrid isolates were not observed.



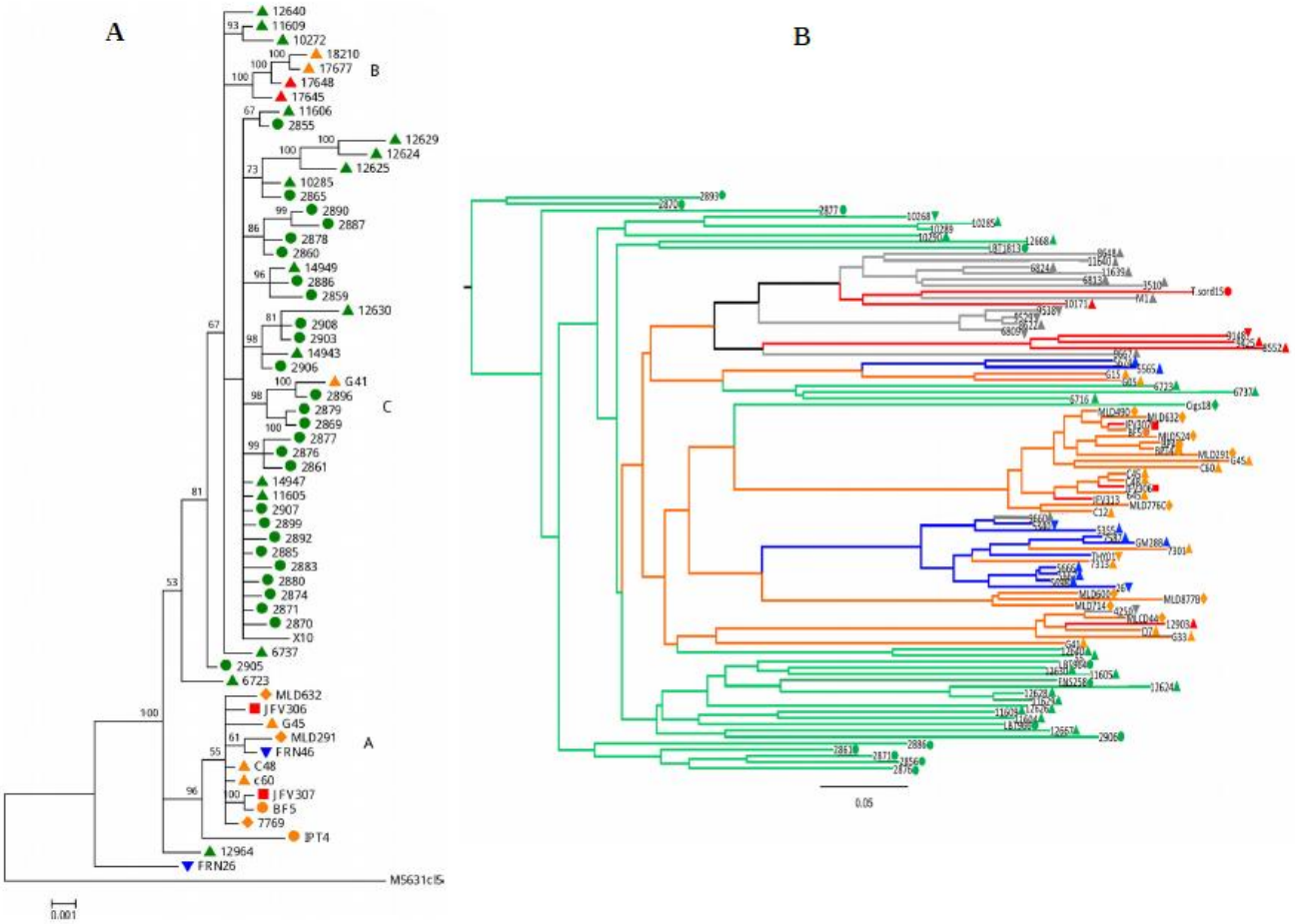
S16 Fig. Haplotypic Bayesian Tree, *RHO1* locus. Haplotypes inferred by PHASE V2.1. Heterozygous hybrid isolates were not observed.



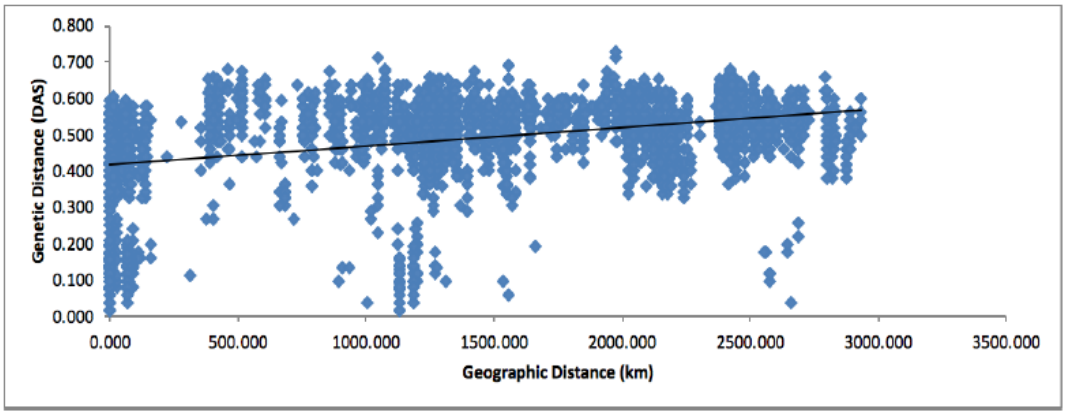
S17 Fig. Comparison between (A) MLST and maxicircle trees (B). Asterisk represent inter-lineage introgression events.



S18 Fig. Nuclear genetic clustering among 92 Brazilian sylvatic TcI strains. Multidimensional scaling plot based on discriminant analysis of principal component (DAPC) analysis for five population clusters defined via *K*-means clustering algorithm (10^9 iterations, over five independent runs, retaining 22 principal components, representing 80% of total variation in the dataset). Individual isolates are represented by dots



S19 Fig. Comparation between (A) MLST and (B) MLMT trees. Biomes are colors coded; red, Cerrado; greed, Amazon; orange, Atlantic Forest; blue, Pantanal; gray, Caatinga.



S20 Fig. Nuclear spatial genetic analysis of *T. cruzi* I isolates from five Brazilian biomes.

S1 Table. *Trypanosoma cruzi* I isolates used in the study

Isolates	Host/vector	Municipality/State	Biome	Year	Latitude	Longitude	MLST	Maxicircle (COII)	MLMT	Genetic cluster based on DAPC
G41	*Didelphis marsupialis*	Guapimirim/RJ	Atlantic Forest	2003	-22.532	-42.990	X	X	X	3
17677	*Didelphis aurita*	Rio de Janeiro/RJ	Atlantic Forest	2012	-22.563	-43.242	X	X		
MLD 291	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	1996	-22.659	-42.383	X	X	X	3
G45	*Didelphis aurita*	Silva Jardim/RJ	Atlantic Forest	2000	-22.659	-42.383	X	X	X	3
MLD 776 (7769)	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	2001	-22.659	-42.383	X	X	X	5
C60	*Philander frenatus*	Teresópolis/RJ	Atlantic Forest	2009	-22.399	-43.011	X	X	X	5
2877	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X	X	3
BF5	*Rhodnius prolixus*	Teresópolis/RJ	Atlantic Forest	1995	-22.399	-43.011	X	X	X	5
MLD 632	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	1996	-22.659	-42.383	X	X	X	5
C48	*Philander frenatus*	Teresópolis/RJ	Atlantic Forest	1994	-22.400	-43.011	X		X	5
2890	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.499	-48.491	X	X		
18210	*Didelphis aurita*	Rio de Janeiro/RJ	Atlantic Forest	2012	-22.940	-43.403	X	X		
IPT4	*Rhodnius prolixus*	Teresópolis/RJ	Atlantic Forest	1992/1994	-22.400	-43.011	X		X	3
2908	*Rhodnius robustus*	Belem/PA	Amazon	2011	-1.488	-48.438	X			
JFV 307	*Phyllostomus albicola*	Arraias/TO	Cerrado	2008	-12.926	-46.935	X	X	X	2
JFV 306	*Carolia perspicillata*	Arraias/TO	Cerrado	2008	-12.926	-46.935	X	X	X	5
17648	*Didelphis marsupialis*	Ananas/TO	Cerrado	2012	-6.405	-48.095	X	X		
17645	*Didelphis* sp	Ananas/TO	Cerrado	2012	-6.405	-48.095	X	X		
FRN 26\26	*Oecomys mamorae*	Aquidauana/MG	Pantanal	2002	-19.575	-56.246	X	X	X	2
FRN 46	*Oecomys mamorae*	Aquidauana/MG	Pantanal	2002	-19.582	-56.246	X	X		
2903	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.488	-48.438	X	X		
2892	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.500	-48.491	X	X		
2878	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.500	-48.491	X	X		
2870	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.500	-48.491	X	X	X	4
2907	*Rhodnius robutus*	Belem/PA	Amazon	2011	-1.488	-48.438	X	X		
2874	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
12640 (55)	*Didelphis marsupialis*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872	X	X	X	3
2885	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
2886	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.499	-48.490	X	X	X	3
2880	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
2905	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.504	-48.449	X	X		
2859	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.499	-48.490	X			
14947	*Philander opossum*	Belem/PA	Amazon	2011	-1.491	-48.451	X	X		
2861	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X	X	3
11609	*Philander opossum*	Abaetetuba/PA	Amazon	2008	-1.718	-48.883	X	X	X	3
2906	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.488	-48.438	X	X	X	3
2896	*Rhodnius robusto*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
2883	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
2899	*Rhodnius robusto*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
109 (LBCE12624)	*Philander opossum*	Abaetetuba/PA	Amazon	2008	-1.776	-48.893	X		X	3
2865	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
11606	*Didelphis marsupialis*	Abaetetuba/PA	Amazon	2008	-1.718	-48.883	X	X		
2879	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
2860	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.499	-48.490	X			
2887	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.501	-48.460	X	X		
14943	*Philander opossum*	Belem/PA	Amazon	2011	-1.499	-48.490	X	X		
2871	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.492	-48.451	X	X	X	3
12630	*Philander opossum*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872	X	X	X	3
88 (LBCE 6723)	*Didelphis marsupialis*	Itupiranga/PA	Amazon	2004	-5.173	-49.366	X		X	3
10285	*Didelphis marsupialis*	Cachoeira do Arari/PA	Amazon	2006	-1.004	-48.957	X	X	X	3
11605	*Philander opossum*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872	X	X	X	3
14949	*Philander opossum*	Belem/PA	Amazon	2011	-1.489	-48.437	X	X		
2876	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.492	-48.451	X	X	X	3
2855	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.492	-48.451	X	X		
2869	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.492	-48.451	X	X		
10272	*Didelphis marsupialis*	Cachoeira do Arari/PA	Amazon	2006	-1.004	-48.957	X	X		
70 (LBCE 12964)	*Didelphis marsupialis*	Curralinho/PA	Amazon	2009	-0.536	-49.184	X			
12629	*Philander opossum*	Abaetetuba/PA	Amazon	2008	-1.758	-49.057	X	X		
2 (LBCE 6737)	*Didelphis marsupialis*	Itupiranga/PA	Amazon	2004	-5.173	-49.366	X		X	3
12625	*Didelphis marsupialis*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872	X	X		
23 (6716)	*Didelphis marsupialis*	Itupiranga/PA	Amazon	2004	-5.173	-49.366			X	3
645	*Didelphis marsupialis*	Teresópolis/RJ	Atlantic Forest	1992	-22.399	-43.011			X	5

2856	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.491	-48.451			X	3
2868	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.491	-48.451		X		
2884	*Rhodnius* sp	Belem/PA	Amazon	2011	-1.491	-48.451		X		
2902	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.489	-48.437		X		
2904	*Rhodnius pictipes*	Belem/PA	Amazon	2011	-1.489	-48.437				
7313	*Didelphis aurita*	Navegantes/SC	Atlantic Forest	2005	-26.899	-48.656			X	5
10290	*Didelphis marsupialis*	Cachoeira do Arari/PA	Amazon	2006	-1.004	-48.957		X	X	
C12	*Philander frenatus*	Teresópolis/RJ	Atlantic Forest	1992/1994	-22.399	-43.011		X	X	3
C45	*Philander frenatus*	Teresópolis/RJ	Atlantic Forest	1992/1994	-22.399	-43.011		X	X	2
G15	*Didelphis marsupialis*	Silva Jardim/RJ	Atlantic Forest	2003	-22.532	-42.990			X	5
JFV 313	*Phyllostomus hastatus*	Arraias/TO	Cerrado	2008	-12.926	-46.935		X	X	5
12628	*Didelphis marsupialis*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872			X	3
G05	*Didelphis* sp	Silva Jardim/RJ	Atlantic Forest	2003	-22.532	-42.990			X	5
Cigs 18	*Saguinus bicolor*	Manaus/AM	Amazon	2000	-3.100	-60.045			X	3
2893	*Rhodnius robusto*	Belem/PA	Amazon	2011	-1.492	-48.450			X	3
9660	*Monodelphis domestica*	Redencao/CE	Caatinga	2006	-4.179	-38.730			X	2
12667*	*Didelphis marsupialis*	Curralinho/PA	Amazon	2009	-0.536	-49.184			X	3
10268*	*Proechimys sp*	Cachoeira do Arari/PA	Amazon	2006	-1.004	-48.957			X	3
10171*	*Didelphis albiventris*	São Raimundo Nonato/PI	Cerrado	2006	-9.967	-45.717		X	X	1
10289*	*Oryzomys sp.*	Cachoeira do Arari/PA	Amazon	2006	-1.004	-48.957			X	3
12668*	*Didelphis marsupialis*	Curralinho/PA	Amazon	2009	-0.536	-49.184			X	3
LBT1813*	*Rhodnius pictipes*	Belém/PA	Amazon	2009	-1.379	-48.476			X	3
8648*	*Didelphis albiventris*	Jaguaruana/CE	Caatinga	2005	-4.831	-37.781			X	1
11640*	*Didelphis albiventris*	Russas/Ceará	Caatinga	2008	-4.939	-37.979			X	1
6824*	*Didelphis albiventris*	Jaguaruana/CE	Caatinga	2004	-4.831	-37.781		X	X	1
11639*	*Didelphis albiventris*	Russas/CE	Caatinga	2008	-4.939	-37.979			X	1
6813*	*Didelphis albiventris*	Jaguaruana/CE	Caatinga	2004	-4.831	-37.781			X	1
3510*	*Didelphis albiventris*	Jaguaruana/CE	Caatinga	2001	-4.831	-37.781			X	1
T sord*	*Triatoma sordida*	Posse/GO	Cerrado	2008	-14.086	-46.371			X	4
M1*	*Didelphis albiventris*	Coronel José Dias/PI	Caatinga	1998	-8.825	-42.506			X	4
9538*	*Rattus rattus*	Jaguaruana/CE	Caatinga	2006	-4.831	-37.781			X	1
9529*	*Rattus rattus*	Jaguaruana/CE	Caatinga	2006	-4.831	-37.781			X	1
8622*	*Didelphis albiventris*	Jaguaruana/CE	Caatinga	2005	-4.831	-37.781			X	1
6809*	*Rattus rattus*	Jaguaruana/CE	Caatinga	2004	-4.831	-37.781			X	1
9148*	*Gracilinanus sp*	Aporé/GO	Cerrado	2006	-18.949	-51.909			X	1
9425*	*Didelphis albiventris*	Aporé/GO	Cerrado	2006	-18.949	-51.909			X	1
8552*	*Didelphis albiventris*	Aporé/GO	Cerrado	2005	-18.949	-51.909			X	5
9667*	*Monodelphis domestica*	Redenção/CE	Caatinga	2006	-4.226	-38.731		X	X	1
5674*	*Monodelphis domestica*	Corumbá/MS	Pantanal	2003	-19.010	-57.655			X	2
5565*	*Didelphis aurita*	Capitão Andrade/MG	Atlantic Forest	2003	-19.070	-41.863			X	3
MLD490*	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	1996	-22.659	-42.383			X	4
MLD524*	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	1996	-22.659	-42.383			X	5
BP4*	*Rhodnius prolixus*	Teresópolis/RJ	Atlantic Forest	1992/1994	-22.399	-43.011			X	3
7301*	*Didelphis aurita*	Navegantes/SC	Atlantic Forest	2005	-26.899	-48.656			X	2
THY01*	*Thylamys macrurus*	Corumbá/MS	Pantanal	2005	-18.992	-56.631			X	2
MLD600*	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	1999	-22.659	-42.383			X	5
MLD877*	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	2003	-22.659	-42.383		X	X	5
MLD714*	*Leontopithecus rosalia*	Silva Jardim/RJ	Atlantic Forest	1998	-22.659	-42.383			X	2
4250*	*Thrichomys apereoides*	São Raimundo Nonato/PI	Caatinga	2001	-9.005	-45.711			X	1
MLCD44*	*Leontopithecus chrysomela*	Ilhéus/BA	Atlantic Forest	2003	-15.269	-39.067			X	4
12903*	*Gracilinanus sp.*	Dianópolis/TO	Cerrado	2009	-11.628	-46.821			X	1
D7*	*Didelphis aurita*	Silva Jardim/RJ	Atlantic Forest	1996	-22.659	-42.383			X	5
G33*	*Didelphis marsupialis*	Silva Jardim/RJ	Atlantic Forest	2000	-22.532	-42.990			X	5
LBT964*	*Rhodnius pictipes*	Abaetetuba/PA	Amazon	2011	-0.536	-49.184			X	3
11629*	*Philander opposum*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872			X	1
12626*	*Micoureus demerarae*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872			X	3
11604*	*Marmosops murina*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872			X	3
LBT966*	*Monodelphis domestica*	Redencao/CE	Caatinga	2008	-4.939	-37.979			X	1
5340*	*Oecomys sp*	Aquidauana/MS	Pantanal	2003	-19.681	-57.338			X	2
5355*	*Monodelphis domestica*	Aquidauana/MS	Pantanal	2003	-19.139	-56.796			X	2
7587*	*Gracilinanus agilis*	Corumbá/MS	Pantanal	2005	-19.010	-57.655			X	2
GM288*	*Nasua nasua*	Corumbá/MS	Pantanal	2007	-19.010	-57.655			X	2
5666*	*Gracilinanus agilis*	Corumbá/MS	Pantanal	2003	-19.139	-56.796			X	2
5667*	*Gracilinanus agilis*	Corumbá/MS	Pantanal	2003	-19.139	-56.796			X	2
5698*	*Gracilinanus sp*	Corumbá/MS	Pantanal	2004	-19.139	-56.796			X	3
FNS258*	*Canis familiaris*	Abaetetuba/PA	Amazon	2008	-1.730	-48.872			X	3

**T. cruzi* I isolates previously published (Lima *et al.* 2014).

MS, Mato Grosso o Sul; **CE**, Ceara; **PA**, Para; **RJ**, Rio de Janeiro; **TO**, Tocantins; **BA**, Bahia; **PI**, Piaui; **SC**, Santa Catarina; **MG**, Minas Gerais; **AM**, Amazonas

S2 Table. MLST gene targets

Gene ID	Chromosome number	Primer sequence (5'3')	Amplicon size (bp)	Fragment length (bp)
CoAR	32	AGGAGGCTTTTGAGTCCACA (20)	554	514
		TCCAACAACACCAACCTCAA (20)		
GTP	12	TGTGACGGGACATTTTACGA (20)	561	521
		CCCCTCGATCTCACGATTTA (20)		
LAP	27	TGTACATGTTGCTTGGCTGAG (21)	444	402
		GCTGAGGTGATTAGCGACAAA (21)		
PDH	40	GGGGCAAGTGTTTGAAGCTA (20)	491	451
		AGAGCTCGCTTCGAGGTGTA (20		
Rb19	29	GCCTACACCGAGGAGTACCA (20)	408	340
		TTCTCCAATCCCCAGACTTG (20)		
RHO1	8	AGTTGCTGCTTCCCATCAAT (20)	455	415
		CTGCACAGTGTATGCCTGCT (20)		

S3 Table. Panel of microsatellite loci and primers.

Chromosome*	Primer code	Repeat type	Forward/Reverse Primer (5'-3')
6	6529$(CA)_a$	$(CA)_n$	TGTGAAATGATTTGACCCGA
			AGAGTCACGCCGCAAAGTAT
10	6855(TA)(GA)	$(TA)_n(GA)_n$	TGTGATCAACGCGCATAAAT
			TTCCATTGCCTCGTTTTAGA
15	11863(CA)	$(CA)_n$	AGTTGACATCCCCAAGCAAG
			CCCTGATGCTGCAGACTCTT
19	TcUn3	Unknown	CTTAAAGAGATACAAGAGGGAAGG
			CTGTTATTTCAATAACACGGGG
19	10101(TA)	$(TA)_n$	AACCCGCGCAGATACATTAG
			TTCATTTGCAGCAACACACA
24	8741(TA)	$(TA)_n$	TGTAACGGTAGGTCTCAATTCG
			TTGCACTTGTGTATCTCGCC
27	10101(TC)	$(TC)_n$	CGTACGACGTGGACACAAAC
			ACAAGTGGGTGAGCCAAAAG
27	10101$(CA)_c$	$(CA)_n$	GTGTCGTTGCTCCCAAACTC
			AAACTTGCCAAATGTGAGGG
27	10101$(CA)_a$	$(CA)_n$	GTCGCCATCATGTACAAACG
			CTGTTGGCGAATGGTCATAA
34	6559(TC)	$(TC)_n$	CGCTCTCAAAGGCACCTTAC
			ATATGGACGCGTAGGAGTGC
37	10187(TTA)	$(TTA)_n$	GAGAGAGATTCGGAAACTAATAGC
			CATGTCCCTTCCTCCGTAAA
37	10187(CA)(TA)	$(CA)_n(TA)_n$	CATGTCATTAAGTGGCCACG

			GCACATGTTGGTTGTTGGAA
37	10187(GA)	$(GA)_n$	GTCACACCACTAGCGATGACA
			ACTGCACAATACCCCCTTTG
37	TcUn2	Unknown	AACAAAATCTAGCGTCTACCATCC
			GGTGTTGGCGTGTATGATTG
37	TcUn4	Unknown	ATGCTCCGCAACATATTACTCA
			GTCGAGCTTCTGTTGTTCCC
39	6925$(TG)_b$	$(TG)_n$	GAAACGCACTCACCCACAC
			GGTAGCAACGCCAAACTTTC
39	6925(CT)	$(CT)_n$	CATCAAGGAAAAACGGAGGA
			CGGTACCACCTCAAGGAAAG
39	7093(TC)	$(TC)_n$	CCAACATTCAACAAGGGAAA
			GCATGAATATTGCCGGATCT
39	7093$(TA)_c$	$(TA)_n$	CGTGTGCACAGGAGAGAAAA
			CGTTTGGAGGAGGATTGAGA
39	7093(TA)b	(TA)n	GGAAACACATCACGCAAAGA
			CTCTCATCTTTTGTTGTGTCCG
39	6925$(TG)_a$	$(TG)_n$	TCGTTCTCTTTACGCTTGCA
			TAGCAGCACCAAACAAAACG
39	7093(TCC)	$(TCC)_n$	AGACGTTCATATTCGCAGCC
			AGCCACATCCACATTTCCTC
40	11283(TCG)	$(TCG)_n$	ACCACCAGGAGGACATGAAG
			TGTACACGGAACAGCGAAG
40	11283$(TA)_b$	$(TA)_n$	AACATCCTCCACCTCACAGG

			TTTGAATGCGAGGTGGTACA
41	10359 (CA)(GA)	$(CA)_n(GA)_n$	AGTCCTACTGCCTCCTTGCA
			CTGTTGGCGAATGGTCATAA

	MS1	MS2	MS3	MS4	MS5	MS6	MS7	MS8	MS9	MS10	MS11	MS12	MS13	MS14	MS15	MS16	MS17	MS18	MS19	MS20	MS21	MS22	MS23	MS24	MS25
2870	104	159	160	111	103	172	183	100	185	147	110	172	173	118	160	154	182	128	140	107	116	147	131	115	169
	104	159	167	115	103	172	185	100	256	151	110	180	173	118	160	154	184	128	140	144	116	149	131	115	169
G45	106	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	134	131	133	171
	106	161	169	111	115	172	196	100	259	147	110	176	178	118	160	152	186	128	140	135	116	134	131	133	171
C60	104	159	160	105	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	171
	106	163	167	111	115	172	196	100	259	147	110	176	178	118	160	152	186	128	140	135	134	136	131	133	171
55	104	159	167	109	103	172	179	100	259	143	110	172	170	118	160	150	182	128	140	107	108	117	131	115	169
	104	163	169	115	105	172	183	100	259	145	110	172	170	118	160	160	188	128	140	132	136	154	131	133	169
2877	104	159	160	115	103	172	183	100	256	145	110	172	166	118	160	152	188	128	140	107	108	117	131	115	169
	104	159	160	115	103	172	183	100	259	151	110	184	176	118	160	154	188	128	140	138	116	149	131	115	169
2886	104	159	160	109	103	172	183	100	255	151	110	180	166	118	160	154	182	128	140	107	108	154	131	115	169
	104	163	174	115	109	172	185	100	259	151	110	180	166	118	160	154	188	128	140	144	143	156	131	133	169
2906	106	159	160	105	103	172	183	100	256	145	110	182	168	118	160	154	184	128	127	132	116	117	134	115	169
	106	159	167	105	109	172	187	100	259	151	110	182	172	118	160	154	184	128	140	138	138	149	134	133	169
2856	104	159	172	115	103	172	183	100	189	147	110	180	166	118	160	154	188	128	140	132	132	152	131	115	169
	104	159	172	115	109	172	188	100	255	151	110	180	166	118	160	154	188	128	140	144	143	156	131	115	169
2876	106	159	158	111	103	172	183	100	255	145	110	180	166	118	160	154	188	128	140	132	116	147	131	115	169
	106	159	172	115	109	172	188	109	255	151	110	180	166	118	160	160	188	128	150	138	116	156	131	115	169
26	104	159	167	105	107	168	183	100	197	143	110	172	175	118	160	154	188	128	140	132	116	117	131	115	169
	104	159	177	105	111	172	198	100	275	147	110	172	178	118	160	154	188	128	140	132	132	145	131	115	169
2861	104	159	160	105	103	172	183	100	255	147	110	182	166	118	160	154	188	128	140	107	116	117	131	115	169
	104	159	172	105	109	172	188	100	255	151	110	182	166	118	160	160	188	128	140	132	116	147	131	115	169
2871	104	159	172	105	103	172	185	100	255	147	110	180	166	118	160	154	188	128	127	132	116	147	131	115	169
	104	159	172	115	109	172	188	100	255	151	110	180	166	118	160	154	188	128	150	144	116	156	131	115	169
2893	104	159	160	111	103	172	183	100	256	147	110	170	173	118	160	154	182	128	127	132	116	117	131	115	169
	104	159	169	115	103	172	183	100	256	151	110	178	175	118	160	160	184	128	140	144	116	147	131	115	169
MLD600	104	159	172	111	107	172	183	100	ND	147	110	168	178	118	160	158	190	128	140	132	116	136	131	115	169
	104	159	176	111	107	172	187	100	285	147	110	168	184	118	160	162	190	128	140	135	116	147	131	115	169
THY01	104	159	167	109	107	167	183	100	273	143	110	172	175	118	160	154	186	128	140	132	116	145	131	115	169
	104	159	177	111	111	170	198	100	275	147	110	172	178	118	160	162	186	128	140	132	116	147	131	115	169
MLD776C	104	159	167	111	113	172	185	100	251	139	110	174	178	118	160	150	182	128	140	132	116	136	131	115	171
	106	159	169	111	115	172	196	100	259	147	110	174	178	118	160	150	186	128	140	135	116	136	131	115	171
MLD632	104	157	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	171
	106	161	169	111	115	172	196	100	259	147	110	176	178	118	160	152	186	128	140	135	116	136	131	115	171
5698	104	159	167	109	107	168	183	100	273	143	110	172	175	118	160	154	188	128	140	132	116	145	131	115	169
	104	159	177	111	111	172	198	100	275	147	110	172	178	118	160	154	188	128	140	132	116	147	131	115	169
645	104	159	167	111	113	172	185	100	251	139	110	174	178	118	160	152	182	128	140	132	116	136	131	115	173
	106	163	169	111	115	172	196	100	259	147	110	174	178	118	160	162	186	128	140	135	116	136	131	115	173
MLD714	104	157	172	111	107	172	183	100	ND	147	110	172	178	118	160	158	190	128	140	132	116	136	131	115	169

	104	159	176	111	107	172	187	100	285	147	110	172	181	118	160	158	190	128	140	135	116	147	131	115	169
D7	106	157	166	111	103	172	183	100	255	143	110	180	170	118	160	156	184	125	138	107	116	142	131	115	169
	106	163	169	111	105	172	183	100	259	147	110	180	170	118	160	160	188	128	142	135	134	142	131	115	169
9425	104	161	172	109	109	172	182	100	251	137	106	170	166	118	160	152	192	128	136	141	116	154	131	115	163
	104	164	172	109	113	172	187	100	ND	143	106	178	166	118	160	156	192	128	142	144	116	156	131	115	169
BF5	104	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	171
	106	161	169	111	115	172	196	100	259	147	110	176	178	118	160	160	186	128	140	135	116	136	131	115	171
7587	104	157	167	109	107	168	183	100	273	143	110	172	175	118	160	154	188	128	140	132	116	145	131	115	169
	104	157	177	109	111	172	198	100	273	147	110	172	178	118	160	154	188	128	140	132	116	147	131	115	169
BP4	104	157	167	111	113	172	185	100	251	139	110	174	178	118	160	152	182	128	140	132	116	136	131	115	171
	106	157	169	111	113	172	196	100	259	147	110	174	178	118	160	152	186	128	140	135	116	136	131	115	171
10289	104	155	174	109	103	170	183	100	256	141	110	180	176	118	160	154	184	128	140	107	116	147	131	112	169
	104	164	174	115	109	172	188	100	259	151	110	182	178	118	160	154	188	128	144	132	116	149	131	115	169
BPT4	104	157	167	111	113	172	185	100	251	139	110	174	178	118	160	152	182	128	140	132	116	136	131	115	171
	106	157	169	111	115	172	196	100	259	147	110	174	178	118	160	152	186	128	140	135	116	136	131	115	171
MLD877B	104	159	172	111	107	172	183	100	251	147	110	166	178	127	160	158	190	128	140	132	116	136	131	115	169
	104	159	176	111	107	172	187	100	ND	147	110	172	181	127	166	162	190	128	140	135	116	147	131	115	169
G33	106	157	166	111	103	170	183	100	255	143	110	178	169	118	160	156	184	125	138	107	116	142	131	115	169
	106	163	169	111	105	170	183	100	259	147	110	178	169	118	160	158	188	128	142	135	116	142	131	115	169
7301	104	157	167	109	107	167	183	100	273	143	110	172	176	118	160	154	186	128	140	132	116	145	131	115	169
	104	157	177	109	111	170	198	100	275	147	110	172	178	118	160	154	186	128	140	132	130	147	131	115	169
8552	104	161	172	109	109	170	181	100	251	137	106	170	166	118	160	156	190	128	136	107	116	122	131	115	163
	104	164	172	111	113	170	187	100	ND	151	108	174	166	118	160	156	190	128	136	141	116	154	131	115	169
G05	104	161	169	109	107	172	183	100	255	143	110	180	175	118	160	158	182	128	144	129	116	136	131	115	169
	106	159	169	111	107	172	187	100	255	153	110	180	175	118	160	158	182	128	144	135	116	141	141	115	169
7313	104	159	158	109	107	168	183	100	273	143	110	172	175	118	160	154	178	128	140	132	116	145	131	115	169
	104	159	167	109	111	172	198	100	281	147	110	172	178	118	160	162	186	128	140	132	116	147	131	115	169
12668	104	157	158	115	103	172	185	100	256	147	110	174	156	118	160	152	180	128	144	107	116	145	131	115	167
	104	161	172	117	103	172	188	100	259	151	110	182	169	118	160	154	184	128	160	132	116	145	131	115	169
5674	104	157	169	109	109	170	183	100	255	143	110	180	175	118	160	154	182	128	142	107	116	136	131	115	169
	106	161	169	115	109	170	187	100	261	153	110	189	175	118	160	154	182	128	144	129	116	136	131	115	169
GM288	104	157	167	109	107	167	183	100	273	143	110	172	175	118	160	154	188	128	140	132	116	145	131	115	169
	104	157	177	109	111	167	198	100	273	147	110	172	178	118	160	154	188	128	140	132	116	147	131	115	169
5355	104	159	167	109	107	172	183	100	261	147	110	172	175	118	160	154	188	128	130	132	130	145	131	115	169
	104	159	177	111	111	172	198	100	275	147	110	172	178	118	160	162	188	128	140	132	130	147	131	115	169
5565	104	159	169	109	109	170	183	100	255	141	110	180	175	118	160	156	182	128	142	107	116	136	131	115	169
	104	161	169	115	109	170	187	100	ND	153	110	182	175	118	160	156	182	128	144	129	126	136	131	115	169
T.SORD15	104	159	166	111	113	172	182	100	251	149	106	178	170	118	160	153	184	125	140	107	116	120	131	115	169
	104	163	166	111	113	172	182	100	251	149	106	178	170	118	160	153	196	128	142	132	116	120	131	115	169
MLD524	106	159	169	111	113	172	185	100	251	139	110	174	178	118	160	152	182	128	140	132	116	136	131	115	171

	106	161	167	111	115	172	196	100	259	147	110	174	178	118	160	152	186	128	140	135	116	136	131	115	171
9667	104	161	172	111	109	170	181	100	261	151	110	176	168	118	160	154	184	128	127	107	116	120	131	115	169
	104	161	172	111	109	170	181	100	261	151	110	176	168	118	160	154	184	128	127	132	116	122	131	115	169
12640	104	159	169	109	105	170	179	100	259	143	110	172	170	118	160	160	182	128	140	132	116	149	131	115	169
	104	161	167	115	105	170	183	100	259	145	110	172	170	118	160	162	188	128	140	138	116	154	131	115	169
JFV306	104	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	173
	106	163	169	111	115	172	196	100	259	147	110	176	178	118	160	162	186	128	140	135	116	136	131	115	173
JFV307	104	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	171
	106	161	169	111	115	172	196	100	259	147	110	176	178	118	160	162	186	128	140	135	116	136	131	115	171
5666	104	159	167	109	107	168	183	100	273	143	110	172	175	118	160	154	188	128	140	132	116	145	131	115	169
	104	159	177	111	111	172	198	100	275	147	110	172	178	118	160	162	188	128	140	132	116	147	131	115	169
5667	104	159	167	109	107	168	183	100	273	143	110	172	175	118	160	154	188	128	140	132	116	145	131	115	169
	104	159	177	111	111	172	198	100	275	147	110	172	178	118	160	160	188	128	140	132	116	147	131	115	169
6809	104	159	167	111	107	172	181	100	259	149	110	178	170	118	160	152	182	128	140	107	116	120	131	115	169
	104	163	172	111	109	172	187	100	ND	151	112	186	180	118	160	158	184	128	142	132	116	149	131	115	169
6824	104	163	172	111	109	172	181	100	259	149	110	178	170	118	160	154	184	128	140	107	116	122	131	115	169
	104	163	172	111	113	172	181	100	261	149	110	178	170	118	160	154	184	128	142	132	116	156	131	115	169
8622	104	159	167	111	107	172	181	100	259	149	110	178	170	118	160	152	182	128	140	107	116	120	131	115	169
	104	163	172	111	109	172	187	100	ND	151	112	186	180	118	160	152	184	128	142	138	116	149	131	115	169
9660	104	159	167	109	107	172	183	100	261	147	110	172	175	118	160	154	188	128	132	132	116	145	131	115	169
	104	159	177	111	111	172	183	100	275	149	110	172	178	118	160	154	188	128	140	132	116	147	131	115	169
11605	104	159	167	111	103	172	183	100	ND	141	106	180	168	118	160	154	184	128	132	132	108	154	131	115	169
	104	159	167	111	109	172	188	100	257	151	112	180	169	118	160	162	184	128	140	138	108	154	141	115	169
10171	104	163	166	111	109	172	181	100	259	149	110	182	170	118	160	154	184	125	140	107	116	152	131	115	169
	104	163	172	111	113	172	181	100	261	149	110	182	170	118	160	156	196	128	140	138	116	154	131	115	169
5340	104	159	167	109	107	172	183	100	261	147	110	172	175	118	160	154	188	128	132	132	116	145	131	115	169
	104	159	177	111	111	172	198	100	275	147	110	172	178	118	160	154	188	128	140	132	116	147	131	115	169
6716	104	163	167	111	109	172	183	100	253	139	110	170	168	118	160	152	182	128	142	107	116	136	131	115	169
	104	163	167	113	109	172	185	100	255	149	110	170	173	118	160	154	186	128	144	135	116	152	141	115	169
6723	104	159	169	113	109	172	183	100	257	143	110	170	169	118	160	152	182	128	140	107	116	136	141	112	169
	104	161	169	113	109	172	185	100	261	149	110	180	176	118	160	153	184	128	142	135	116	145	141	112	169
LBT964	104	159	167	111	103	172	179	100	255	147	110	170	168	118	160	150	182	128	130	107	116	154	131	115	169
	104	164	167	119	109	172	188	100	257	153	112	180	170	118	160	156	184	128	140	138	136	156	141	115	169
11604	104	159	167	111	103	172	183	100	257	141	110	182	169	118	160	154	184	128	130	138	116	154	131	112	169
	104	159	167	119	107	172	188	100	259	141	110	182	169	118	160	154	188	128	130	138	116	156	131	115	169
FNS258	104	159	167	111	109	172	183	100	255	141	106	182	168	118	160	154	182	128	130	107	116	156	131	115	169
	104	164	167	111	109	172	183	100	259	149	112	182	168	118	160	162	184	128	138	138	116	156	131	152	169
LBT966	106	159	167	111	109	172	183	100	257	151	110	172	169	118	160	154	182	128	130	107	116	145	131	115	169
	108	161	172	119	111	172	194	100	259	151	110	172	169	118	160	160	184	128	130	138	116	145	131	115	169
C12	104	159	169	111	113	172	185	100	253	139	110	174	178	118	160	152	182	128	140	132	116	136	131	115	169

	106	159	167	111	115	172	196	100	259	147	110	174	178	118	160	152	186	128	140	135	116	136	131	115	171
12626	104	159	167	109	109	172	183	100	256	145	110	172	168	118	156	150	184	128	140	132	116	152	131	115	169
	106	159	169	111	109	172	183	100	256	149	112	180	170	118	160	154	184	128	146	138	116	156	131	115	169
12624	104	167	100	159	105	172	183	100	257	151	106	174	168	118	160	150	182	128	130	107	116	156	131	115	169
	106	167	111	163	109	172	194	100	259	151	112	180	168	129	160	154	188	128	146	138	116	156	131	115	169
12628	104	159	167	100	105	172	183	100	257	151	106	174	168	118	160	150	182	128	130	107	116	145	131	115	169
	106	163	167	111	109	172	194	100	259	151	112	180	168	118	160	154	188	128	144	138	116	156	131	115	169
11640	104	163	172	111	109	172	181	100	257	143	106	178	170	118	160	154	184	125	140	132	116	122	131	115	169
	104	163	172	111	109	172	181	100	259	145	110	182	176	118	160	156	196	128	142	132	116	154	131	115	169
11639	104	163	172	111	109	172	181	100	259	149	106	178	170	118	160	154	184	128	142	107	116	122	131	115	169
	104	163	172	111	113	172	181	100	259	149	106	178	170	118	160	162	184	128	142	132	116	154	131	115	169
11629	104	159	167	100	105	172	183	100	257	151	106	174	168	118	160	150	182	128	130	107	116	156	131	115	169
	106	163	167	111	109	172	194	100	259	151	112	180	168	118	160	154	188	128	146	138	116	156	131	115	169
JFV313	104	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	169
	106	163	169	111	115	172	196	100	259	147	110	176	178	118	160	160	186	128	140	135	116	136	131	115	173
6813	104	161	172	111	109	172	181	100	259	149	106	178	170	118	160	152	184	128	146	107	116	120	131	115	169
	104	161	172	111	109	172	181	100	259	149	110	178	170	118	160	154	184	128	146	132	116	154	131	115	169
9538	104	159	167	111	107	172	181	100	259	147	110	178	170	118	160	152	182	128	140	132	116	120	131	115	169
	104	163	172	111	109	172	187	100	ND	149	112	186	180	118	160	152	184	128	142	138	116	149	141	115	169
8648	104	163	172	111	109	172	181	100	251	151	106	176	170	118	160	154	182	125	140	107	116	152	131	115	169
	104	163	172	111	111	172	181	100	251	151	106	178	170	118	160	154	184	128	142	132	116	154	131	115	169
3510	104	163	172	111	109	172	181	100	251	143	110	178	170	119	160	152	184	128	142	107	116	120	131	115	169
	104	163	172	111	109	172	181	100	259	143	110	178	170	119	160	152	184	128	142	132	116	154	131	115	169
9529	104	159	167	111	107	172	181	100	259	147	110	178	170	118	160	152	182	128	140	107	116	120	131	115	169
	104	163	172	111	109	172	187	100	ND	149	112	186	180	118	160	152	184	128	142	138	116	149	131	115	169
4250	106	157	166	111	103	172	183	100	255	143	110	178	170	118	160	156	184	125	138	107	116	142	131	115	169
	106	163	169	111	105	172	183	100	259	147	110	178	170	118	160	160	188	128	142	135	116	142	131	115	169
M1	104	159	166	111	111	172	181	100	251	149	110	176	170	118	160	152	196	125	142	107	116	122	131	115	169
	104	163	166	111	113	172	187	100	259	149	110	178	170	118	160	156	196	128	142	138	116	154	131	115	169
C48	104	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	173
	106	163	169	111	115	172	196	100	259	147	110	176	178	118	160	152	186	128	140	135	116	136	131	115	173
MLD291	104	159	167	111	113	172	185	100	251	139	110	166	178	117	160	152	182	128	140	132	116	136	131	115	171
	106	161	169	111	115	172	196	100	259	147	110	166	178	129	160	152	186	128	140	135	116	136	131	115	171
G15	104	159	169	109	107	172	183	100	255	141	110	180	175	118	160	156	182	128	142	129	116	141	131	115	169
	106	161	169	113	109	172	187	100	259	151	110	180	175	118	160	158	182	128	144	135	116	141	141	115	169
10290	104	159	167	109	103	172	183	100	255	151	110	184	169	118	160	150	184	128	138	107	116	147	131	115	169
	104	164	172	109	109	172	183	100	255	151	110	184	176	118	160	154	190	128	144	132	116	147	131	115	169
Cigs18	106	152	167	109	101	170	183	100	257	135	110	174	159	118	158	152	184	125	140	132	116	136	131	115	169
	106	155	172	119	107	174	185	100	259	145	110	176	166	118	160	158	186	128	140	135	116	149	131	115	169
6737	104	164	167	113	109	170	183	100	253	147	110	172	169	117	160	154	186	128	142	107	116	147	141	112	169

	106	164	167	113	109	172	185	100	261	147	110	176	169	117	160	162	186	128	144	132	116	152	141	115	169
MLCD44	106	159	166	111	103	172	183	100	255	143	110	178	170	118	160	156	184	125	138	107	116	142	131	115	169
	106	163	169	111	105	172	183	100	259	147	110	178	170	117	160	160	188	128	142	135	116	142	131	115	169
G41	104	157	167	109	103	172	183	100	256	141	110	172	173	117	160	150	182	125	140	107	116	142	131	115	169
	106	164	167	111	105	172	190	100	259	151	110	184	178	117	160	156	184	128	140	132	116	149	131	115	169
C45	104	161	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	173
	106	159	169	111	115	172	196	100	259	147	110	176	178	118	160	152	186	128	140	135	116	136	131	115	173
12630	104	159	167	111	105	172	183	100	257	149	110	174	169	118	160	150	184	128	130	132	116	147	131	115	169
	106	161	167	111	109	172	194	100	257	149	112	178	169	118	160	154	184	128	140	138	116	154	141	115	169
11609	104	159	167	119	103	172	179	100	257	145	110	176	170	118	160	154	184	128	130	107	116	154	131	115	169
	104	159	167	119	105	172	183	100	257	151	110	184	170	118	160	154	184	128	130	138	116	156	131	115	169
12903	106	155	166	111	103	170	183	100	255	143	110	180	170	118	160	156	184	125	138	107	116	142	131	115	169
	106	161	169	111	105	170	183	100	259	147	110	180	170	118	160	160	188	128	142	135	116	142	131	115	169
10285	104	155	174	109	103	170	183	100	256	141	110	180	176	118	160	154	184	128	140	107	116	147	131	112	169
	104	164	174	115	109	170	188	100	257	151	110	182	178	118	160	154	188	128	144	132	116	149	131	115	169
10268	104	159	167	109	107	170	183	100	256	151	110	180	180	118	160	154	186	128	140	107	116	147	131	115	169
	104	159	169	115	109	170	188	100	259	151	110	184	180	118	160	154	188	128	144	132	116	154	131	115	169
12667	106	159	169	111	103	172	191	100	256	139	110	182	156	118	160	154	186	128	142	107	116	145	131	115	169
	106	159	172	117	109	172	191	100	259	151	110	180	168	118	160	154	188	128	154	138	116	152	141	115	169
MLD490	104	159	167	111	113	172	185	100	251	139	110	176	178	118	160	152	182	128	140	132	116	136	131	115	169
	106	161	169	111	115	172	196	100	259	147	110	176	178	118	160	152	186	128	140	135	116	136	131	115	171
LBT1813	104	157	167	115	103	172	190	100	255	145	110	182	168	118	160	156	182	128	140	132	116	149	131	115	169
	104	157	172	115	103	172	190	100	259	151	110	186	175	118	160	158	186	128	142	138	116	156	131	115	169
9148	104	161	172	109	107	170	181	100	251	137	106	170	166	118	160	152	192	128	138	141	116	154	131	115	163
	104	164	172	111	109	170	187	100	ND	143	110	176	166	118	160	156	192	128	142	144	116	154	131	115	169

ND=no data

S5 Table. Population genetic parameters for *a posteriori* sylvatic populations of TcI in Brazil

Population*	G/N	PL	PA/L ± SE	Ar ± SE	D_{AS} ± SD	Ho	He	%PL He	% PL Hd	F_{IS}±SE
1	17/17	20	0.61 ± 0.17	2.68 ± 0.31	0.403 ± 0.138	0.34	0.41	5	35	0.224 ± 0.08
2	15/15	18	0.72 ± 0.28	2.14 ± 0.27	0.331 ± 0.172	0.34	0.32	33.3	11.1	0.043 ± 0.14
3	38/38	25	1.26 ± 0.20	3.46 ± 0.27	0.491 ± 0.092	0.42	0.52	4	48	0.201 ± 0.04
4	5/5	15	0.06 ± 0.067	1.76 ± 0.39	0.459 ± 0.079	0.42	0.31	0	6.7	-0.397 ± 0.36
5	17/17	17	0.27 ± 0.12	1.74 ± 0.17	0.378 ± 0.208	0.37	0.27	52.9	29.4	-0.207 ± 0.19

***Population designation based on *a posteriori* DAPC cluster assignment.**

N: number of isolates in population; G: number of multilocus genotypes (MLGs) per population based on microsatellite data of 25 loci analyzed; PL: number of polymorphic loci out of 25 loci analysed; PA/L: mean number of private alleles per locus ± SE, calculated in HP-Rare (Kalinowski, 2005); Ar: allelic richness as a mean over loci ± SE, calculated in FSTAT 2.9.3.2 (Goudet, 1995); D_{AS}: mean pairwise allele sharing ± SD, calculated in MICROSAT v1.5d (Minch, 1997); Ho: mean observed heterozygosity across all loci, calculated in Arlequin v3.11 (Excoffier, 2005); He: mean expected heterozygosity across all loci, calculated in Arlequin v3.11 (Excoffier, 2005); %PL He: proportion of polymorphic loci showing a significant excess in heterozygosity after a sequential Bonferroni correction (Rice, 1989), calculated in Arlequin v3.11 (Excoffier, 2005); %PL Hd: proportion of polymorphic loci showing a significant deficit in heterozygosity after a sequential Bonferroni correction (Rice, 1989), calculated in Arlequin v3.11 (Excoffier, 2005); F_{IS}: mean fixation index ± SE, calculated in FSTAT 2.9.3.2 (Goudet, 1995).

S6 Table. F_{ST} values in a five- way comparison between *a posteriori* populations (P<0.001)

	Population 1	Population 2	Population 3	Population 4	Population 5
Population 1	*				
Population 2	0.332	*			
Population 3	0.153	0.177	*		
Population 4	0.317	0.456	0.225	*	
Population 5	0.388	0.422	0.289	0.508	*

S7 Table. Pairwise F_{ST} values for microsatellite data grouped according to the parasites' hosts.

	D_albiventris	*D_aurita*	*D_marsupialis*	Leontopithecus	Monodelphis	*P_opossum*	*P_frenatus*	Chiroptera	Rhodnius	Gracilinanus	*R_rattus*
D_albiventris		0.31	0.18	0.27	0.29	0.26	0.39	0.35	0.24	0.14	0.19
D_aurita	0.31		0.14	0.22	0.11	0.33	0.37	0.33	0.20	0.11	0.36
D_marsupialis	0.18	0.14		0.13	0.15	0.17	0.20	0.15	0.10	0.10	0.18
Leontopithecus	0.27	0.22	0.13		0.22	0.30	0.20	0.11	0.20	0.19	0.25
Monodelphis	0.29	0.11	0.15	0.22		0.28	0.37	0.33	0.17	0.13	0.34
P_opossum	0.26	0.33	0.17	0.30	0.28		0.42	0.38	0.18	0.25	0.29
P_frenatus	0.39	0.37	0.20	0.20	0.37	0.42		0.01	0.29	0.31	0.40
Chiroptera	0.35	0.33	0.15	0.11	0.33	0.38	0.01		0.26	0.25	0.40
Rhodnius	0.24	0.20	0.10	0.20	0.17	0.18	0.29	0.26		0.18	0.25
Gracilinanus	0.14	0.11	0.10	0.19	0.13	0.25	0.31	0.25	0.18		0.25
R_rattus	0.19	0.36	0.18	0.25	0.34	0.29	0.40	0.40	0.25	0.25	
median	0.27	0.26	0.15	0.21	0.25	0.29	0.34	0.30	0.20	0.18	0.27
average	0.26	0.25	0.15	0.21	0.24	0.29	0.30	0.26	0.21	0.19	0.29
	D_albiventris	***D_aurita***	***D_marsupialis***	**Leontopithecus**	**Monodelphis**	***P_opossum***	***P_frenatus***	**Chiroptera**	**Rhodnius**	**Gracilinanus**	***R_rattus***
	Cerrado	Atlantic Forest	Atlantic Forest	Atlantic Forest	Pantanal	Amazon	Atlantic Forest	Cerrado	Atlantic Forest	Pantanal	Caatinga
	Caatinga		Amazon		Caatinga				Amazon	Cerrado	

FDR = 0,016

* 0,016 < p < 0,05

** 0,001 < p < 0,016

*** p < 0,001

5 DISCUSSÃO

Trypanosoma cruzi é um parasito heterogêneo paninfectivo, cuja origem é estimada como tendo ocorrido há aproximadamente 80 a 100 milhões de anos, coincidente com a separação do supercontinente Gondwana, que incluía África, América do Sul, Antártida e Austrália (Stevens *et al.*, 1999; Stevens e Gibson, 1999; Stevens *et al.*, 2001). As subpopulações do parasito foram organizadas em 6 genótipos ou Unidades Discretas de Tipagem ou DTU (*Discrete Typing Units*), TcI-TcVI, além de uma provável linhagem TcVII, TcBat, cuja ecologia ainda é desconhecida. Provavelmente, como consequência da heterogeneidade intra DTU e ecologia de outras DTUs, apresentam alguns resultados controversos. A possibilidade de observar essas diferenças genéticas existentes em *T. cruzi* e outros parasitos, foi devido ao incremento do poder analítico das ferramentas moleculares. Esse foi o caso da DTU I de *T. cruzi*, para o qual, a alta diversidade vem sendo demonstrada há vários anos (Herrera *et al.*, 2007; Falla *et al.*, 2009; Llewelyn *et al.*, 2011, Messenger *et al.*, 2012; Ramirez *et al.*, 2013).

Por se tratar da DTU com a mais ampla distribuição na natureza, a diversidade genética de *T. cruzi* I vem sendo estudada por vários autores cuja hipótese propõe uma subdivisão das populações de TcI, de acordo com áreas geográficas, reservatórios, vetores, ciclos de transmissão e até manifestações clínicas da doença humana (Ramirez *et al.*, 2013; Ramirez e Hernandez 2017). Com o objetivo de testar essa hipótese, o primeiro passo desses autores foi o sequenciamento da região intergênica dos genes mini-exon de isolados de TcI (Herrera *et al.*, 2007). Essa análise que incluiu isolados do ciclo silvestre e do ciclo domiciliar da Colômbia, resultou na proposta de subdividir TcI em quatro haplótipos. A partir daí a série de análises que foram realizadas utilizando como marcadores genéticos o citocromo b, PCR-RFLP, MLST, MLMT (Herrera *et al.*, 2007; Llewellyn *et al.*, 2009; Diosque *et al.*, 2014) geraram resultados não conclusivos. Ramirez e Hernandes em 2017, concluíram que a alta diversidade observada em TcI, tornava pouco provável que se pudesse estabelecer qualquer tipo de associação robusta com alguma das variáveis mencionadas acima.

As análises moleculares de alta resolução que foram aplicadas a um grande painel de isolados de TcI derivados de um amplo espectro de mamíferos silvestres de cinco biomas, apresentados nos dois trabalhos que compõem essa tese, confirmam a grande diversidade genética presente no genótipo TcI de *T. cruzi.*

A nossa hipótese em relação à ausência de associações de subgrupos de TcI com biomas ou espécies de hospedeiros, foi parcialmente confirmada, devido a que não foram

observadas associações claras, embora os testes estatísticos mostraram que eventuais associações não podem ser descartadas de todo.

A competência de Didelphis sp. como bioacumulador dos genótipos de TcI foi confirmada pelas análises de MLMT, reforçando a importância deste táxon como reservatório de *T. cruzi* e principalmente de TcI e de suas subpopulações. Outro ponto muito discutido é a estrutura populacional de *T. cruzi*. Nossos resultados mostraram a presença de eventos de introgressão mitocondrial e incongruências entre árvores filogenéticas geradas com genes constitutivos. No entanto, os resultados sobre os eventos de intercâmbio genético não foram robustos, o que sugere que esses eventos são raros embora possam ocorrer.

5.1 Diversidade genética intra TcI

As análises aqui apresentadas sobre a diversidade genética de isolados brasileiros de *T. cruzi* I confirmam a expressiva heterogeneidade e diversidade em DTU I de *T. cruzi*. A comparação entre genes de taxa de evolução rápida (gene do maxicírculo e MLMT) e genes de evolução lenta (MLST), foram realizados pela primeira vez em um mesmo painel de amostras de TcI do Brasil.

O poder de discriminação dos genes constitutivos diferiu entre eles e entre os resultados de outros autores (Yeo *et al*., 2011; Ramirez et al., 2013; Diosque *et al.*, 2014). Os fragmentos de genes analisados mostraram divergência em relação à diversidade genética entre eles e, ainda mais, em relação aos resultados de outros autores, mesmo sendo genes constitutivos de evolução lenta. Isso indica que esses genes constitutivos não podem ser considerados como marcadores universais para estudo da diversidade intra TcI.

Fragmentos de genes que, em trabalhos anteriores foram identificados como sendo altamente variáveis, e.g. genes *DHFR-TS* de acordo com Yeo *et al.*, (2011) e *CoAR* de acordo com Diosque *et al.*, (2014), em nossos estudos apresentaram baixos valores de eficiência de tipificação (TE) e poder de discriminação (DP), além de apresentar baixa variabilidade genética em relação aos demais genes estudados.

Os genes *RB19*, *GPI* e *LAP*, foram considerados não informativos em estudos de diversidade genética das seis DTUs de *T. cruzi* (Yeo *et al.*, 2011; Diosque *et al.*, 2014). Contrariamente ao esperado, o *RB19* foi um marcador altamente informativo em isolados de TcI do Brasil, evidenciando diversidade de subpopulações de TcI. As variações no TE são geralmente os resultados de pressões seletivas em loci individuais, deriva gênica ou diferenças nas taxas de mutações. Nossos resultados não permitem explicar as diferenças do poder de discriminação da diversidade de TcI dos fragmentos de genes constitutivos estudados

utilizados pelos diferentes autores. O que poderia explicar os nossos resultados é que o enorme universo que compõe a subpopulação *T. cruzi* I, sua ampla dispersão por uma diversidade de ambientes e espécies hospedeiras, torna questionável a representatividade das amostras.

O aumento dos valores de suporte observados nas árvores geradas no segundo trabalho, se deve provavelmente à duplicação dos SNPs e a análise bayesiana. Quando foram geradas árvores com o método *average state* e NJ em MLSTest, foram observadas diferenças nesses valores de suporte. Portanto, para estudos da diversidade genética em TcI, o método de *average state* e NJ, utilizado para organismos diploides, são recomendados para evitar valores de suporte e clusters fictícios. No entanto, as análises mostraram clusters semelhantes tanto em árvores com os fragmentos individuais como a árvore com os fragmentos concatenados.

Quanto ao fragmento de gene do maxicirculo, o baixo nível de diversidade observado foi surpreendente, uma vez que se trata de um gene de evolução rápida. Essa baixa diversidade é consistente com a presença de eventos de introgressão, reportados em várias populações de TcI em América Latina (Llewellyn *et al.*, 2011; Carrasco *et al.*, 1996).

5.2 Intercâmbio genético em TcI

A capacidade de recombinação em *T cruzi*, e particularmente em TcI, tem sido motivo um debate extenso, intenso e de décadas, (Tibayrenc *et al.*, 1990; Tibayrenc and Ayala, 1991; 2012; 2013; 2014[a]; Ramírez e Llewellyn, 2014; Tomasini *et al.*, 2014[a]; 2014[b]). O modelo de reprodução reconhecido em *T cruzi* é predominantemente clonal, embora, a utilização de marcadores polimórficos para a análises de populações levaram a alguns autores à conclusão de que intercâmbios genéticos ocorrem em condições experimentais (Gaunt *et al.*, 2003; Carrasco *et al.*, 1996).

Um dos indicadores clássicos da provável ocorrência de eventos de intercâmbio genético, são as incongruências entre as topologias das árvores geradas com os genes individuais. As incongruências topológicas já haviam sido observadas em isolados TcI por outros autores (Dingle *et al.*, 2001; Ramirez & Hernandez *et al.* 2017).

Em nossos dois trabalhos aqui apresentados, foram observados fortes indícios de eventos de intercâmbio genético, como mostra a presença das incongruências topológicas nas árvores filogenéticas. As incongruências topológicas observadas eram moderadas, consistentes com uma estrutura populacional de tipo 2, segundo os parâmetros de Tomasini *et al.*, (2014). Esse tipo de estrutura populacional, inclui árvores com incongruência em alguns

clusters, mas com uma topologia que não pode ser totalmente resolvida na análise de MLST (Tomasini *et al.*, 2014). As politomias nas árvores geradas com os fragmentos de genes individuais constitutivos, não permitiram resolver totalmente as topologias das árvores filogenéticas. A presença das politomias pode ser explicada, como proposto por Tomasini *et al.* (2014), pelo baixo número de polimorfismos informativos dos fragmentos de genes analisados e também devido à ausência de dados que permitam identificar o relacionamento dessas subpopulações.

A presença de eventos de intercâmbio genético é também reforçada pela presença de prováveis alelos recombinantes e os potenciais parentais como demonstrado nos dois trabalhos que compõem esta tese. No entanto, esses alelos recombinantes devem ser analisados com cuidado, uma vez que as sequências foram testadas utilizando o programa PHASE, que tem sido utilizado para análises de amostras de origem humana e que aprioristicamente assume a recombinação genética como padrão.

A frequência de eventos de intercâmbio genético em *Trypanosoma* spp. é um aspecto muito controvertido. Assim, alguns autores consideram que intercâmbios genéticos ocorrem apenas raramente (Tibayrenc *et al.*, 2015; Tomasini *et al.*, 2014) e outros autores consideram que o intercâmbio genético é frequente (Ramirez e Llewellyn *et al.*, 2014; Messenger *et al.*, 2015). O conjunto de nossos resultados (incongruência topológica, isolados heterozigotos e eventos de introgressão) apoia a hipótese de que os eventos de intercâmbio genético são raros, desde que eles não foram conclusivos, já que mosaicos não foram observados mesmo com um número total de próximo a 100 isolados além da pouca resolução das árvores individuais na análise de MLST.

5.3 Distribuição geográfica dos genótipos TcI

Embora não tenhamos observado associações robustas de subpopulações de TcI com biomas, nós observamos: (1) um cluster que incluiu todos os isolados do bioma Amazônia com alguns isolados dos biomas Mata Atlântica e Cerrado, e (2) um cluster que incluiu isolados de hospedeiros mamíferos que utilizam o dossel. São duas as possíveis explicações. Em relação ao cluster da Amazônia, nenhum dos genótipos encontrados nesse bioma foi encontrado em outras áreas, o que pode ser devido, ao menos em parte, à enorme distância dos locais de coleta neste bioma, em relação aos demais locais e/ou às condições ecológicas peculiares. A presença, no cluster da Amazônia, de alguns genótipos de TcI observados dos biomas Mata Atlântica e Cerrado, é indicativo do expressivo potencial de dispersão desses genótipos, natural ou artificialmente.

Vale mencionar que, embora tenham sido analisados maior número de isolados de TcI do bioma Amazônia, estes apresentaram baixos níveis de fluxo gênico, além de uma extensa diversidade genética. No segundo trabalho nós observamos que clusters mais próximos coincidiram com ambientes próximos.

Ainda no bioma Amazônia, os índices de agrupamento evidenciaram pouca perturbação da área. Além disso, os isolados de Amazónia apresentaram a maior riqueza alélica dentre os biomas analisados, o que pode ser devido à evolução clonal em um ambiente relativamente estável. Amazônia é o bioma que a apresenta a maior biodiversidade, em relação aos demais biomas (Paglia *et al.*, 2012). Essa diversidade é provavelmente acompanhada também pela diversidade de parasitas.

O cluster formado por genótipos similares encontrados em espécies de hospedeiros que frequentam o dossel sugere um ciclo de transmissão ocorrendo de modo independente nesse ambiente.

A presença de isolados idênticos, coletados em ambientes diferentes (um do bioma Amazônia e um do bioma Mata Atlântica) e espaços de tempo diferentes reforçam a existência da ampla distribuição geográfica de genótipos TcI. Adicionalmente, a presença de genótipos iguais em áreas separadas por barreiras geográficas, mostra a ampla dispersão dos genótipos de TcI na natureza. Este foi o caso das localidades Abaetetuba e Cachoeira do Arari, que estão separadas pela Baía de Marajó. Certamente, fatores bióticos e abióticos, inclusive as atividades humanas, estão relacionados com a ampla dispersão natural dos genótipos de TcI em áreas distantes. Um dos fatores bióticos mais importantes a serem considerados é a composição faunística da área em estudo e coleta representativa. De fato, um estudo inicial (no primeiro trabalho) realizado com um número pequeno de isolados do bioma Cerrado, mostrou que esses compunham um único cluster, sugerindo baixa diversidade. No entanto, quando o número de amostras de áreas do bioma Cerrado foi aumentado, se observou mais genótipos que inclusive em outros biomas, a saber, Mata Atlântica e Pantanal. Isto resulta extremamente importante na hora de gerar conclusões em relação às associações de genótipos com áreas geográficas ou hospedeiros. Apesar do enorme esforço de captura de animais que vêm sendo realizados no campo, é importante ressaltar que se trabalha com uma sub amostragem de populações de *T. cruzi* I, já que o universo do parasito é muito maior do que podemos acessar, e que qualquer tipo de associação que poderiam ser observadas, devem ser analisadas com cautela.

A mesma situação foi observada na análise por MLMT de isolados de *T. cruzi* dos biomas Caatinga, onde foi observada maior diversidade genética quando se aumentou o número de isolados.

A riqueza e a dispersão das subpopulações de TcI provavelmente refletem a diversidade de biocenoses nos diferentes biomas bem como à conectividade entre a maioria deles o que permite o fluxo gênico entre as subpopulações de TcI.

Nos biomas Mata Atlântica, Cerrado e Caatinga, a estrutura populacional de *T. cruzi* se caracterizou por apresentar alto nível de fluxo gênico e *subclusters* intrapopulacionais O fluxo genético pode ser explicado por dispersão aérea, especificamente pelo deslocamento de quirópteras e triatomíneos. É sabido que os morcegos albergam uma grande diversidade de genótipos de *T. cruzi*, mas o seu papel na biogeografia e dispersão do parasito não é muito conhecida.

A análise de MLST, realizada no segundo trabalho, foi parcialmente congruente com a análise do gene do maxicírculo. No entanto, existem diferenças substanciais como, por exemplo, o isolado G41 que apresentou uma topologia diferente nessa árvore. A discordância entre as filogenias nuclear e mitocondrial é indicativo de uma associação prolongada e contínua entre populações de localidades muito distantes (Messenger *et al.,* 2011). Adicionalmente, a incongruência entre as árvores observada é consistente com a proposta de que a recombinação de *T. cruzi* envolve o intercâmbio independente do material genético nuclear e mitocondrial. Os múltiplos eventos de introgressão observados entre grupos que abrangem uma extensa área geográfica mostram que estes eventos são frequentes.

Associações de subpopulações de TcI com espécies hospedeiras ou espécies de triatomíneos permanecem ainda sem respostas consistentes. Essa é uma questão que nossos resultados também não conseguiram esclarecer de modo categórico: embora as análises por MLMT e MLST não tenham mostrado associações evidentes entre subtipos de TcI e espécies de hospedeiro, as análises estatísticas não permitiram descartar de todo esta possibilidade. Se por um lado não foi possível estabelecer uma associação de genótipos de TcI com espécie de hospedeiro, o encontro de um cluster que agrupou animais que frequentam o dossel remete a uma tendência de um conjunto de genótipos de TcI determinado pelo uso de estrato florestal. A presença de todos os isolados da Amazônia em um cluster teoricamente indicativo de uma associação de genótipos de TcI com este bioma, é desafiado pela presença de isolados dos biomas Mata Atlântica e Cerrado. Nossos resultados mostram que, mesmo sendo uma única DTU de *T. cruzi*, esta apresenta enorme complexidade epizootiologica e que propostas de associações de subpopulações de *T cruzi* com biomas ou hospedeiros devem ser analisadas com cautela.

Didelphis spp. hospedeiros que frequentam todos os estratos florestais, também integraram esse cluster, como confirmaram as análises de MLST, MLMT e a árvore do gene de maxicírculo. A presença de isolados de *T. cruzi,* obtidas de didelfídeos, em todos os

clusters e associado aos baixos valores de Fst, é compatível com a nossa hipótese de seu papel como bioacumuladores da diversidade dos genótipos *T. cruzi* (Jansen *et al.*, 2015).

Reservatórios sinantrópicos como didelfídeos, são ecléticos quanto a habitats (Olifiers *et al.,* 2005) e se alimentam de restos orgânicos descartados pelos humanos, bem como de frutos, insetos e pequenos vertebrados nas áreas silvestres. Por outro lado, didelfideos são predados por animais mesopredadores ou topo de cadeia, dentro da rede trófica o que pode explicar as frequentes infecções mistas.

O segundo trabalho desta tese, reforça o importante papel desempenhado por *Didelphis marsupialis* na dispersão de genótipos TcI (Jansen *et al.*, 2015), como evidenciado pelos baixos valores de Fst em comparação com isolados obtidos de outras espécies de hospedeiros. No entanto, não podemos afirmar que essa característica seja um atributo específico de *D. marsupialis*, uma vez que, o número de amostragem das outras espécies do gênero foi menor.

5.4 *T. cruzi* e o perfil epidemiológico emergente de doença de Chagas

Várias tentativas de associações com características morfológicas bioquímicas, moleculares, ciclos de transmissão e manifestações clínicas foram realizadas desde a descrição da espécie (Andrade *et al.* 1983; Batista *et al.* 2009; Corrales *et al.* 2009; del Puerto *et al.* 2010). Na Colômbia, TcI_{Dom} e TcI silvestre foram propostos como associados aos ciclos de transmissão doméstico e silvestre, respectivamente (Herrera *et al.,* 2009; Ramirez *et al.*, 2013). Alguns autores propuseram que TcI silvestre era responsável pelas formas graves da doença de Chagas o que não foi confirmado por outros autores (Hernández *et al*., 2014).

Adicionalmente, não foi confirmado TcI_{Dom} como sendo exclusivamente transmitido no intradomicílio (Ramirez & Hernandez *et al.,* 2017). De fato, foi demonstrado posteriormente que TcI_{Dom} também é transmitido em ciclos silvestres e não é encontrado somente no ciclo de transmissão doméstico. Análises realizados por Ramirez *et al.,* (2017) indicaram que, embora em Colômbia sejam encontrados dois grupos distintos de cepas de TcI (Dom e Silvestre), a associação com determinados ciclos de transmissão não foi clara.

O genótipo denominado TcI_{Dom}, associado ao ambiente doméstico, foi encontrado sendo transmitido entre animais de copa de palmeiras no ambiente silvestre, e TcI silvestre não demonstrou apresentar uma associação específica com nenhuma das áreas amostradas (Ramirez e Hernández 2017).

De acordo com os dois trabalhos que compõem esta tese, não foram observados isolados de TcI peculiares a áreas de surto, uma vez que os clusters observados nas árvores filogenéticas independeram das áreas de surto. Além disso, foram observados baixos valores de *bootstrap* mostrando que os *subclusters* dos isolados da Amazônia não foram robustos.

O conjunto de nossas observações nos levam a propor que, provavelmente, os surtos e casos de DCA na Amazônia estão muito mais relacionados aos hábitos dos moradores do que a algum fator ligado ao parasito. De fato, o hábito de processar o fruto do Açaí para obter o suco no crepúsculo vespertino ou matutino em equipamento desprotegido, fora do domicilio e sob iluminação artificial, constitui provavelmente um dos muitos fatores de risco, ao lado de branqueamento realizado em temperaturas insuficientes para esterilizar os frutos entre outros. Com base nisso, sugerimos que apenas um programa educativo constante que considere as peculiaridades culturais de cada localidade será capaz de impedir que novos casos de DCA aconteçam na Amazônia.

Em relação às áreas onde isolados TcI foram obtidos, é importante considerar que os ambientes são em geral sobrepostos e separações dificilmente podem ser reconhecidas, já que o ambiente doméstico, e peridomiciliar geralmente estão muito próximos se não até incluídos no ambiente silvestre.

Trypanosoma cruzi é um parasito geneticamente diverso, mas associações de subpopulações com ambientes, manifestações clinicas da doença humana ou espécie hospedeira permanecem controvertidas, muito provavelmente devido à complexidade dos fatores envolvidos na transmissão ou subamostragem do parasito.

6 CONCLUSÕES

- A unidade taxonômica TcI de *T. cruzi* que circula nos biomas brasileiros é altamente diversa;
- A diversidade em TcI demonstrada pelos fragmentos de genes *RHO1* e *Rb19* sugerem uma diversificação antiga desse genótipo;
- As diferentes sub-populações de TcI estão dispersas pelos biomas brasileiros, independente do tempo;
- A diversidade mais expressiva da DTU I de *T. cruzi* foi observada no bioma Amazônia;
- Isolados de TcI obtidos no bioma Amazônia evidenciaram baixo nivel de fluxo genético;
- As subpopulações de TcI obtidas de vetores e mamíferos da Caatinga, Cerrado e Mata Atlântica, mas não da Amazônia, mostraram alto nível de fluxo genético;
- Associações estritas entre subpopulações de TcI e áreas geográficas não foram observadas;
- Análises moleculares mostraram a ausência de associações de subpopulações de TcI com espécies de vetores ou mamíferos, no entanto, testes estatísticos mostraram que essa possibilidade não pode ser descartada;
- A ausência de genótipos da Amazônia nos demais biomas sugere um ciclo de transmissão de TcI independente naquele bioma;
- Não foi possível estabelecer uma relação entre perfil molecular de isolados TcI e ocorrência de surto de doença de Chagas aguda;
- Genótipos de TcI obtidos de hospedeiros que frequentam o dossel formaram um *cluster*, sugerindo um ciclo de transmissão independente acontecendo nesse estrato florestal;
- A constatação de heterozigotos, assim como de incongruências entre árvores filogenéticas dos genes individuais, sugerem intercâmbio genético em subpopulações de TcI;
- A introgressão mitocondrial em subpopulações de TcI constitui um evento frequente;
- Análises filogenéticas através de MLMT, MLST e o gene de maxicírculo mostram a mesma capacidade de resolução;
- Os fragmentos de genes constitutivos *COAR, GTP, LAP, PDH, RB19, LYT, DHFR-TS, METIII e RHO1*, não são marcadores universais da diversidade de TcI.

- Didelfideos demostraram ser os hospedeiros capazes de manter alta diversidade de populações de TcI, podendo ser considerados como bioacumuladores desta DTU.

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8 ANEXO

PLOS ONE

RESEARCH ARTICLE

Expanding the Knowledge of the Geographic Distribution of *Trypanosoma cruzi* TcII and TcV/TcVI Genotypes in the Brazilian Amazon

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Abstract

Trypanosoma cruzi infection is a complex sylvatic enzooty involving a wide range of animal species. Six discrete typing units (DTUs) of *T. cruzi*, named TcI to TcVI, are currently recognized. One unanswered question concerning the epidemiology of *T. cruzi* is the distribution pattern of TcII and hybrid DTUs in nature, including their virtual absence in the Brazilian Amazon, the current endemic area of Chagas disease in Brazil. Herein, we characterized biological samples that were collected in previous epizootiological studies carried out in the Amazon Basin in Brazil. We performed *T. cruzi* genotyping using four polymorphic genes to identify *T. cruzi* DTUs: mini-exon, 1f8, histone 3 and gp72. This analysis was conducted in the following biological samples: (i) two *T. cruzi* isolates obtained by culturing of stools from the triatomine species *Rhodnius picttipes* and (ii) five serum samples from dogs in which trypomastigotes were observed during fresh blood examination. We report for the first time the presence of TcII and hybrid DTUs (TcV/TcVI) in the Amazon region in mixed infections with TcI. Furthermore, sequencing of the constitutive gene, gp72, demonstrated diversity in TcII even within the same forest fragment. These data show that TcII is distributed in the five main Brazilian biomes and is likely more prevalent than currently described. It is very probable that there is no biological or ecological barrier to the transmission and establishment of any DTU in any biome in Brazil.

Introduction

Trypanosomiasis by *Trypanosoma cruzi* is primarily an ancient sylvatic enzooty involving a wide range of mammalian species and triatomine vectors in the Americas. Humans were likely included in the transmission cycle as soon as they arrived in the Americas approximately 20,000 years before present (bp) [1]. Since the discovery of the parasite and its cycle by Carlos Chagas (1909), the high morphologic, biologic, biochemical, and more recently molecular variability of *T. cruzi* isolates, has been observed and discussed [2]. The currently employed molecular tools allow for the recognition of six discrete typing units (DTUs), named TcI to TcVI [3]. Nevertheless, the complexity of *T. cruzi* remains unresolved, considering, for example, the recent discovery of an additional *T. cruzi* genotype in bats [4] and the recognition of heterogeneity within TcI [5].

T. cruzi is diploid, genetically very polymorphic, and has a clonal structure that manifests a lack of (or very restricted) sexuality [6–7]. Nevertheless, a large body of evidence points to the importance of hybridization events as the cause of the extensive heterogeneity of the taxon [8]. The two more divergent lineages are TcI and TcII, whose separation time is still under debate, as it ranges from 3 to 88 million years bp [9, 10]. The more recently diverged DTUs are TcV and TcVI, which resulted from at least one hybridization event that is estimated to have occurred 0.9 million years bp [11].

The epidemiology of the *T. cruzi* DTUs remains a challenging subject. Large gaps in knowledge concerning the distribution of the distinct DTUs are due mainly to difficulties obtaining representative samples of such a widely distributed enzootic taxon as *T. cruzi*. Currently, TcI has been reported as the most frequently isolated of all mammalian taxa throughout the geographic range of the parasite in an ample variety of biomes and habitats [3]. In comparison, DTUs III and IV are isolated less frequently, although they are also widely distributed [3, 12–14]. Very little is known about the wild hosts of the hybrid DTUs V and VI, which until now, have been primarily isolated from humans or from domiciliated triatomines [3, 15–16]. In Brazil, there is one single report of TcV infecting a wild host, the caviomorph rodent species *Thrichomys laurentius* [17].

The sylvatic hosts and distribution of TcII in the wild also need to be clarified. This parental and ancient *T. cruzi* DTU, which was previously associated primarily with human infection, has been reported to occur in the central belt of South America, which covers the countries of Brazil, Chile, Colombia, Bolivia, Uruguay and Paraguay [3, 15–16]. Above the Amazon region, TcII was found to infect *Triatoma dimidiata* in Guatemala [16]. In Brazil, this DTU has been found to infect a broad range of mammalian species in the Atlantic Forest, Caatinga, Pantanal and Savannah biomes [13, 18–21]. Despite its ability to infect a large variety of wild host species, TcII has been isolated from a smaller number of animals, and therefore, it has been proposed that this DTU occurs in more focal cycles [19]. In recent decades, *T. cruzi* isolates from the Amazon region have been subjected to several studies involving molecular characterization. However, only the TcI, TcIII and TcIV DTUs have been reported in this biome [22–24].

Altogether, the emergence of Chagas disease in the Amazon region and the generally sparse knowledge about this biome led us to study the enzootic transmission cycle of *T. cruzi* since 2006 in this region [25–27]. Herein, we extend these studies and provide new data on the distribution of Tc hybrids and TcII among triatomines and dogs.

Materials and Methods

Study area

This study was conducted in three municipalities/localities in the state of Pará. These were Abaetetuba/Ajuaí (01°43′24″S; 48°52′54″W) and Belém/Val-de-Cans (01°27′21″S; 48°30′16″W) in the northeastern mesoregion of the state and Monte Alegre/Setor 11 (01°38′20″S; 54°14′32″W) in the lower Amazon mesoregion of the state [26–27]. The common climate is characterized as tropical humid with regular rainfall and winds and temperatures between 27°C and 36°C. In most of the collection areas, the original native vegetation (Amazonian forest) is being replaced by an extensive açaí fruit monoculture, with a few remaining patches of the original vegetation at river banks (Fig. 1). No specific permissions were required for these locations.

Biological samples

For this study of the ecology of *T. cruzi* DTUs in the Amazon region, dogs (N=422) and triatomine insects (N=495) were examined [25–27]. *T. cruzi* isolates derived from two *Rhodnius pictipes* and five serum samples of dogs with patent parasitemia (positive blood slide smears) from rather distant regions inside Pará were characterized. The two *T. cruzi* isolates obtained from *R. pictipes* were captured in palm trees (*Attalea phalerata*) in Rio Ajuaí (LBT 1458), Abaetetuba municipality, and Val de Cans (LBT 1814) localities (Table 1). *T. cruzi* isolates were obtained by culturing the intestinal contends of the triatomine in NNN+LIT biphasic medium supplemented with 10% fetal bovine serum. When cultures of the two isolates reached the exponential phase of growth, they were subjected to DNA extraction using the phenol-chloroform method, as described elsewhere [28], and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, Fiocruz – COLTRYP (COLTRYP 00458 and COLTRYP 00060, respectively).

The owners of the dogs from which we obtained the serum samples were from a rural locality in the Monte Alegre municipality (Table 1). The scarce population of this area lives off of subsistence agriculture and uses dogs for hunting. Notably, the dogs were autochthonous and never moved to other sites. Canine infections were detected by fresh blood smear examination and confirmed as *T. cruzi* by PCR of the variable region of the kDNA [27]. Unfortunately, we did not have the opportunity to perform hemocultures from these dogs. The DNA from the five canine serum samples, LBT 1818, LBT 1819, LBT 1820, LBT 1821 and LBT 1822

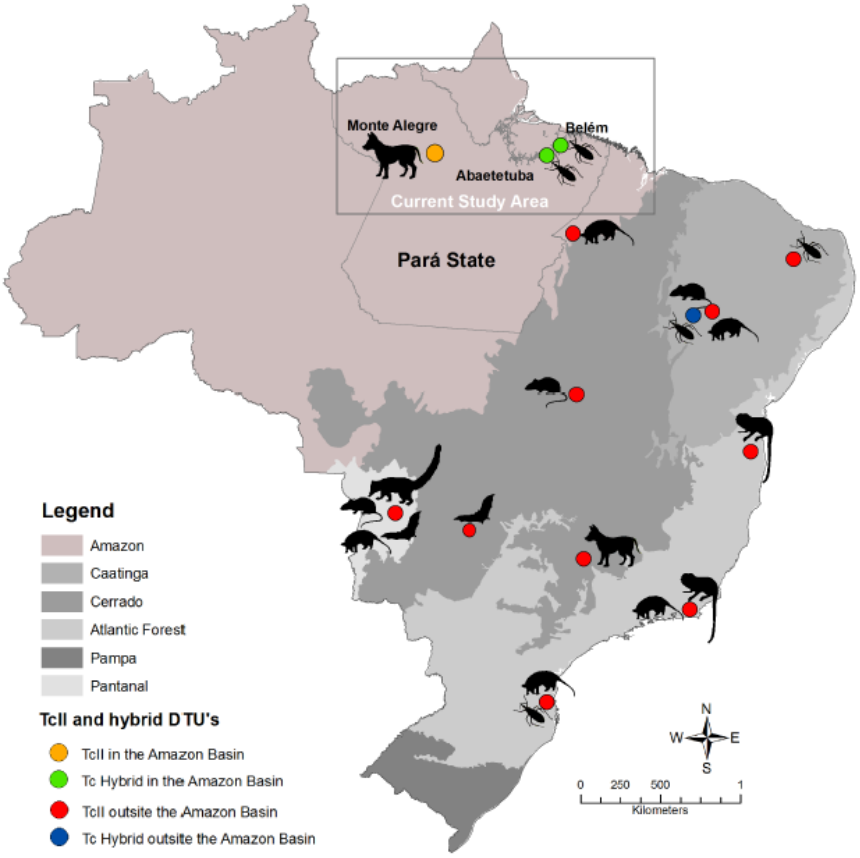

Fig. 1. The distribution of *Trypanosoma cruzi* TcII and hybrid genotypes in the Amazon (present study) and in Brazil (published data). Red circles indicate the location of TcII isolates, blue circles indicate the localization of hybrid isolates, and black symbols represent mammalian and vector host species. The upper right figure shows the localization of TcII and hybrid DTUs from this study in the state of Pará. Footnotes: Atlantic Forest biome [19, 37–38]; Caatinga biome [17, 20]; Cerrado biome [21]; Pantanal biome [13].

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was extracted using the phenol-chloroform method, as for parasite culture extraction, except without SDS in the pre-treatment (Fig. 2).

Mini-exon assay and cloning of mixed *T. cruzi* DTUs samples

All *T. cruzi* samples were initially subjected to multiplex PCR of the mini-exon gene performed as described by Fernandes [29], to identify three *T. cruzi* DTU groups and *T. rangeli*: TcI (200 basepairs-bp), Tc2 (TcII/TcV/TcVI–250 bp), zymodeme 3 (TcIII/TcIV–150 bp) and *T. rangeli* (100 bp) (Fig. 2).

Table 1. *Trypanosoma cruzi* molecular characterization in naturally infected hosts from the state of Pará, Brazil.

T. cruzi samples (biological material)	Host species	Municipality	Mini-exon assay[b]	Other Genotyping protocols	*T. cruzi* characterization
LBT 1458 (isolate)	*Rhodnius pictipes*	Abaetetuba	TcI and TcII/V/VI	-	TcI and TcII
LBT 1458 (clone 5)		Abaetetuba	TcII-V-VI	1f8 gene[c]	TcII
LBT 1458 (clone 7)		Abaetetuba	TcII-V-VI	1f8 gene[c]	TcII
LBT 1814 (isolate)	*Rhodnius pictipes*	Belém	TcI and TcII/V/VI	Histone 3 gene[d]	TcI and TcII
LBT 1818[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI	-	TcI
LBT 1819[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI and TcII/V/VI	-	TcI and TcII/TcV/TcVI
LBT 1820[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI	-	TcI
LBT 1821[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI	-	TcI
LBT 1822[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI and TcII/V/VI	Histone 3 gene[d] and mini-exon sequencing[e]	TcI and TcV/TcVI

(-) Not available;
[a]*Trypanosoma cruzi* kDNA positive serum samples from Xavier et al. [27];
[b]Mini-exon assay according to Fernandes et al. [29];
[c]PCR-RFLP assay of the 1f8 gene according to Rozas et al. [31];
[d]PCR-RFLP assay of the histone 3 gene according to Westenberger et al. [32];
[e]Sequence of a 250 bp fragment from the mini-exon gene. GenBank accession number KJ402456.

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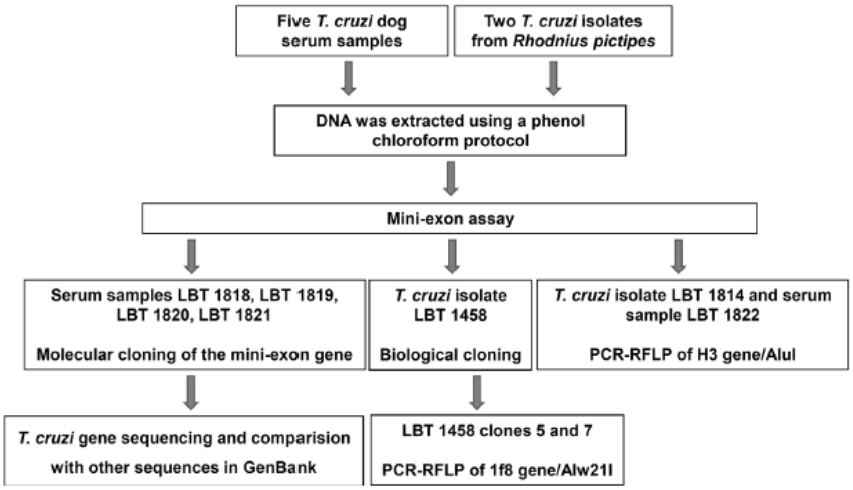

Fig. 2. Schematic representation of the methodology used for *Trypanosoma cruzi* typing of distinct biological samples. Serum and feces derived respectively from five naturally infected dogs and axenic cultures of feces obtained from two *Rhodnius pictipes*.

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The LBT 1458 isolate presented controversial results in the mini-exon assay when samples from a new extraction were typed using the same method and conditions. Therefore, to assess the true subpopulation composition of this isolate, biological cloning was performed. For this purpose, we used solid phase medium tecnique in a Petri dish [30] and selected two Tc2 clones (Fig. 2).

PCR products from canine serum samples (LBT 1819 and LBT 1822) that showed mixed *T. cruzi* DTUs by mini-exon assay were cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) following the manufacturer's protocol. Each colony grown corresponded to one individual clone containing an insert (amplicon) of both DTUs. The colonies (clones) were randomly collected and subjected to PCR of the mini-exon gene [29] to select clones with 250 bp fragments corresponding to genotypes TcII, TcV or TcVI.

PCR-RFLP for *T. cruzi* DTU characterization

The following protocols for genotyping the mixed samples were performed depending on the nature of the biological samples: (a) to characterize LBT1458 clones, we performed restriction fragment length polymorphism (RFLP) analysis of the nuclear 1f8 gene after digestion with the Alw21I enzyme, which distinguishes DTUs TcII from TcV and TcVI [31] and (b) PCR-RFLP of the gp72 gene and analysis of fragments digested with the TaqI enzyme were performed as a confirmatory marker to discriminate TcII and to distinguish TcV from TcVI [31] (Fig. 2).

For genotyping the original *T. cruzi* isolate (LBT 1814) and canine serum sample LBT 1822, we used PCR-RFLP of the histone H3 gene and the digestion with the AluI enzyme because this marker distinguishes TcII from TcV and TcVI without overlapping fragments (TcV and TcVI) [21, 32]. Each reaction included negative and positive controls from representative DTUs. The PCR and RFLP results were visualized in 3% agarose gels stained with ethidium bromide under UV illumination (Fig. 2).

T. cruzi gene sequencing of mini-exon and gp 72 genes

Mini-exon gene

To compare the mini exon gene with other sequences in GenBank and to identify the *T. cruzi* DTU, we sequenced a 250 bp fragment of the mini-exon of sample LBT 1822. This fragment was obtained after molecular cloning of this gene. The primers used were Tc2 (TcII/TcV/TcVI) and Exon, the same primers that were used for the amplification of this fragment in the multiplex assay.

Glycoprotein 72 gene (gp72 gene)

To clarify the unusual profile exhibited by the RFLP protocol for gp72 in LBT 1458 cl 5 and cl 7 we extended the study by testing this protocol in twenty isolates from COLTRYP that were previously identified as TcII by a combination of PCR-RFLP of HSP60, GPI loci and 24S αDNA AFLP markers [33–34] and GPI

Table 2. *Trypanosoma cruzi* II isolates subjected to gp72 gene sequencing.

T. cruzi II samples	Host species	Municipality-State[a]/Biome	PCR-RFLP gp72 gene profile[b]	GenBank accession number
MLD 564b	*Leontopithecus rosalia*	Silva Jardim-RJ/Atlantic Forest	A	KJ402453
MLD 832	*Leontopithecus rosalia*	Silva Jardim-RJ/Atlantic Forest	A	KJ402451
MLD 840	*Leontopithecus rosalia*	Silva Jardim-RJ/Atlantic Forest	A	KJ402452
MLCD 92	*Leontopithecus chrysomelas*	Uma-BA/Atlantic Forest	A	KJ402448
JCA3	*Triatoma brasiliensis*	João Costa-PI/Caatinga	A	KJ402446
LBT 1458 clone 5	*Rhodnius pictipes*	Abaetetuba-PA/Amazon	B	KJ402454
LBT 1458 clone 7	*Rhodnius pictipes*	Abaetetuba-PA/Amazon	B	KJ402455
MLD 594b	*Leontopithecus rosalia*	Silva Jardim-RJ/Atlantic Forest	B	KJ402450
CD 621	*Canis lupus familiaris*	São Roque de Minas-MG/Cerrado	B	KJ402444
CD 640	*Canis lupus familiaris*	São Roque de Minas-MG/Cerrado	B	KJ402445
MLD 1025	*Leontopithecus rosalia*	Silva Jardim-RJ/Atlantic Forest	B	KJ402449
MLCD 82	*Leontopithecus chrysomelas*	Uma-BA/Atlantic Forest	B	KJ402447

[a]RJ - Rio de Janeiro; BA - Bahia; PI - Piauí; PA - Pará; MG - Minas Gerais;
[b]A - according to profile described by Rozas et al. [31]; B - profile that was distinct form that described by Rozas et al. [31].

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sequencing [32]. We then sequenced these two clones in addition to five TcII isolates with patterns described by Rozas et al. [31] and five isolates exhibiting a similar c5 and c7 pattern (Table 2). The analysis of this locus also allowed examination of a possible phylogenetic significance of this difference. The amplicons (1290 bp) were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) and subjected to cycle sequencing reactions with the Big Dye Terminator v 3.1 commercial kit (Applied Biosystems, Foster City, California, USA). The products were sequenced in a 3100 automatic sequencer (Applied Biosystems) using the same primers used for amplification.

Sequence analysis

Sequence editing, alignment and phylogenetic tree construction were performed using Chromas v. 1.45 (School of Health Sciences, Griffith University, Queensland, Australia) and the Mega v 5.1 free program [35]. We used the Kimura-2-parameter model and the neighbor-joining statistical methods for phylogenetic reconstruction. The bootstrap was acquired from 1,000 replicate trees. We included analysis of six sequences from GenBank and three TcI isolates as the outgroup in this phylogenetic reconstruction (Table 2). The BLAST (Basic Local Alignment Search Tool) option Nucleotide was used to compare sequences acquired from canine serum with mini-exon *T. cruzi* sequences from GenBank.

Ethics statement

All dog manipulation procedures were performed in accordance with COBEA (Brazilian College of Animal Experimentation) following the guidelines of the Animal Ethics Committee (CEUA) protocol of FIOCRUZ (Oswaldo Cruz Institute Foundation), Ministry of Health, Brazil. All procedures followed protocols approved by the FIOCRUZ Committee of Bioethics (license 0015-07). In all cases, written consent from the dog owners was obtained, and the owners who also helped us to handle the animals during sampling to avoid incidents. Blood was collected from dogs in non-heparinized vacutainer tubes by puncture of the cephalic vein. No specific permissions were required for conducting this study in the aforementioned locations. We did not manipulate endangered or protected species during our field study.

Results

T. cruzi II and V/VI in the Amazon region

The characterization of the samples for *T. cruzi* is summarized in Table 1 and Fig. 3, demonstrating the occurrence of TcII and a Tc hybrid (TcV or TcVI) in the Amazon region in two *Rhodnius pictipes* (respectively LBT 1458 and LBT 1814) and one dog (Fig. 3C, D and Table 1). Another dog (LBT 1819) was found to be infected with Tc2 (TcII/TcV/TcVI) in the mini-exon assay, but due to an insufficient amount of DNA, we were unable to perform additional assays. This is the first report of these genotypes in the Amazonian region.

These three samples were in a mixed infection with DTU TcI as demonstrated by the mini-exon assay. This fact was observed in isolate LBT 1458: the first amplification of the mini-exon gene presented a TcI/Tc2 mixed infection, and in subsequent amplifications of DNA derived from a new extraction, there was only a Tc2 (TcII/TcV/TcVI) profile (Fig. 3B). After cloning, we typed ten of the obtained biological clones and observed that three of them were TcI and seven were Tc2 (TcII/TcV/TcVI) (Fig. 3C). Nevertheless, the PCR-RFLP of the gp72 gene did not allow the characterization of LBT 1458 clones 5 and 7. The identification of TcII in the mixed isolate LBT 1814 was possible using the H3/AluI RFLP protocol (Fig. 3D). We obtained the sequence for the 250 bp fragment (of the mini-exon Tc2 (TcII/TcV/TcVI) group) from the LBT 1822 dog sample and then subjected it to BLAST analyses from NCBI. The sequence obtained showed 100% coverage and 99% identity with three hybrid strains (TcV- SC43 and MN and TcVI - CL Brener). Only 20 to 40% coverage and 100% similarity were observed among the five TcII sequences, although one single TcII strain (Tu18) presented 100% coverage and 99% identity. The profile exhibited by RFLP of the H3 gene using AluI as a restriction enzyme confirmed the hybrid genotype (TcV/TcVI) in dog LBT 1822, but the intensity of the bands was very weak (data not shown).

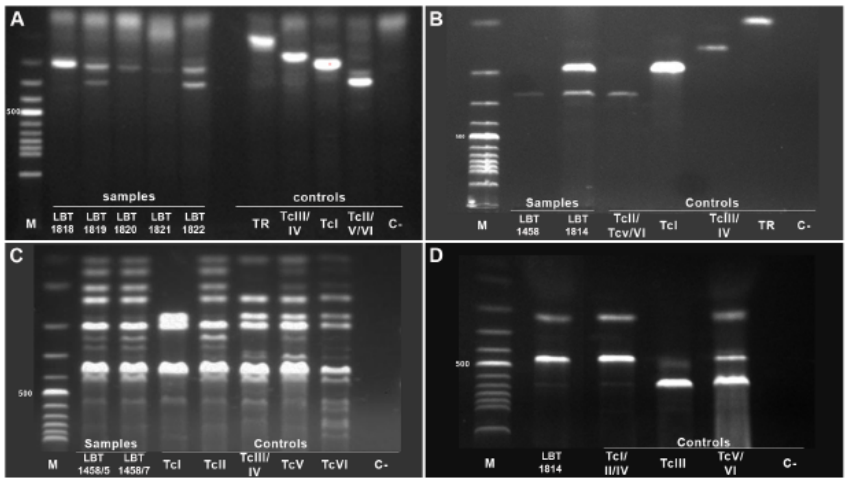

Fig. 3. *Trypanosoma cruzi* genotyping from naturally infected dogs and triatomines from the Amazon Biome. (A) PCR product size polymorphisms of the non-transcribed intergenic region of the SL-RNA mini-exon (mini-exon assay) of *T. cruzi* DNA from dog sera from Monte Alegre in the state of Pará, samples: 1- dog LBT 1818, 2- dog LBT 1819, 3- dog LBT 1820, 4- dog LBT 1821, and 5- dog LBT 1822 (B) Mini-exon assay of *Rhodnius pictipes* isolates from Abaetetuba (LBT 1458) and Belém (LBT 1814) in the state of Pará, Samples: 1- LBT 1458 and 2- LBT 1814 (C) *T. cruzi* genotyping profiles for PCR-RFLP with the 1f8 gene/Alw21I restriction enzyme of LBT 1458 clones 5 and 7, Samples: 1- LBT 1458 clone 5 and 2- LBT 1458 clone 7 (D) *T. cruzi* genotyping profiles for PCR-RFLP with histone 3/AluI restriction enzyme for the LBT 1814 isolate: Sample 1- LBT 1814 isolate. The *T. cruzi* DTUs, *T. rangeli* (H-14) and negative controls are indicated in the figure. DTU reference strains: I - Sylvio X/10 cl 1; II – Esmeraldo cl3; III – M5631 cl5; IV–92122102R; V – SC43 cl1; and VI – CL Brener. Agarose gel 3%, stained with ethidium bromide.

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Gp72 locus diversity

Our TcII isolates were genetically diverse, as revealed by sequencing of gp72 (Fig. 4). The topology of the generated neighbor-joining tree shows the lack of correlation between TcII isolates, considering both geographic and genetic distances (Table 2, Fig. 4). The sequencing analysis showed even more diversity within TcII than was previously observed, highlighting the two patterns of isolates. The two distinct patterns of the gp72 locus by RFLP analysis are due to an SNP (single nucleotide polymorphism) in one of the restriction sites for the enzyme TaqI. Isolates with a thymine to guanine mutation are not cleaved by the enzyme.

Discussion and Conclusion

Trypanosomiasis caused by *T. cruzi* is a zoonosis that is widely dispersed in nature in the Americas. Due to a the large increase in the occurrence of this parasite difficulties involving the capture and transport of biological materials and parasite isolation from wild mammals and vectors, data on the distribution of *T. cruzi* genotypes are aggregated and do not reflect all habitats where this parasite occurs. The greatest barrier to understanding the transmission cycle ecology of *T. cruzi* DTUs is the challenge inherent to fieldwork. As a consequence, there are still

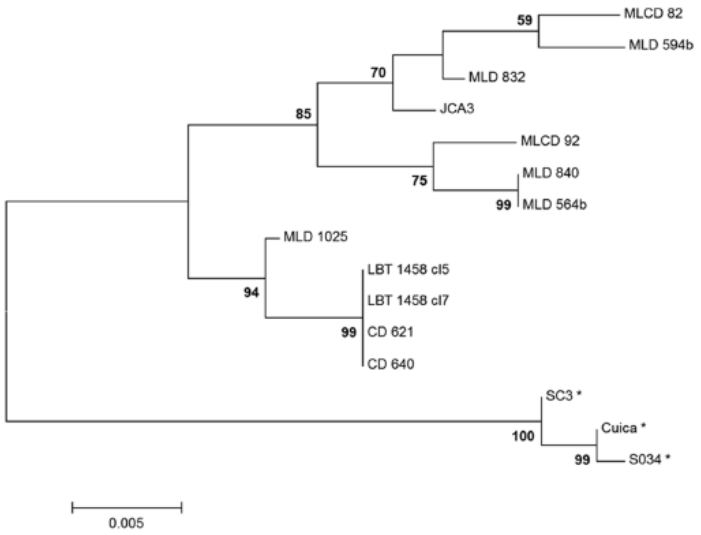

Fig. 4. Phylogenetic relationships between *Trypanosoma cruzi* II sylvatic isolates and TcII clones assessed using the gp 72 gene. The tree was constructed using the neighbor-joining method with Kimura-2-parameter distances. Bootstrap values are shown above major clades. MLD: *Leontopithecus rosalia* from Poço das Antas Biologic Reserve in the Atlantic Forest of the state of Rio de Janeiro: MLCD: *L. chrysomelas* from the Una Biologic Reserve in the Atlantic Forest of the state of Bahia, CD: dogs from the Cerrado in the state of Minas Gerais, LBT: *Rhodnius pictipes* from the Amazon Biome and isolate JCA3 is from a *Triatoma brasiliensis* from the state of Piauí in the Caatinga Biome. *GenBank sequences of strain TcI first published by Flores-López et al. [9].

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numerous areas and mammal species that have been inadequately or never sampled. Additionally, only wild animal isolates from blood culture or xenodiagnosis are typed, and mammals with subpatent infections based on positive serological tests are never considered. Therefore, an important bias is introduced that certainly results in misinterpretations of alleged associations between genotypes of *T. cruzi* with host species. In addition, the peculiarities of interactions among each subpopulation (potentially different DTUs) with their respective hosts may produce distinct selective pressures that result in higher or lower success in the isolation of parasites through the current available methods. This bias may result in misinterpretation of the ecology and biology of this trypanosomatid. The Amazon region biome has especially attracted our attention in recent years due to an increase in cases of Chagas disease transmitted by the oral route and the scarcity of data concerning the ecology of *T. cruzi* genotypes in this large and diverse biome [2, 3, 22]. The apparent difference in the prevalence of the *T. cruzi* DTUs in nature is intriguing and may be influenced by the different patterns of infection among each *T. cruzi* DTU and its mammalian hosts. Although these strategic differences have not yet been fully clarified, all *T. cruzi* DTUs were found to be successful because of their maintenance in nature. Most

likely, each DTU establishes a particular interaction pattern with their several host species. After a short period of high TcII parasitemia, which is detectable in hemocultures, for example, we observed that although the hosts remained serologically positive, it was no longer possible to recover the parasites by hemoculture [21]. This suggests that the transmission strength of these animals is restricted to a short period of approximately two months [21]. Opossums (*Didelphis aurita*) handle TcII in a different manner: in experimental infections with Y and FL strains, both of which are TcII, these mammals were able to control parasitemia even when very young and still dependent on the marsupium [36]. Nevertheless, naturally infected *Didelphis* spp are able to maintain TcII in the wild. In contrast, the four-eyed opossum (*Philander frenata*) was able to maintain long-lasting infections with a high prevalence of positive hemocultures when experimentally infected with the Y strain (TcII) [37]. A long-lasting TcII transmission cycle in the wild has been described in two tamarin species, *Leontopithecus rosalia* and *L. chrysomelas*, in two fragments of the Atlantic Forest Biome [38]. Additionally, the carnivore *Nasua nasua* in the Pantanal biome was demonstrated to be a suitable wild TcII host [13].

In this study, we show for the first time the presence of *T. cruzi* DTU TcII and one hybrid DTU (TcV or TcVI) in the state of Pará in the Brazilian Amazon. These data together with previous data from our group show that TcII is present in wild transmission cycles in all Brazilian biomes (Fig. 1) and is not restricted to areas below the Amazonian Basin, as has always been assumed [2, 3]. Additionally, the presence of infected triatomine bugs and dogs by TcII and hybrid DTUs imply that other mammals and triatomine bugs should also be infected and involved in the transmission cycle of these DTUs in Pará. Notably, before this study, a hybrid DTU was only reported in wild mammals in Brazil once, in a sylvatic rodent species, *Thrichomys laurentius,* in the northeastern region of the country [17], demonstrating that we are still far from understanding the distribution and hosts of hybrid DTUs. The report of a Tc hybrid in the Amazon leads to even more questions, mainly due to the large gap in reports of these DTUs in Brazil, as most of the isolates that we obtained were from the southern part of the country and derived from humans, and only one isolate was reported in a wild rodent from the northeastern Caatinga [17].

The characterization of *T. cruzi* by multiplex PCR fo the mini-exon gene, developed by Fernandes et al. [29] and validated by Aliaga et al. [39], was employed as the first step to type *T. cruzi* DTUs. This method allows a reliable characterization of TcI and distinguishes two DTU groups, TcIII/TcIV and TcII/TcV/TcVI. It is a valuable method because *T. cruzi* isolates derived from the wild environment very often include more than one DTU. Due to the nature and the peculiarities of the samples, it became necessary to use different typing techniques. Therefore, because serum samples that contain small quantities of *T. cruzi* DNA consequently result in low DNA recovery rates, our technique of choice was multiplex PCR of the mini-exon gene because this gene occurs in multiple copies per cell (approximately 200),thereby increasing our chances of success. Moreover, all attempts to type this material using other targets, such as H3, were

unsuccessful because these are single copy genes. We used the same rationale employed for assays using ancient DNA – using multi-copy genes [1, 40–41]. The presence of both TcI and Tc2 (TcII/TcV/TcVI) fragments in the serum samples led us to perform molecular cloning of the amplicons to select and subsequently sequence these TcII clones. Another rationale for this technique is that the same parasite may contain minor nucleotide variations, which results in double peaks in the chromatogram. Obtaining a single sequence allows a comparison between gene sequences that are already deposited in the gene bank.

A different situation was presented by isolates 1814 and 1458, which were derived from *R. pictipes* that displayed mixed TcI and Tc2 profiles, respectively, when typed using the mini-exon gene. Once the cultures were available, the amount of DNA was not a limiting factor, and it was possible to type the DTUs using single-copy genes such as H3 and 1f8. The decision to perform biological cloning was made because the first typing using the mini-exon gene showed a mixed profile. Later typings of the same material, however, revealed only Tc2. Biological cloning has allowed us to choose and use 1f8/Alw21I to type 1458 because this target displays a unique profile for TcII, as shown in Fig. 3C. In the case of isolate 1814, biological cloning did not appear to be necessary because we had no conflicting results when we re-characterized the material as in the previous case. Thus, we were certain that this was an isolate containing TcI and Tc2. This allowed us to choose another option for typing mixed TcI/TcII isolates, which was to eliminate the possibility of dealing with the hybrid genotypes (TcV and TcVI). In the latter case, we used the same method described by Rocha et al. [21].

The isolation of TcII from *R. pictipes* demonstrates the ability of this triatomine species to maintain this genotype, in contrast with previous reports. Moreover, these studies were conducted in experimentally infected *Rhodnius prolixus* [42–44] and were based on observations of only three TcII strains (Y, AF-1, Tu18), which do not represent the diversity of this genotype in the wild. Although natural infections by *T. cruzi* in *Rhodnius* sp in the Amazonian biome have been studied intensely over the past few decades, the majority of these studies did not employ molecular genotyping tools [22]. Studies of natural infection with *T. cruzi* in *R. pictipes* in the Amazon region that aimed to characterize the parasite isolates have reported infections only with TcI, TcIV and *T. rangeli* [24, 45].

The greater issue challenging our understanding of the populational structure of the TcII DTU and hybrids is their apparent focal distribution in nature. Among the inherent difficulties of detecting and characterizing mixed infections is the selective pressure exercised by axenic medium. An insufficient understanding of the behavior and dynamics of TcII and Tc hybrid infection in mammals and vector species is likely the consequence of selective pressures on the process of isolation and maintenance and different sensitivities of in diagnostic methods, in addition to collection bias. This was clearly demonstrated in the diagnosis by PCR of a TcII infection in the serum samples of three specimens of *Didelphis aurita* from an outbreak area in the state of Santa Catarina (Maldonado et al., unpublished data), whose hemoculture was characterized as TcI [25]. Another example is a *T. cruzi* isolate obtained from *Didelphis albiventris* from the Caatinga

biome that after several years maintenance in the COLTRYP of our laboratory was always characterized as a single TcI infection by different typing techniques [29, 31] but was then demonstrated to also include TcII after biological cloning was performed (Lima et al., unpublished data). The same event was observed in this study with isolate LBT 1458, which was characterized as mixed TcI/Tc2 in the first mini-exon assays and in a second DNA extraction, demonstrated only the Tc2 (TcII/TcV/TcVI) band pattern (Fig. 3B). Moreover, the presence of TcI in this isolate was subsequently demonstrated by biological cloning. These factors contribute to a misinterpretation of the prevalence of *T. cruzi* DTUs in nature. Additionally, the large number of sylvatic mammals with positive serology that do not result in recovery of parasites by hemoculture (70%) may also contribute to an underestimation of parasite prevalence.

The RFLP of the gp72 gene discriminated two groups among the Brazilian TcII isolates, one with a previously known profile and another with an unusual profile. The sequencing of this locus points to an SNP that is responsible for these two profiles, although a phylogenetic reconstruction showed that the presence of the same SNP does not imply higher phylogenetic relatedness. The genetic diversity among twelve sylvatic isolates and clones from Caatinga, Cerrado, Atlantic Forest and Amazon (Fig. 4) in a constitutive gene reinforces our hypothesis that TcII has a broader distribution and higher prevalence of infection than is currently reported because a common assumption of such an analysis is that diversity is directly related to the size of a given population [46–47]. The absence of a correlation between geographic area and the two TcII sequencing patterns suggests that variants of TcII are also most likely not limited to a group, such as the tamarin species of the Atlantic Forest, but include other mammalian host species in other biomes [35].

Classically the TcII DTU has been associated with the intradomiciliar transmission of *T. cruzi* by *Triatoma infestans* and with severe cases of Chagas disease [1, 3]. Indeed, this was the epidemiological profile that the Southern Cone initiative successfully targeted and controlled, leading to a decrease in the importance of this epidemiological scenario. Additionally, the TcII DTU was classically described as being prevalent in the region just below the Amazon, specifically the Midwest region of Brazil. Moreover, in recent years, increasingly robust evidence of a wide distribution of TcII in free-living wild animals has emerged [13, 37–38] Our present finding of TcII in Amazonia refutes two paradigms: (i) the association of this DTU with a particular biome and host species and (ii) TcII association with human infection and even severe Chagas disease because no single human case of TcII infection in Amazonia has been detected. Altogether, more than 100 years after the description of *T. cruzi* by Carlos Chagas, who even then noted the diversity of this taxon [48], the scientific community still cannot answer one of the first questions asked by that brilliant scientist: "What do the different forms of the parasite suggest?" We dare say that we are still far from deciphering this mystery.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: VSL ACPV SCCX AMJ. Performed the experiments: VSL IFRM. Analyzed the data: VSL ALRR ACPV AMJ. Contributed reagents/materials/analysis tools: ACPV AMJ. Wrote the paper: VSL ALRR AMJ.

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