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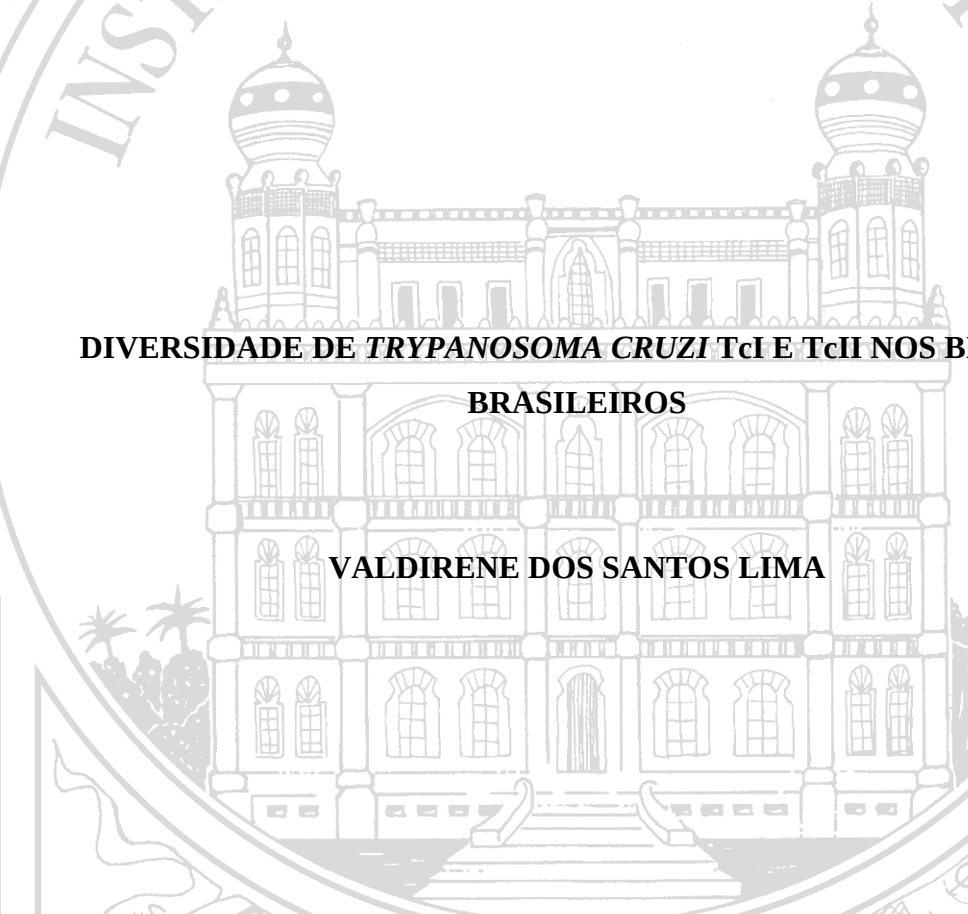
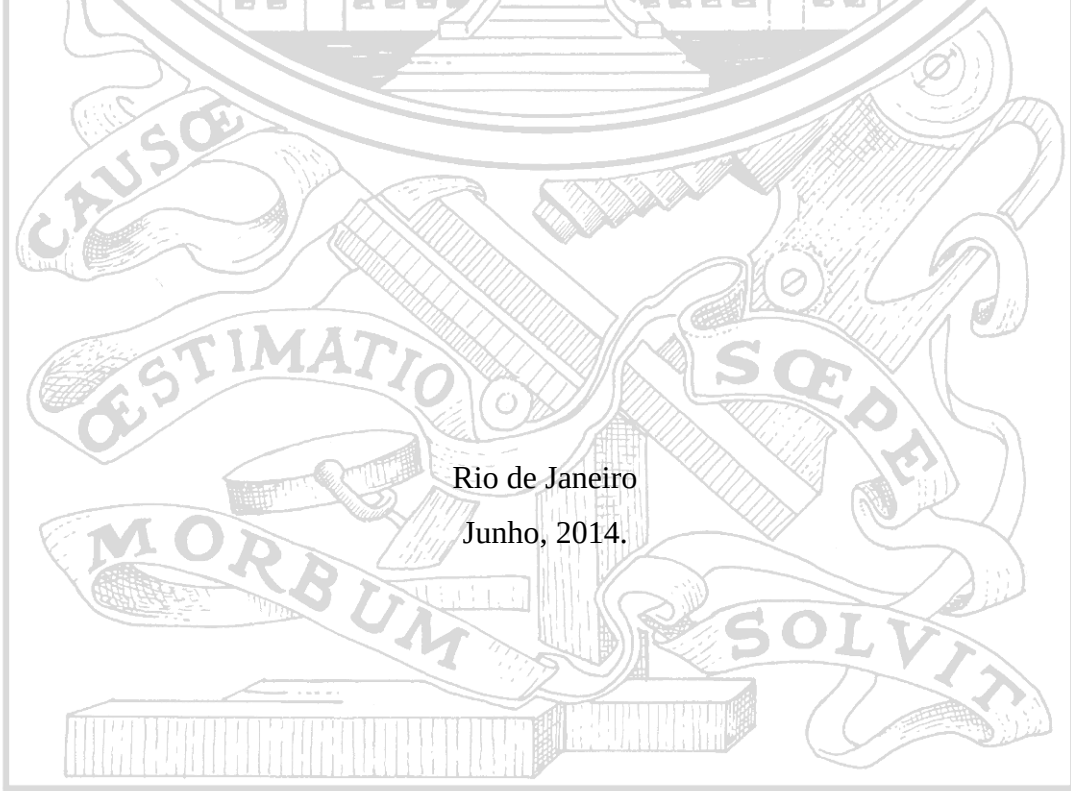


DIVERSIDADE DE *TRYPANOSOMA CRUZI* TcI E TcII NOS BIOMAS BRASILEIROS

VALDIRENE DOS SANTOS LIMA



Rio de Janeiro
Junho, 2014.

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INSTITUTO OSWALDO CRUZ
PÓS-GRADUAÇÃO EM BIOLOGIA PARASITÁRIA

VALDIRENE DOS SANTOS LIMA

DIVERSIDADE DE *TRYPANOSOMA CRUZI* TcI E TcII NOS BIOMAS BRASILEIROS

Orientadora: **Drª. Ana Maria Jansen**

Tese apresentada ao Programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências.

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PÓS-GRADUAÇÃO EM BIOLOGIA PARASITÁRIA

TESE DE DOUTORADO

DIVERSIDADE DE *TRYPANOSOMA CRUZI* TcI E TcII NOS BIOMAS BRASILEIROS

VALDIRENE DOS SANTOS LIMA

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Dedico esse trabalho a meus amores: Ana Clara, Yuri, Arthur, Caio e Clarice.

“A Vida está sempre certa”

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DIVERSIDADE DE *TRYPANOSOMA CRUZI* TcI E TcII NOS BIOMAS BRASILEIROS

Tese de doutorado / Valdirene dos Santos Lima

Resumo

A Tripanossomíase por *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) é uma antiga zoonose amplamente distribuída do Sul dos Estados Unidos ao Sul da Argentina. O Brasil dispõe de seis biomas ecologicamente distintos: Amazônia, Cerrado, Caatinga, Pantanal, Mata Atlântica e Pampa. A transmissão silvestre do *T. cruzi* ocorre ao longo desses biomas, envolvendo uma ampla variabilidade de hospedeiros e vetores. *T. cruzi* é subdividido em sete unidades discretas de tipagem (Discrete Typing Unit - DTU), TcI a TcVI e uma recentemente reconhecida, TcBat. O conhecimento sobre o padrão de distribuição geográfica dessas DTUs ainda é incompleto. TcI é o mais disperso ao longo de toda área de distribuição do parasita inclusive os biomas brasileiros. Analisamos a diversidade de TcI, originado de cinco biomas, exceto do Pampa, com a abordagem MLMT (Multilocus Microsatellite Typing). Foram caracterizados 107 isolados de TcI originados de 29 espécies de mamíferos silvestres e vetores usando vinte e sete loci nucleares de microssatélites e dez loci mitocondriais. Nós comparamos esses dados com isolados TcI de toda a América. A diversidade genética foi alta entre os isolados desse estudo além de se evidenciar um novo clado que se destacou de toda diversidade genética conhecida de TcI nas Américas. Detectamos introgressão mitocondrial ocorrendo através do intercâmbio genético entre a Amazônia e a Caatinga. Observamos similaridades genéticas entre isolados da Mata Atlântica com isolados de todos os outros biomas analisados. A fragmentação da diversidade genética das populações TcI da Mata Atlântica pode estar refletindo o padrão de fragmentação desse bioma. Sugerimos que a diversidade do *T. cruzi* I possa servir como uma sentinela da conservação de ecossistemas. A segunda DTU mais isolada no meio silvestre no Brasil é TcII. O padrão de distribuição de TcII e DTUs híbridas TcV e TcVI na natureza e sua diversidade genética são umas das numerosas lacunas no conhecimento do *T. cruzi*, incluindo a suposta ausência dessas DTUs na Amazônia. Neste estudo analisamos a diversidade genética de 60 isolados TcII pela análise de 19 loci microssatélites nucleares. Alto grau de diversidade foi verificado nesse painel de isolados TcII em pequenas áreas da Mata atlântica e Caatinga e entre hospedeiros geneticamente homogêneos como os *Leontopithecus* spp. Diferentes graus de estruturação populacional foram observados nestes locais. A complexidade de estruturação e diversidade sugere que esse genótipo é mais disperso do que indica sua prevalência nos isolamentos em meio de cultura em uma variedade maior de hospedeiros.A pesquisa da enzootia por *T. cruzi* no estado do Pará levou ao encontro dos genótipos TcII e híbrido (V ou VI) circulando entre triatomíneos e cães, respectivamente. Este achado mostrou que o TcII é presente na Amazônia e portanto nos cinco principais bioma brasileiros ao contrário da clássica distribuição dessa DTU descrita na litertura. A presença de Tc híbrido na Amazônia, é mais intrigante devido a enorme extensão geográfica em que essas DTUs não são descritas no Brasil, e pode sugerir a existência de diferentes estratégias de infecção que estejam prevenindo seu isolamento e subestimando sua real prevalência na natureza.

Diversity and populational estructure of *Trypanosoma cruzi* TcI and TcII in Brazilian biomes

PhD Thesis / Valdirene dos Santos Lima

Abstract

The Trypanosomiasis by Trypanosoma cruzi (Kinetoplastida , Trypanosomatidae) is an ancient zoonosis widely distributed in the southern United States to southern Argentina. Brazil has six ecologically distinct biomes: Amazon, Cerrado, Caatinga, Pantanal, Atlantic Forest and Pampa. The sylvatic transmission of T. cruzi occurs along these biomes, involving a wide variability of hosts and vectors. T. cruzi is divided into seven discrete typing units (DTU), TcI the TcVI and a recently recognized, TcBat. The knowledge about the geographic distribution pattern of these DTUs is still not complete. TcI is more dispersed throughout the distribution area of parasite including Brazilian biomes. We analyze the diversity of TcI, originated five biomes except the Pampa, with MLMT approach (Multilocus Microsatellite Typing). 107 isolates were characterized TcI originated from 29 species of wild mammals and vectors using twenty-seven nuclear microsatellite loci and ten mitochondrial loci. We compare these data with TcI isolates across Americas. Genetic diversity was high among the isolates in this study in addition to evidence of a new clade that stood out from all known TcI genetic diversity in the Americas. We detected mitochondrial introgression occurring through genetic exchange between the Amazon and the Caatinga. Observed genetic similarities among isolates of the Atlantic Forest with isolates of all other biomes analyzed. The fragmentation of genetic diversity of TcI the Atlantic populations may reflect the fragmentation pattern of this biome. We suggest that the diversity of T. cruzi I can serve as a sentinel ecosystem conservation. The second most isolated DTU in the wild environment in Brazil is TCII. The distribution pattern of TCII and hybrid DTUs TCV and TcVI in nature and their genetic diversity are some of the many gaps in the knowledge of T. cruzi, including the supposed absence of these DTUs on Amazon. This study analyzed the genetic diversity of 60 isolates TCII by analysis of 19 nuclear microsatellite loci. High degree of diversity was found in this panel TCII isolates in small areas of the Atlantic Forest and Caatinga and among genetically homogeneous hosts as Leontopithecus spp. Different degrees of population structure were observed at these sites. The complexity and diversity of structure suggests that this genotype is more dispersed than indicating its prevalence in isolates in culture medium in a greater variety of research hospedeiros.A enzootic T. cruzi in the state of Pará led to the meeting of TCII genotypes and hybrid (V or VI) circulating among triatomine bugs and dogs , respectively. This finding showed that TCII is present in the Amazon and therefore the five major Brazilian unlike the classical distribution described in this DTU litertura biome. The presence of Tc hybrid in the Amazon , is more intriguing because of the enormous geographic extent to which these DTUs are not described in Brazil , and may suggest the existence of different infection strategies that are preventing their isolation and underestimating their actual prevalence in nature. Alternative ways to investigate genotypes in biological samples can clear up on its distribution.

Apresentação da tese

Esta tese inclui três temas: o primeiro estudo é sobre a diversidade genética de TcI silvestre em cinco principais biomas brasileiros usando a abordagem de MLMT e gerou um artigo aceito para publicação no periódico Parasites & Vectors, intitulado: Wild *Trypanosoma cruzi* I genetic diversity in Brazil suggests admixture and disturbance in parasite populations from the Atlantic Forest region. O segundo tema é o estudo da diversidade genética de *Trypanosoma cruzi* DTU II oriundos de primatas de dois fragmentos de Mata Atlântica (sudeste e nordeste), e de outros mamíferos da Caatinga e Cerrado também pela abordagem de MLMT.e será encaminhado para publicação. O terceiro tema foi o estudo da dispersão das DTUs de *T. cruzi* II e híbridas (V/VI) na Amazônia, bioma brasileiro ainda sem registro de sua ocorrência e gerou um artigo submetido ao periódico Plos One. Em anexo seguem o artigo aceito para publicação, o artigo submetido e os três artigos em que a aluna é co-autora. Nestes três artigos em co-autoria, a enzootia por *T. cruzi* em municípios dos estados do Pará e Minas Gerais (Serra da Canastra) foi abordado, além de proverem para esta tese, isolados dessas áreas.

Introdução

Trypanosoma cruzi

Trypanosoma cruzi é um protozoário parasita da ordem Kinetoplastida, família Trypanosomatidae, descrito por Carlos Chagas em 1909 e agente etiológico da doença de Chagas que atinge atualmente cerca de 10 milhões de pessoas do sul dos Estados Unidos à Patagônia (Yeo *et al.*, 2005; Schofield *et al.*,2006).

A infecção causada por *T. cruzi* é primariamente uma enzootia complexa, transmitida por dezenas de espécies de triatomíneos vetores e mantida por dezenas de espécies de mamíferos incluídos em 8 ordens. Os humanos foram incluídos nos ciclos de transmissão provavelmente logo que chegaram às Américas, há cerca de 15.000 anos (Guhl *et al.*, 2000).

Há classicamente dois diferentes ciclos de transmissão do parasita, o denominado ciclo silvestre ou enzoótico, relacionado aos triatomíneos vetores e mamíferos silvestres e esporadicamente com a ocorrência de casos humanos. O ciclo doméstico, envolvendo triatomíneos domiciliados, humanos e animais domésticos e/ou domiciliados (Miles *et al.*, 2003). A conexão entre os dois ciclos é mediada por mamíferos sinantrópicos como ratos, camundongos, morcegos e marsupiais, principalmente (Freitas *et al.*, 2006).

O contato infeccioso com o hospedeiro pode ocorrer de diferentes formas, sendo as principais: (a) via de transmissão vetorial que ocorre através da contaminação de mucosa ou lesões na pele com as fezes do vetor infectadas com a forma tripomastigota do parasita, (b) via de transmissão oral através da ingestão de alimentos contaminados com formas tripomastigotas. No meio silvestre essa via de transmissão é a mais provável principalmente entre mamíferos com hábitos insetívoros ou onívoros. (c) via de transmissão congênita tem uma prevalência descrita de 5% dos casos de gestantes portadoras da doença de Chagas. As vias transfusional e por transplante de órgãos são possíveis vias, no entanto na atualidade as medidas de controle em bancos de sangue e na seleção de doadores de órgãos diminuíram quase a zero a prevalência (OMS). Após a interiorização do parasita nos mamíferos hospedeiros, este é capaz de invadir e se multiplicar em quase todos os tecidos.

T. cruzi apresenta uma estrutura populacional predominantemente clonal e suas subpopulações exibem extrema heterogeneidade biológica, genética e bioquímica (Higo *et al.*, 2004; Macedo *et al.*, 2004; Freitas *et al.*, 2006). Um mesmo hospedeiro ou vetor pode abrigar simultaneamente diferentes subpopulações ou clones de *T. cruzi*. Esses clones competem entre si sendo que dadas subpopulações podem ser selecionadas em detrimento de outras em nichos, hospedeiros e áreas geográficas específicas. Diante desse cenário atualmente vem

sendo proposto que a variabilidade de formas clínicas pode ser uma resultante da interação entre o pool genético do hospedeiro, o ambiente e principalmente pela composição das subpopulações do parasita no inóculo (Macedo *et al.*,2004, Souto *et al.*,1996; Zingales *et al.*, 1998; Andrade *et al.*, 2002).

A heterogeneidade em *T. cruzi*

As subpopulações de *T. cruzi* exibem considerável heterogeneidade biológica, genética e bioquímica (Macedo *et al.*, 2004; de Freitas *et al.*, 2006, Revollo *et al.*, 1998). A espécie é subdivida em seis unidades discretas de tipagem (Discrete Typing Units- DTUs) ou genótipos, TcI a TcVI (Brisse *et al.*, 2000; Zingales *et al*., 2009) e recentemente um novo genótipo foi descoberto no Brasil, proposto como sendo associado a quirópteros, TcBat, (Marcili 2009, Zingales *et al.*, 2012).

As duas DTUs parentais e mais divergentes em termos moleculares são TcI e TcII e seu tempo de divergência está ainda sob debate. Diferentes estudos apontam tempos de divergência, variando de 3-10 milhões de anos (Freitas *et al.*, 2006, Machado & Ayala 2001, Lewis *et al.*, 2011) a 88 milhões de anos (Briones *et al.*, 1999). Eventos de hibridação têm sido apontados como envolvidos na diversificação do *T. cruzi*. A origem das DTUs TcIII e TcIV ainda está em debate, alguns estudos apontam evidências de que são originadas de eventos de hibridação ancestrais entre TcI e TcII, estimado em torno de 2,4 milhões de anos (Lewis *et al.*, 2011, Westenberger *et al.*, 2005). Outro estudo sugere essas duas DTUs serem um único grupo ancestral, baseado no fato delas compartilharem um genoma mitocrondrial totalmente diverso de TcI e TcII (Freitas *et al.*, 2006). No entanto, é consenso a natureza híbrida das DTUs TcV e TcVI. Todos os estudos baseados na comparação de sequências nucleotídicas, confirmam que essas duas DTUs são fruto da combinação dos genomas nucleares das DTUs TcII e TcIII (Freitas *et al.*, 2006, Westenberg *et al*., 2005, Machado & Ayala 2001, Florés-Lopes *et al.*, 2011). Não há consenso, portanto, sobre o tempo estimado desse evento de hibridação variando de 900 mil anos (Flores-Lopes *et al.*, 2011) a uma origem antropogênica há apenas 33-60 mil anos (Lewis *et al.*, 2011).

Estudos demonstrativos in vitro confirmam que a capacidade de hibridação é mantida na espécie (Gaunt *et al.*, 2003) e estudo baseado em marcadores nucleares e mitocondriais tem detectado eventos de recombinação natural entre isolados de TcI (Barnabé *et al.*, 2013, Ramírez *et al.*, 2012, Ocaña *et al.*, 2010) e indícios de hibridação natural entre as DTUs TcI e TcIII e entre TcIII e TcIV (Lewis *et al*., 2011). Barnabé *et al.*, 2013 sugere que esses eventos importante colaboração para a divergência genética do parasita.

Distribuição geográfica das DTUs

O conhecimento sobre o padrão de dispersão das DTUs de *T. cruzi* ainda comporta várias lacunas a ser completadas devido principalmente a amostragem não contemplar todas as áreas e hospedeiros de ocorrência do parasita. São descritas diferenças na distribuição geográfica e importância epidemiológica entre as DTU's ao longo de toda América Latina. O TcI, uma das DTUs parentais é o mais frequentemente isolado em todos os taxa de mamíferos através de toda área de ocorrência do parasita cobrindo uma ampla diversidade de biomas e habitats (Zingales *et al.*, 2012). Comparativamente ao padrão de dispersão do TcI, as DTUs III e IV apesar de serem amplamente distribuídas no meio silvestre, são isoladas menos frequentemente (Zingales 2012, Herrera *et al.*, 2008, Lisboa 2009). TcIII é distribuído ao longo de toda América do Sul (Zingales 2012, Lisboa 2009, Herrera 2008, Llewellyn 2009a), TcIV alcançam o sul dos estados Unidos até o sul do Brasil (Zingales *et al.*, 2012, Abolis *et al.*, 2011) e ambos não raramente são isolados de casos humanos na Amazônia e TcIV na Venezuela e sul do Brasil (Abolis *et al.*, 2011). Muito pouco é conhecido sobre os hospedeiros silvestres das DTUs híbridas TcV e TcVI, que até o momento têm sido principalmente isoladas de humanos e de triatomíneos domiciliados no sul do Cone Sul (Zingales *et al.*, 2012, Breniere *et al.*, 2012, Toledo *et al.*, 2013). No Brasil, há um único registro de TcV infectando uma espécie de roedor silvestre, *Thrichomys a. laurentius* (Araújo *et al.*, 2011).

TcII, como dito acima é uma das DTUs parentais, portanto antiga, certamente com milhões de anos circulando entre a fauna das Américas. No entanto, seus hospedeiros silvestres e sua distribuição são aparentemente mais restritos comparado aos do TcI. Esta DTU tem sido classicamente associada à infecção humana e é descrito ocorrer em uma faixa central na América do Sul, abaixo da bacia Amazônica, incluindo os países: Brasil, Chile, Colômbia, Bolívia, Uruguai e Paraguai (Zingales *et al.*, 2012, Breniere *et al.*, 2012, Toledo et a., 2013). Acima da região Amazônica, o único registro de TcII foi infectando triatomíneos domiciliados da espécies *Triatoma dimidiata* em uma área endêmica da Guatemala (Pennington *et al.*, 2009). No Brasil, esta DTU é a segunda em prevalência de isolamento e tem sido detectada infectando uma variedade de animais silvestres, tais como: marsupiais (3 espécies), primatas (5 espécies), carnívoros (2 espécies), roedores (3 espécies) e triatomíneos (4 espécies) nos biomas Mata Atlântica, Caatinga, Pantanal e Cerrado (Herrera *et al.*, 2008, Lisboa *et al.*, 2000, Xavier *et al.*, 2007, Rocha *et al.*, 2013). Em uma análise do banco de dados da Coleção de *Trypanosoma* sp de Mamíferos Silvestres, Domésticos e Vetores da Fundação Oswaldo Cruz (ColTryp) esse número de espécies representam 1/3 da diversidade

de espécies de hospedeiros com hemocultivo positivo de TcI e cerca de 1/4 do número total de isolados TcI Além dessa disparidade em prevalência e diversidade de hospedeiros, de acordo com os locais de origem desses isolados TcII, tem se atribuído a essa DTU uma distribuição em ciclos focais (Rocha *et al.*, 2013). Paralelo à característica focal na sua distribuição, outra característica observada em TcII é a competência de algumas espécies de mamíferos em manter populações TcII. Dois robustos ciclos de transmissão do genótipo TcII foram estudados pelo nosso grupo tanto do ponto de vista da enzootia quanto do perfil ecológico em dois fragmentos da Mata Atlântica, nas Reservas Biológicas de Poço das Antas, no Rio de Janeiro, e a de Una na Bahia (Lisboa *et al*., 2000; 2006; Monteiro *et al*., 2006). Esses ciclos envolveram exclusivamente primatas das espécies *Leontopithecus rosalia* e *L. chrysomelas*, do Rio de Janeiro e Bahia respectivamente. A infecção desses primatas foi caracterizada por parasitemias patentes e estáveis, observadas por hemocultivos positivos por até 10 anos (Lisboa *et al*., 2006). As espécies *Philander frenatus* (cuíca) e *Nasua nasua* (quati) também se mostraram competentes em manter populações TcII (Jansen *et al*., 1991; Herrera *et al*., 2008).

A aparente diferença na prevalência das DTUs de *T. cruzi* na natureza é intrigante. A eficiência em se obter isolados de hemocultivo de animais capturados é aproximadamente de 10% (comunicação pessoal A. M. Jansen). Sales-Campos *et al.*, 2014 sugere que a impossibilidade de detecção de algumas subpopulações pode ser devido a baixa parasitemia do parasita durante a fase crônica de infecção, baixa sensibilidade dos métodos atualmente usados para isolamento do parasita e a ausência de periodicidade circadiana. Portanto, não se identifica a(s) DTU(s) de *T. cruzi* que está causando infecção em uma parcela importante de animais silvestres. Um outro aspecto a ser considerado são as prováveis diferenças nas estratégias de cinética de infecção e transmissão observadas entre as DTUs, que pode interferir na disponibilidade de populações aos métodos de isolamento atuais. Fernandes *et al.*, 2006 verificaram diferentes mecanismos envolvidos na invasão celular in vitro entre as DTUs TcI e TcII. Se há diferenças nas estratégias entre as DTUs, essas aparentemente são bem sucedidas uma vez que as DTUs mais ou menos frequente são mantidas na natureza desde há milhões de anos até o presente. Portanto, não se pode descartar a possibilidade de viés imbutido nos dados de distribuição das DTUs.

Diversidade da DTU TcI

Estudos prévios apontam para diversidade intra-DTU em TcI distribuídos ao longo das Américas, em análises por RAPD, MLEE, ITS, gene de Calmodulina, polimorfismos em

minicírculos e no gene de miniexon (Souto *et al.*, 1996; Brisse *et al.*, 2000; Brandão *et al.*, 2007 Salazar *et al.*,2006; Herrera *et al.*, 2007; O`Connor *et al.*, 2007). Em análises de diversidade em TcI com base no polimorfismo da região intergênica do gene de miniexon, Herrera *et al*., 2007 propuseram a existência de quatro haplótipos Ia-Id associados a ciclos de transmissão na Colômbia. Cura *et al*., 2010 analisando a mesma região gênica, descreveram um novo haplótipo TcIe associado ao ciclo doméstico no Chile e no cilco silvestre na Bolívia. Ramírez *et al*., 2012 utilizando o Multilocus Sequencing Typing do genoma mitocondrial e Multilocus Microsatelite Typing (MLMT) nuclear no estudo de clones de isolados TcI da Colômbia identificaram um consistente agrupamento de de isolados associados à infecção humana que denominaram TcI_{DOM} e que correspondem aos anteriormente denominados $TcIa/VEN_{DOM}$ (Herrera *et al*., 2007; Llewellyn *et al*., 2009b).

A abordagem de Multilocus Microsatelite Typing (MLMT) utilizada para estudo de genética populacional de *T. cruzi* a partir de 2009 elucidou aspectos relacionados à estrutura populacional de TcI. Llewellyn *et al.*, 2009b em uma escala continental abrangendo 8 países nas Américas e isolados TcI de 18 espécies de hospedeiros e vetores, analisando 48 loci de DNA microssatélite, observou grande diversidade nesta DTU e uma estruturação espacial dos genótipos encontrados, além de dados que permitiram sugerir diferentes origens dos isolados humanos em surtos por via oral ou vetorial na Venezuela. Outros estudos se seguiram do mesmo grupo, agora com estudos populacionais em escala menor tanto geograficamente quanto em diversidade de espécies de hospedeiros de TcI, especificamente no Equador (Ocaña *et al.*, 2010), Colômbia (Ramírez *et al.*, 2012) e na Venezuela (Segovia *et al.*, 2013). A abordagem do MLMT nestes estudos permitiu a elucidação da diferencial amplitude de dispersão entre os ciclos de transmissão silvestre e doméstico e os hospedeiros portadores dos genótipos transmitidos para humanos (Ocaña *et al.*, 2010, Segovia *et al.*, 2013). A combinação de dados de MLMT e genoma mitocondrial se mostrou uma ferramenta útil para se evidenciar eventos de hibridação entre isolados TcI (Ocaña *et al.*, 2010, Ramírez *et al.*, 2012).

Diversidade da DTU TcII

A diversidade genética da DTU TcII é pouco conhecida. Estudos realizados com isolados originados de humanos observaram heterogeneidade neste grupo (Freitas *et al.*, 2006; Brisse *et al.*, 2000; Venegas *et al.*, 2009). No entanto, sobre a diversidade deste genótipo no meio silvestre, tradicionalmente associado com infecção humana no Brasil, nada se sabe. Somente o conhecimento da diversidade genética e estrutura populacional de TcII no

ambiente silvestre é que permitirá inferir sobre a estratégias dispersão, tamanho populacional e estruturação por hospedeiros e geográfica (Lewis *et al.*, 2009; Llewellyn *et al.*, 2009a,b). A Mata Atlântica é um cenário singular para análises populacionais desse genótipo no Brasil, onde encontramos representativo número de isolados originados de duas espécies de primatas endêmicos em suas respectivas áreas. Outra peculiaridade nessa análise é a homogeneidade genética de cada uma dessas duas espécies de hospedeiros, que teoricamente conferiria um mesmo potencial em manter populações TcII, permitindo avaliar a capacidade desses hospedeiros como mantenedores de diversidade TcII ou também de seletores.

Esse estudo somado a dados ecológicos desses hospedeiros, como padrão de constância e migração entre grupos (Lisboa *et al.*, 2000; Lisboa *et al.*, 2006) poderão contribuir para um melhor entendimento sobre a dinâmica de dispersão do genótipo TcII que circula nestas localidades.

Biomas brasileiros

O impacto dos ciclos de aquecimento e resfriamento ocorridos no período Pleistoceno, sobre os biomas e diversidades ecológica e de espécies da América Latina e em particular Brasil são matéria de longo debate (Knapp, 2003). No entanto, há evidências que esses ciclos históricos de expansão florestal, contração e fragmentação tiveram impacto sobre a atual relação entre animais e ambientes no Brasil, incluindo distribuição de pequenos mamíferos e diversidade (Costa 2003). Amazônia e Mata Atlântica foram provavelmente contíguas no passado tornando-se separadas pelo aumento na aridez durante o período terciário. Na região referente ao bioma Caatinga registros palinológicos do final do pleistoceno (~11.000 anos antes do presente) indicam ter havido vegetação comum a Amazônia e Mata Atlântica. (De Oliveira *et al.*, 1999). A extensão de influência dessas mudanças na vegetação e na fauna de pequenos mamíferos é uma questão central (Costa 2003). Atualmente o Brasil é composto por seis distintos biomas ou ecorregiões (Costa 2003) o maior deles é a Amazônia ao norte, fazendo fronteira ao leste com o Cerrado e Pantanal ao sul. O nordeste do país é dominado pelo bioma Caatinga composto predominantemente por vegetação xérica. Ao longo da costa brasileira, o bioma Mata Atlântica se extende do sul de Pernambuco até o sul do Rio Grande do Sul, caracterizado por uma floresta tropical úmida. A diversidade de mamíferos silvestres, hospedeiros em potencial do *T. cruzi*, ao logo desse mosaico paisagístico é maior na Amazônia, seguida da Mata Atlântica, Cerrado, Caatinga e Pampa (Paglia *et al.*, 2012).

A tripanossomíase americana por *T. cruzi* no homem (doença de Chagas) foi muito prevalente no Brasil, especialmente em uma faixa abrangendo as regiões nordeste e centro-

oeste e sul do país (Schofield & Dias 1999). Na verdade, a doença de Chagas foi provavelmente endêmica em populações humanas no Brasil desde a entrada do homem nas Américas em torno de 15.000 anos atrás (Guhl *et al.*, 2000). Mesmo o homem não sendo considerado bom reservatório pra a transmissão do *T. cruzi* sua inclusão nas discussão sobre dispersão da diversidade do parasita é importante devido a sua abundância, presença em todos os biomas brasileiros e capacidade migratória podendo carrear animais infectados.

Diversidade parasitária em nível de espécie é reconhecida como um marcador para a persistência, produtividade, organização e resiliência do ecossistema (Marcogliese 2005). Portanto, ecossistemas em que organismos hospedeiros são parasitados por uma variedade de diferentes espécies de parasitas, são considerados mais saudáveis. Além disso, parasitas, com seus ciclos de vida curtos e taxas de mutação mais rápidas em relação aos seus hospedeiros, podem ser úteis como sentinelas em escala fina de análises de dispersão populacional e diferenciação (Wirth *et al.*, 2005). Há evidências de que a fragmentação do habitat afeta tanto a diversidade de *T. cruzi* quanto as taxas de infecção (Wirth *et al.*, 2005; Vaz *et al.*, 2007; Ramírez *et al.*, 2012). Na verdade diversidade genética em um parasita multi-hospedeiro como o *T. cruzi* pode ser avaliado como substituto para a diversidade de espécies de parasitas proposta no modelo (Marcogliese *et al.*, 2005), e na avaliação do estado de preservação de um ecossistema.

Objetivo geral

Estudar a diversidade genética, estrutura populacional e dispersão das duas DTUs de *T. cruzi* mais isoladas no Brasil, TcI e TcII, em cinco biomas.

Objetivos específicos

Objetivo específico 1: Estudar a diversidade genética e estrutura populacional de isolados de *T. cruzi* TcI de cinco diferentes biomas brasileiros usando a abordagem de Multilocus Microsatellite Typing (MLMT). Avaliar a existência de troca genética entre esses isolados através da comparação de reconstrução filogenética a partir dos genomas nuclear e mitocondrial.

Objetivo específico 2: Estudar a diversidade genética e estrutura populacional de isolados de *T. cruzi* TcII originados de mamíferos e vetores de vida livre de dois fragmentos de Mata Atlântica (sudeste e nordeste), Caatinga e Cerrado pela abordagem de MLMT.

Objetivo específico 3: Avaliar a presença de DTUs ainda não descritas *T. cruzi* circulando em animais silvestres em áreas de surto de doença de Chagas no estado do Pará, Amazônia brasileira.

Materiais e métodos

Objetivo específico 1 - Estudar a diversidade genética e estrutura populacional de isolados de *T. cruzi* TcI de cinco diferentes biomas brasileiros usando a abordagem de Multilocus Microsatellite Typing (MLMT). Avaliar a existência de troca genética entre esses isolados através da comparação de reconstrução filogenética a partir dos genomas nuclear e mitocondrial.

Isolados de T. cruzi e clonagem biológica

Um total de cento e sete isolados, a grande maioria originada de hospedeiros mamíferos silvestres ao longo de cinco biomas brasileiros, Amazônia, Mata Atlântica, Cerrado, Caatinga e Pantanal foram selecionados (Tab. 1). O genótipo TcI desses isolados foi confirmado através da técnica de multiplex PCR do gene de miniexon (Fernandes *et al*., 2001) e também da combinação dos resultados dos marcadores nucleares HSP60, GPI e 24Sα rDNA(LSU rDNA) descrita por Lewis *et al.*, 2009. Este protocolo se baseia na análise de polimorfismo de produtos de PCR do domínio D7 do gene 24Sα rDNA e análises do perfil de restrição por enzimas dos genes GPI e HSP60 (RFLP).o que permite a discriminação dos seis genótipos de *T. cruzi*. O sequenciamento de um fragmento do gene GPI (Lauthier *et al.*, 2012) em alguns isolados com perfil TcI foi feito para confirmação devido ao grupamento desses isolados em um novo clado distinto dos clados previamente descritos em isolaos das américas. Um total de 14 isolados originados dos cinco biomas foram selecionados para clonagem biológica usando técnica de clonagem em placa descrita por Yeo *et al.* 2007. O critério utilizado foi a presença de mais de uma subpopulação com base no número de alelos nas análises de microssatélites.

Análise por microssatélites da diversidade isolados de T. cruzi TcI

Um painel de 27 loci de microssatélites, distribuídos ao longo de oito cromossomos, foi amplificado em 107 isolados silvestres seguindo protocolo previamente descrito por Llewellyn *et al.*, 2009b. Deste primeiro painel, 19 loci de microssatélites foram empregados para avaliar diversidade entre um maior número de amostras (161) que incluíram isolados originais (107), clones derivados dos isolados originais (24) e perfis MLMT de trinta isolados previamente publicados (Messenger *et al.*, 2011). Parâmetros de diversidade em genética populacional do perfil dos 27 loci foram primeiramente calculados baseados no grupamento das amostras de acordo com a origem geográfica e por bioma. O primeiro parâmetro de

diversidade genética a nível populacional calculado foi riqueza alélica (Ar) usando correção por tamanho de amostra no programa FSTAT 2.9.3.2 (Goudet 1995). Secundariamente, para uma melhor medida de sub-agrupamento intra-população, a medida de diversidade par-a-par DAS e o desvio padrão associado foi também avaliado por população. O índice de fixação FIS, uma medida de distribuição de heterozigosidade dentro e entre indivíduos, foi estimado por locus por população em FSTAT 2.9.3.2 (Goudet 1995). Testes por população do desvio do equílibrio de Hardy Weinberg nos loci específicos foram calculados no programa ARLEQUIN v3.1 e níveis de significância associada para p valor derivado depois de correção sequencial de Bonferroni para minimizar a probabilidade de ocorrência de erro Tipo 1 (Rice 1989). Para os conjuntos de dados de ambas as análises MLMT com 27 e 19 loci, a topoplogia de cada isolado ou clone foi definida pela árvore filogenética do tipo neighbour-joining baseada nas distâncias par-a-par entre os genótipos multilocus MLGs [avaliado usando DAS (1 –proporção de alelos compartilhados em todos os loci/n)] calculado no programa MICROSAT (Minch *et al.*, 1995). Para o conjunto de dados gerados a partir de 27 loci nós adicionalmente definimos a composição genética via um algoritmo de grupamento do tipo K (K-means clustering algorithm) implementado em adegenet (Jombart 2008), o qual define o número ótimo de populações por referência do critério de informação Bayesiana (Bayesian Information Criterion, BIC). Essse grupamentos foram subsequentemente submetidos a análise discriminante de componentes principais (Jombart 2010).

Análises de DNA do Maxicírculo de clones TcI

Fragmentos de dez genes que compõe os maxicírculos foram amplificados e sequenciados de 14 clones de *T. cruzi* seguindo protocolo previamente descrito (Messenger *et al.*, 2011). As sequências de nucleotídeos dos fragmentos foram concatenadas em cada em cada amostra e alinhado baseando-se em sequências previamente publicados (Messenger *et al.*, 2011). Filogenias foram inferidas usando o método de Máxima Verossimilhança (ML) implementado em PhyML (categoria de taxa de 4 substituições) (Guindon *et al.*, 2010). O melhor modelo de substituição nucleotídica foi selecionado de 88 modelos e sua significância avaliada de acordo com o critério de informação Akaike (Akaike Information Criterium, AIC) em jMODELTEST 1.0 (Posada & Crandall 1998). O melhor modelo selecionado para este conjunto de dados foi GTR+I+G. O suporte de Bootstrap para a topologia dos clados foi estimada depois da geração de 1000 pseudo-replicatas de árvores desse conjunto de dados. Análise filogenética Bayesiana foi realizada usando MrBAYES v3.1 (Ronquist & Huelsenbeck 2003) (definições de acordo com jMODELTEST 1.0). Cinco análises

independentes foram processadas usando uma aleatória árvore inicial com três cadeias quentes e uma cadeia fria sobre 10 milhões de gerações com amostragem a cada 10 simulações (25% burn-in).

Tabela 1. Isolados brasileiros de *Trypanosoma cruzi* I silvestres avaliados por MLMT, com seus hospedeiros, biomas e municípios e estados de origem .

Código do isolado	Hospedeiro	Bioma	Município/ Estado	Latitude	Longitude	Código da população atribuído (DAPC)	População *A priori*
3510	*Didelphis albiventris*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	10	Ceará
6809	*Rattus rattus*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	7	Ceará
6812	*Didelphis albiventris*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	10	Ceará
6813	*Didelphis albiventris*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	10	Ceará
6824	*Didelphis albiventris*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	10	Ceará
8622	*Didephis albiventris*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	7	Ceará
8648	*Didelphis albiventris*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	10	Ceará
9529	*Rattus rattus*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	7	Ceará
9531	*Rattus rattus*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	7	Ceará
9538	*Rattus rattus*	Caatinga	Jaguaruana/Ceará	-4.8308	-37.7814	7	Ceará
9667	*Monodelphis domestica*	Caatinga	Redenção/Ceará	-4.2261	-38.7311	10	Ceará
11629	*Didephis albiventris*	Caatinga	Russas/Ceará	-4.9392	-37.9786	6	Ceará
11639	*Didelphis albiventris*	Caatinga	Russas/Ceará	-4.9392	-37.9786	10	Ceará
11640	*Didelphis albiventris*	Caatinga	Russas/Ceará	-4.9392	-37.9786	10	Ceará
8552	*Didephis albiventris*	Cerrado	Aporé/Goiás	-18.9489	-51.9086	1	Goiáis
9148	*Gracilinanus sp*	Cerrado	Aporé/Goiás	-18.9489	-51.9086	1	Goiáis
9149	*Didephis albiventris*	Cerrado	Aporé/Goiás	-18.9489	-51.9086	1	Goiáis
9425	*Didephis albiventris*	Cerrado	Aporé/Goiás	-18.9489	-51.9086	1	Goiáis
10268	*Proechimys sp*	Amazônia	Cachoeira do Arari/Pará	-1.0044	-48.9572	8	Norte Pará
10272	*Didelphis marsupialis*	Amazônia	Cachoeira do Arari/Pará	-1.0044	-48.9572	8	Norte Pará
10285	*Didelphis marsupialis*	Amazônia	Cachoeira do Arari/Pará	-1.0044	-48.9572	8	Norte Pará
10288	*Oecomys sp.*	Amazônia	Cachoeira do Arari/Pará	-1.0044	-48.9572	8	Norte Pará
10289	*desconhecido*	Amazônia	Cachoeira do Arari/Pará	-1.0044	-48.9572	8	Norte Pará
10290	*Didelphis marsupialis*	Amazônia	Cachoeira do Arari/Pará	-1.0044	-48.9572	8	Norte Pará
11604	*Marmosops murina*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará
11605	*Philander opossum*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará

Código do isolado	Hospedeiro	Bioma	Município/ Estado	Latitude	Longitude	Código da população atribuído (DAPC)	População *A priori* (continuação)
11609	*Philander opossum*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará
11611	*Philander opossum*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	8	Norte Pará
12624	*Philander opossum*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	6	Norte Pará
12625	*Didelphis marsupialis*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	8	Norte Pará
12626	*Micoureus demerarae*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	32	Norte Pará
12628	*Didelphis marsupialis*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	6	Norte Pará
12630	*Philander opossum*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará
12631	*Philander opossum*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará
12640	*Didelphis marsupialis*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	8	Norte Pará
12667	*Didelphis marsupialis*	Amazônia	Curralinho/Pará	-0.5364	-49.1842	8	Norte Pará
12668	*Didelphis marsupialis*	Amazônia	Curralinho/Pará	-0.5364	-49.1842	8	Norte Pará
12964	*Didelphis marsupialis*	Amazônia	Curralinho/Pará	-0.5364	-49.1842	8	Norte Pará
FNS258	*Canis familiaris*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	6	Norte Pará
LBT1812	*Rhodnius pictipes*	Amazônia	Belém/Pará	-1.3789	-48.4756	8	Norte Pará
LBT1813	*Rhodnius pictipes*	Amazônia	Belém/Pará	-1.3789	-48.4756	8	Norte Pará
LBT918	*Canis familiaris*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	6	Norte Pará
LBT964	*Rhodnius pictipes*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará
LBT966	*Rhodnius pictipes*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	4	Norte Pará
LBT967	*Rhodnius pictipes*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	8	Norte Pará
LBT969	*Rhodnius pictipes*	Amazônia	Abaetetuba/Pará	-1.7297	-48.8719	8	Norte Pará
5324	*Oecomys sp*	Pantanal	Aquidauana/Mato Grosso do Sul	-19.6806	-57.3378	2	Pantanal
5340	*Oecomys sp*	Pantanal	Aquidauana/Mato Grosso do Sul	-19.6806	-57.3378	2	Pantanal
5355	*Monodelphis domestica*	Pantanal	Aquidauana/Mato Grosso do Sul	-19.1393	-56.7958	2	Pantanal
FRN46	*Oecomys sp*	Pantanal	Aquidauana/Mato Grosso do Sul	-19.6806	-57.3378	11	Pantanal
5666	*Gracilinanus agilis*	Pantanal	Corumbá/Mato Grosso do Sul	-19.1393	-56.7958	2	Pantanal
5667	*Gracilinanus agilis*	Pantanal	Corumbá/Mato Grosso do Sul	-19.1393	-56.7958	2	Pantanal
5674	*Monodelphis domestica*	Pantanal	Corumbá/Mato Grosso do Sul	-19.0097	-57.6547	9	Pantanal

Código do isolado	Hospedeiro	Bioma	Município/ Estado	Latitude	Longitude	Código da população atribuído (DAPC)	População *A priori* (Continuação)
5679	*Thylamys macrurus*	Pantanal	Corumbá/Mato Grosso do Sul	-19.1393	-56.7958	2	Pantanal
5698	*Gracilinanus sp*	Pantanal	Corumbá/Mato Grosso do Sul	-19.1393	-56.7958	2	Pantanal
7587	*Gracilinanus agilis*	Pantanal	Corumbá/Mato Grosso do Sul	-19.0097	-57.6547	2	Pantanal
GM288	*Nasua nasua*	Pantanal	Corumbá/Mato Grosso do Sul	-19.0097	-57.6547	2	Pantanal
GM295	*Nasua nasua*	Pantanal	Corumbá/Mato Grosso do Sul	-19.0097	-57.6547	2	Pantanal
THY01	*Thylamys macrurus*	Pantanal	Corumbá/Mato Grosso do Sul	-18.9919	-56.6313	2	Pantanal
4250	*Thrichomys apereoides*	Caatinga	São Raimundo Nonato/Piauí	-9.0053	-45.7114	5	Piaui
4262	*Thrichomys apereoides*	Caatinga	São Raimundo Nonato/Piauí	-8.4167	-42.3331	5	Piaui
6183	*Didelphis albiventris*	Caatinga	São Raimundo Nonato/Piauí	-9.0053	-45.7114	10	Piaui
FNS1	*Triatoma brasiliensis*	Caatinga	João Costa/Piauí	-8.5103	-42.4200	5	Piaui
M1	*Didelphis albiventris*	Caatinga	Coronel José Dias/Piauí	-8.8250	-42.5064	10	Piaui
M3	*Didelphis albiventris*	Caatinga	Coronel José Dias/Piauí	-8.8250	-42.5064	7	Piaui
645	*Didelphis marsupialis*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
762	*Didelphis*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.4117	-42.9667	9	Mata Atlântica
5563	*Nectomys squamipes*	Mata Atlântica	Capitão Andrade/Minas Gerais	-19.0700	-41.8633	3	Mata Atlântica
5565	*Didelphis aurita*	Mata Atlântica	Capitão Andrade/Minas Gerais	-19.0700	-41.8633	9	Mata Atlântica
5574	*Didelphis aurita*	Mata Atlântica	Capitão Andrade/Minas Gerais	-19.0700	-41.8633	9	Mata Atlântica
BF5	*Rhodnius prolixus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
BP4	*Rhodnius prolixus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
BPT4	*Rhodnius prolixus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
C12	*Philander frenatus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
C45	*Philander frenatus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
C48	*Philander frenatus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
C60	*Philander frenatus*	Mata Atlântica	Teresópolis/Rio de Janeiro	-42.9667	-42.9667	11	Mata Atlântica
D7	*Didelphis aurita*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	5	Mata Atlântica
D8	*Didelphis marsupialis*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	5	Mata Atlântica
G05	*Didelphis sp.*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.5319	-42.9897	9	Mata Atlântica
G15	*Didelphis marsupialis*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.5319	-42.9897	9	Mata Atlântica

Código do isolado	Hospedeiro	Bioma	Município/ Estado	Latitude	Longitude	Código da população atribuído (DAPC)	População *A priori*
G33	*Didelphis marsupialis*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.5319	-42.9897	5	Mata Atlântica
G41	*Didelphis marsupialis*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.5319	-42.9897	8	Mata Atlântica
MLCD44	*Leontopithecus chrysomela*	Mata Atlântica	Ilhéus/Bahia	-15.2694	-39.0666	5	Mata Atlântica
MLD291	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	11	Mata Atlântica
MLD490	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	11	Mata Atlântica
MLD524	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	11	Mata Atlântica
MLD600	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	3	Mata Atlântica
MLD632	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	11	Mata Atlântica
MLD714	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	3	Mata Atlântica
MLD776c	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	11	Mata Atlântica
MLD877b	*Leontopithecus rosalia*	Mata Atlântica	Silva Jardim/Rio de Janeiro	-22.6592	-42.3831	3	Mata Atlântica
7301	*Didelphis aurita*	Mata Atlântica	Navegantes/Santa Catarina	-26.8989	-48.6558	2	Santa Catarina
7313	*Didelphis aurita*	Mata Atlântica	Navegantes/Santa Catarina	-26.8989	-48.6558	2	Santa Catarina
7344	*Olygoryzomys nigripes*	Mata Atlântica	Jaborá/Santa Catarina	-27.1703	-51.7375	2	Santa Catarina
6716	*Didelphis marsupialis*	Amazônia	Itupiranga/Pará	-5.1733	-49.3656	8	Sul Pará
6723	*Didelphis marsupialis*	Amazônia	Itupiranga/Pará	-5.1733	-49.3656	9	Sul Pará
6737	*Didelphis marsupialis*	Amazônia	Itupiranga/Pará	-5.1733	-49.3656	8	Sul Pará
13100	*Didelphis marsupialis*	Amazônia/Cerrado	Augustinópolis/Tocantins	-5.4683	-47.8894	8	Sul Pará
13103	*Phyllostomus hastatus*	Amazônia/Cerrado	Augustinópolis/Tocantins	-5.4683	-47.8894	4	Sul Pará
10171	*Didelphis albiventris*	Cerrado/Caatinga	São Raimundo Nonato/Piauí	-9.9667	-45.7167	10	Tocantins
12903	*Gracilinanus sp.*	Cerrado	Dianópolis/Tocantins	-11.6278	-46.8208	5	Tocantins
JFV297	*Desmodus rotundus*	Cerrado	Arraias/Tocantins	-12.9264	-46.9350	11	Tocantins
JFV306	*Carolia perspicillata*	Cerrado	Arraias/Tocantins	-12.9264	-46.9350	11	Tocantins
JFV307	*Phyllostomus albicola*	Cerrado	Arraias/Tocantins	-12.9264	-46.9350	11	Tocantins
JFV313	*Phyllostomus hastatus*	Cerrado	Arraias/Tocantins	-12.9264	-46.9350	11	Tocantins
T.sord15	*Triatoma sordida*	Cerrado	Posse/Goiás	-1.7297	-48.8719	10	Tocantins

Objetivo específico 2 - Estudar a diversidade genética e estrutura populacional de isolados de *T. cruzi* TcII originados de mamíferos e vetores de vida livre de dois fragmentos de Mata Atlântica (sudeste e nordeste), Caatinga e Cerrado pela abordagem de MLMT.

Isolados TcII de T. cruzi

Selecionamos 60 isolados silvestres de *T. cruzi* previamente tipados como Tc2 (TcII/TcV/TcVI) pelo método de multiplex PCR do gene de miniexon e depositados no crio-banco institucional ColTryp localizado no Laboratório de Biologia de Tripanosomatídeos, Instituto Oswaldo Cruz, Fiocruz. A genotipagem à nível de DTU identificando esses isolados como TcII foi feita com combinação dos seguintes marcadores e respectivas técnicas, PCR-RFLP dos genes HSP60 e GPI e AFLP do gene 24S αDNA (Lewis *et al.*, 2009). O sequenciamento de gene GPI foi utilizado em 40 isolados cujo tamanho de banda do marcador 24S αDNA não foi claro (Llewellyn *et al.*, 2009). Esses isolados são oriundos da Mata Atlântica, Caatinga e um isolado do Cerrado. Os isolados da Mata Atlântica são oriundos de três estados, Rio de Janeiro (40), Bahia (13), e Santa Catarina (3). De outros estados e biomas temos, 7 isolados do Piauí (Caatinga) e 1 do Tocantins (Cerrado). Os dados sobre hospedeiros e biomas de origem estão sumarizados na Tab. 2 e Fig. 1.

Tabela 2. Isolados de *Trypanosoma cruzi* II silvestres avaliados por MLMT, com seus hospedeiros, biomas e estados brasileiros de origem .

Espécies	isolados(n)	bioma	Estado
Leontopithecus rosalia	33	Mata Atlântica	Rio de Janeiro
Philander opossum	1	Mata Atlântica	Rio de Janeiro
Didelphis aurita	1	Mata Atlântica	Rio de Janeiro
Triatoma tibimaculata	3	Mata Atlântica	Santa catarina
Leontopithecus chrysomelas	13	Mata Atlântica	Bahia
Triatoma brasiliensis	6	Caatinga	Piauí
Didelphis albiventris	1	Caatinga	Piauí
Trichomys laurentius	1	Caatinga	Piauí
Olygoryzomys sp	1	Cerrado	Tocantins

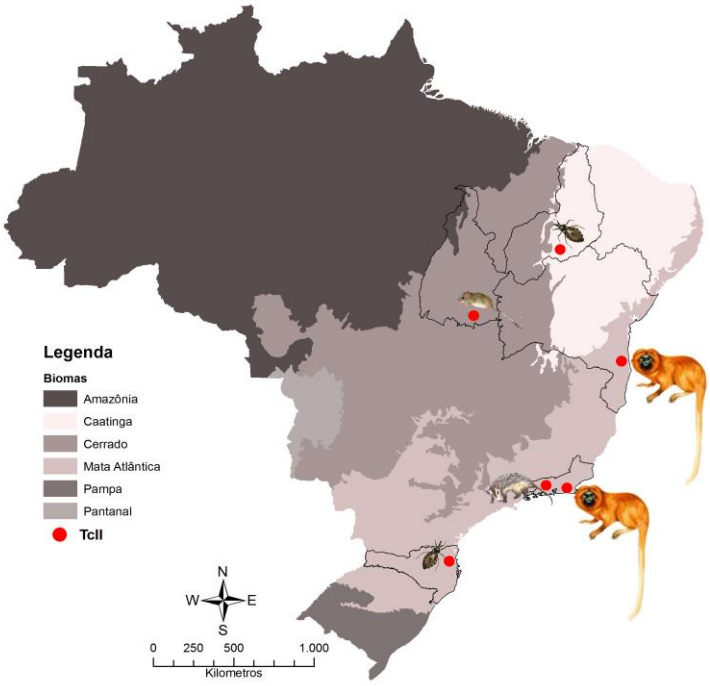

Figura 1. Mapa da localização dos isolados TcII deste estudo com seus respectivos hospedeiros, vetores e biomas de origem.

DNA microssatélites (MLMT) de isolados TcII

Os 60 isolados listados foram analisados quanto ao perfil polimórfico de 26 loci de DNA microssatélite (Tab. 3). Seguimos a metodologia de Llewellyn *et al.*, 2009b, sob supervisão do próprio autor. Após a PCR com os iniciadores específicos para os 26 loci o tamanho dos alelos foi determinado utilizando um sequenciador automático de capilaridade (AB3730, Applied Biosystems, UK). As análises dos dados de tamanhos de fragmentos gerados pelo sequenciados foram feitas no programa GeneMapper. Os loci de microssatélites utilizados neste estudo foram definidos em Llewellyn *et al.*, 2009b.

Tabela 3. Lista de loci microssatélites nucleares avaliados neste estudo.

Loci de microssatélites	
6925TGbNED	10101CAaNED
11283TAbNED	8741TAVIC
10101TAVIC	10187TTAPET
10101TCVIC	6529CAaVIC
10187GATET	10101CAcVIC
6925CTVIC	7093TC
7093TAbNED	7093TCCVIC
10187TAVIC	7093TAcTET
6529TAbPET	6925TGaNED
TcUn4VIC	11863CAVIC
10187CATAVIC	11283TCGFAM
mclf10FAM	6855TAGANED
10359CANED	6559TCTET

Após sequenciamento e análise dos fragmentos, eliminamos 5 loci que, ou apresentaram-se monomórficos e portanto sem utilidade para a análise de diversidade ou apresentaram falhas de amplificação, os loci retirados da análise são os seguintes: 11863 CA VIC, 6855 TA GA NED, 8741 TA VIC, TcUn2 NED e TcUn3 PET.

As estimativas de distância genética entre os 60 isolados baseadas nos perfis polimórficos de DNA microssatélites (MLMT) foram feitas com o programa MSAT2 sob o modelo de infinitos alelos usando DAS (1-proporção de alelos compartilhados em todos os loci/n) e utilizadas para construção do cladograma no programa FigTree v1.1.2 (Rambaut 2008).

Para as análises de diversidade e populacionais utilizamos a estimativa de riqueza alélica calculada no programa FSTAT 2.9.3.2, sendo os valores de cada população corrigidos de acordo com o tamanho da mesma através do método de rarefação de Hurlbert no programa MolKin v3.0. Os índices de heterozigosidade e estimativas de subdivisão (FST) pareados das populações foram feitas em Arlequin 3.0. O cálculo do índice de fixação (subdivisão) de cada população em relação à população total (FST) foi calculado da seguinte forma: FST = 1 – média da heterozigosidade esperada na população/ média da heterozigosidade esperada em todas as populações.

Objetivo específico 3 - Avaliar a presença de DTUs ainda não descritas *T. cruzi* circulando em animais silvestres em áreas de surto de doença de Chagas no estado do Pará, Amazônia brasileira.

Área de estudo

Este estudo foi conduzido com isolados de triatomíneos silvestres e amostras de soro de cães de três municípios e respectivas localidades no estado do Pará: Abaetetuba/Ajuaí (01º43'24'' S; 48º52'54'' W) e Belém/Val-de-Cans (01º27'21''S; 48º30'16''W) são localizadas na mesorregião nordeste do estado e Monte Alegre/Setor 11 (01°38'20"S; 54°14'32"W) é localizado na mesorregião do baixo Amazonas (Roque *et al.*, 2013, Xavier *et al.*, 2012) (Fig. 2). O clima comum dessas regiões se caracteriza como tropical úmido, com chuvas e ventos regulares, e temperatura entre 27°C e 36°C.

Amostras biológicas

Foram caracterizados isolados de *T. cruzi* derivados de dois triatomíneos da espécie *Rhodnius pictipes* e amostras de DNA de *T. cruzi* extraído de soro de cinco cães naturalmente infectados (Fig. 2). Os dois isolados de *T. cruzi*, LBT 1458 e LBT 1814 foram obtidos de *R. pictipes* capturados em palmeiras *Attalea pharelata*, respectivamente nas localidades de Rio Ajuaí e Val de Cans, respectivamente nos municípios de Abaetetuba e Belém no estado do Pará. A obtenção de isolados de *T. cruzi* foi através de cultivo do conteúdo intestinal dos triatomíneos em meio de cultura bifásico NNN+LIT suplementado com 10% de soro fetal bovino. Quando as culturas alcançaram a fase exponencial de crescimento foram submetidas à extração de DNA através do método de fenol-clorofórmio e à criopreservação para depósito na Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores - COLTRYP, da Fundação Oswaldo Cruz.

A seleção dessas cinco amostras de DNA extraído de soro de cães utilizados neste estudo para genotipagem foi baseada na parasitemia patente exibida por esses animais em exame à fresco e a infecção por *T. cruzi* confirmada através de amplificação por PCR da região variável do k-DNA (Xavier *et al.*, 2012). A taxa de infecção na microscopia ótica visualmente indicava a proporção de 50% de parasitas e 50% de hemácias em cada campo. Outro critério para análise dessas amostras foram os sintomas clínicos apresentados por esses cães (febre, edema generalizado, esplenomegalia, rigidez abdominal e palidez nas mucosas) com subsequente mortalidade de 40% (2/5) (Xavier *et al*., 2012). O DNA do soro dos cinco cães, respectivamente, LBT 1818, LBT 1819, LBT 1820, LBT 1821 e LBT 1822 foi extraído

através do método de fenol-clorofórmio, como o usado para extração a partir de cultura, somente excluindo-se o pré-tratamento com SDS (Xavier *et al.*, 2012).Os proprietários dos cães dos quais obtivemos amostras de soro vivem na zona rural (Sector 11) do município de Monte Alegre/PA. A escassa população da área vive de agricultura de subsistência e frequentemente usa os cães para caçar. Vale mencionar que os cães estudados eram autóctones e nunca se deslocaram para outros municípios.

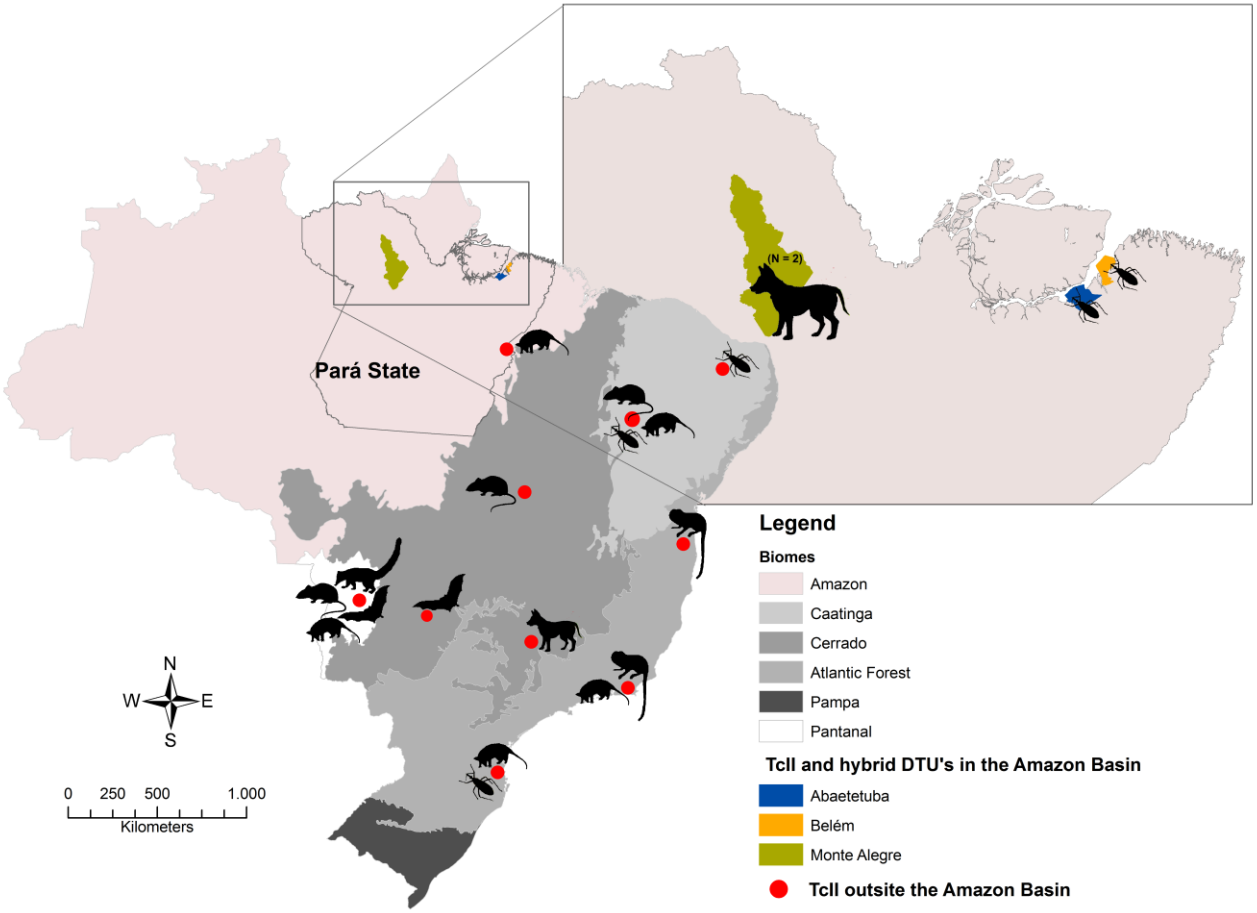

Figura 2. Localização dos genótipos TcII e híbrido no estado do Pará na região Amazônia e distribuição isolados TcII no Brasil. Círculos vermelhos indicam localização de isolados TcII em mamíferos e símbolos pretos representam seus respectivos hospedeiros mamíferos e vetores. Acima à direita da figura, são destacados os municípios deste estudo no estado do Pará. Os dados para distribuição de TcII nos demais biomas brasileiros foram obtidos em Lisboa *et al.*, 2000; Pinho *et al.*, 2000; Roque *et al.*, 2008; Xavier *et al.*, 2007; Rocha *et al.*, 2013 e Herrera *et al.*, 2008).

PCR do gene de Mini-exon

Todas as amostras de *T. cruzi* foram submetidas à etapa inicial de tipagem através da técnica de multiplex PCR do gene de miniexon de acordo com o protocolo descrito por Fernandes *et al.*2001, para identificação de três grupos de DTUs de *T. cruzi,* TcI (200 pares de base-pb), Tc2 (TcII/TcV/TcVI – 250 pb), Zimodema 3 (TcIII/TcIV – 150 pb) além de discriminar *T. rangeli* (100 pb).

Clonagem biológica de isolado de T. cruzi

O isolado LBT 1458 apresentou resultados controversos no ensaio de miniexon, ou seja, sob as mesmas condições metodológicas, primeiramente exibiu perfil de infecção misto TcI/Tc2, com bandas de 200 e 250 pb e nas demais amplificações esse isolado exibiu somente a banda referente a Tc2 (250 pb). Optamos por submetê-lo a clonagem biológica com objetivo de verificar a composição de subpopulações e genotipá-las separadamente pela técnica meio sólido em placas de Petri (Yeo *et al.*, 2007). Nós selecionamos dez clones para tipagem pelo ensaio do gene de miniexon (Fernandes *et al.*, 2001). Obtivemos nessa primeira seleção, três clones TcI e 7 clones Tc2, destes últimos selecionamos dois para completar a genotipagem em DTU (TcII, TcV ou TcVI).

Clonagem molecular do gene de miniexon

Produtos de PCR de amostras de soro dos cães (LBT 1819 e LBT 1822) que apresentaram infecção mistas por DTUs de *T. cruzi* pelo ensaio de miniexon foram clonados usando o Kit pGEM®T Easy Vector System (Promega, Madison, WI, USA) e seguindo o protocolo dos fabricantes. Cada colônia crescida correspondeu a um clone individual contendo um inserto (amplicon 200 ou 250 pb) de uma das DTUs. As colônias (clones) foram coletadas aleatoriamente e submetidas ao ensaio de miniexon (Fernandes *et al.*, 2001) para selecionar os clones com fragmentos de 250 pb correspondente aos genótipos TcII, TcV ou TcVI.

Análise de polimorfismo dos fragmentos de restrição do DNA genômico - RFLP para caracterização de DTUs de T. cruzi

Os seguintes protocolos para genotipagem de amostras mistas foram adotadas dependendo da origem biológica das mesmas: (a) para genotipar os clones do isolado LBT1458, nós realizamos RFLP do gene nuclear 1f8 após digestão pela enzima Alw21I, protocolo que distingue as DTUs TcII das DTUs híbridas TcV e TcVI (Rozas *et al.*, 2007) e

(b) RFLP do gene gp72 pela enzima TaqI foi testado como protocolo confirmatório que apresenta perfis distintos para as DTUs TcII, TcV e TcVI (Rozas *et al.*, 2007). Contudo, este protocol não permitiu a caracterização dos clones selecionados do LBT 1458, clone 5 e 7. Para genotipagem do isolado LBT 1814 e da amostra de soro de cão LBT 1822 foi utilizado o protocolo de PCR-RFLP do gene Histona 3/enzima AluI que distingue TcII das DTUs híbridas TcV e TcVI sem sobreposição de fragmentos (Westenberger *et al.*, 2005; Rocha *et al.*, 2013). Cada reação incluiu controles negative e positivos representativo das DTUs. Os resultados da PCR e RFLP foram visualizados em gel de agarose 3% corado com brometo de etídio sob luz UV.

Sequenciamento dos genes de Miniexon e gp72 de T. cruzi

Gene de Mini-exon

Foi sequenciado o fragmento de 250 pb amplificado da região do Miniexon da amostra de DNA de soro de cão LBT 1822. A sequência foi comparada com sequências depositadas no GenBank para identificação da DTU de *T. cruzi* presente nesta amostra. Este fragmento foi obtido a partir de clonagem molecular da amplificação pelo ensaio de multiplex PCR do gene de miniexon. Os iniciadores usados para sequenciamento foram os específicos para a região de 250 pb (Fernandes *et al.*, 2001).

Gene glicoproteína 72 (gene gp72)

Para tentar entender o distinto perfil exibido pelo protocolo de RFLP do gene gp72/TaqI nos clones 5 e 7 do isolado LBT 1458 nós ampliamos o estudo testando este protocolo em vinte isolados depositados na ColTryp, previamente identificados como TcII pela combinação de PCR-RFLP dos genes HSP60 e GPI e AFLP do gene 24S αDNA (Lewis *et al.*, 2009) além do sequenciamento de gene GPI (Llewellyn *et al.*, 2009b). Foram sequenciados os dois clones do isolado LBT 1458 além de cinco isolados TcII com padrão RFLP do gp72 igual ao descrito por Rozas *et al.*, 2007 para isolados TcII e cinco isolados TcII exibindo padrão distinto como os clones LBT 1458 (Tab. 4). As análises deste locus também permitiram verificar a possível concordância destes diferentes perfis no agrupamento dos isolados. Os amplicons de 1290 bp foram purificados usando o kit de purificação comercial Illustra GFX PCR DNA and Gel Band (GE Healthcare Life Sciences,Little Chalfont, Buckinghamshire, UK) e submetidos à reação de sequenciamento com o Kit Big Dye Terminator v 3.1 (Applied Biosystems, Foster City,California, USA). Os produtos foram sequenciados em um sequenciador automático Sequencer 3100 (Applied Biosystems) da

Plataforma de sequenciamento institucional da Fiocruz, usando os mesmos primers da amplificação (Rozas *et al.*, 2007).

Tabela 4. Isolados Trypanosoma cruzi II submetidos ao sequenciamento do gene gp72, seus hospedeiros de origem, município, estado e bioma de origem

Isolados *T. cruzi* II	**Espécies de Hospedeiros**	**Município-Estado[a]/ Bioma**	**Perfil PCR-RFLP do gp72 gene [b]**	**Número de acesso no GenBank**
MLD 564b	*Leontopithecus rosalia*	Silva Jardim-RJ/ Mata Atlântica	A	KJ402453
MLD 832	*Leontopithecus rosalia*	Silva Jardim-RJ/ Mata Atlântica	A	KJ402451
MLD 840	*Leontopithecus rosalia*	Silva Jardim-RJ/ Mata Atlântica	A	KJ402452
MLCD 92	*Leontopithecus chrysomelas*	Una-BA/ Mata Atlântica	A	KJ402448
JCA3	*Triatoma brasiliensis*	João Costa-PI/ Caatinga	A	KJ402446
LBT 1458 clone 5	*Rhodnius pictipes*	Abaetetuba-PA/ Amazônia	B	KJ402454
LBT 1458 clone 7	*Rhodnius pictipes*	Abaetetuba-PA/ Amazônia	B	KJ402455
MLD 594b	*Leontopithecus rosalia*	Silva Jardim-RJ/ Mata Atlântica	B	KJ402450
CD 621	*Canis familiaris*	São Roque de Minas-MG/ Cerrado	B	KJ402444
CD 640	*Canis familiaris*	São Roque de Minas-MG/ Cerrado	B	KJ402445
MLD 1025	*Leontopithecus rosalia*	Silva Jardim-RJ/ Mata Atlântica	B	KJ402449
MLCD 82	*Leontopithecus chrysomelas*	Una-BA/ Mata Atlântica	B	KJ402447

[a] RJ - Rio de Janeiro; BA - Bahia; PI - Piauí; PA - Pará; MG - Minas Gerais;

A – de acordo com perfil descrito por Rozas *et al* (2007); B – perfil distinto.

Análise de sequências

A edição de sequências, alinhamento e construção de árvore filogenética foram realizados usando os softwares Chromas v. 1.45 (School of Health Sciences, Griffith University, Queensland, Australia) e Mega v 5.1 (Tamura *et al.*, 2011). Foram usados o modelo Kimura-2-parametros e o método estatístico para reconstrução filogenética Neighbor-joining. O nível de confiança representado pelos valores de bootstrap foi atingidos a partir de

1.000 pseudo-árvores. Foram incluídas nas análises de reconstrução filogenética como grupo externo, três sequências TcI do GenBank (Tab. 4)

Resultados

Objetivo específico 1 - Estudar a diversidade genética e estrutura populacional de isolados de *T. cruzi* TcI de cinco diferentes biomas brasileiros usando a abordagem de Multilocus Microsatellite Typing (MLMT). Avaliar a existência de troca genética entre esses isolados através da comparação de reconstrução filogenética a partir dos genomas nuclear e mitocondrial.

Os loci de microssatélites nucleares demonstraram alta diversidade genética entre os 107 isolados TcI estudados. A fim de comparação, os isolados foram agrupados *a priori* de acordo com ambas, origem geográfica e por bioma (Fig. 3). A partir desse agrupamento, 11 populações foram definidas. Os dados de cada isolado estão representados na Tab. 1 e parâmetros de genética populacional associados a essas onze populações estão expostos na Tab. 5. O cálculo de riqueza alélica (Ar) se deu após correção por tamanho de amostras. Valores de Ar é mais alta entre isolados das populações PARÁ NORTE e PARÁ SUL no leste da Amazônia (Ar = 2.027 e 2.134), bem como nos isolados da Mata Atlântica (Ar = 2.010) e Tocantins, no bioma Cerrado (Ar = 1.959). Enquanto Ar é uma medida útil da diversidade genética dentro da população, a diversidade estruturada dentro de uma população também foi calculada pela média pareada de alelos compartilhados (DAS) entre genótipos MLMT (MLGs) em cada população (Tab. 5). Em resumo, a subestruturação da diversidade genética entre algumas populações parece consideravelmente maior do que em outras. A média pareada dos valores de DAS e seus desvios padrões parecem refletir este fenômeno (Tab. 5). Diversas populações com elevados desvios padrões (por exemplo, Mata Atlântica – 0.369 ± 0.199, Tocantins - 0.362 ± 0.221) provavelmente possuem sub-agrupamentos intra-população. Em contraste, a diversidade genética é uniformemente distribuída entre isolados dentro das populações com baixos desvios padrões sobre as médias DAS (PARÁ NORTE - 0.445 ± 0.082, PARÁ SUL - 0.416 ± 0.053). A heterozigosidade observada variou consideravelmente entre as populações. Contudo, em populações com número de indivíduos N>10 a interpretação é mais passível de ser significativa, valores positivos de FIS prevaleceram, e por inferência o déficit de heterozigosidade comparado às expectativas de Hardy-Weinberg. O agrupamento de amostras baseado na distância genética nuclear pareada fornece compreensão dentro dos padrões idiossincráticos de diversidade genética observada nas populações. A presença de genótipos pertencentes a diferentes biomas em um mesmo clado ocorreu em múltiplos agrupamentos. Este fenômeno é melhor representado pela

composição das barras adjacentes aos agrupamentos plotados em escala multidimensional na Fig.3. Isolados da Mata Atlântica e Tocantins agruparam entre grupos múltiplos e divergentes. Entretanto TcI das populações PARÁ NORTE PARÁ SUL e Ceará se agrupam nos mesmos clados ou em clados intimamente relacionados (Fig. 3). Os demais grupos representam situações intermediárias entre esses dois extremos. Dado o intenso grau de mistura de genótipos e substrutura em várias populações nós decidimos não calcular os índices de desequilíbrio de ligação específico de cada população. É sabido que a substrutura em uma população infla tais medidas e aumenta a probabilidade de erro do tipo 1 (Maynard Smith *et al.*, 1993). Em vez disso, nós optamos em avaliar a congruência entre genoma nuclear e mitocondrial ou o diferencial agrupamento como evidência de eventos raros de intercâmbio genético. Para tais comparações nós incorporamos dados previamente publicados de DNA nuclear e DNA mitochondrial em nosso conjunto de dados (Messenger *et al.*, 2011). A Fig. 4 mostra as árvores resultantes e o único recombinante que detectamos entre os 14 clones testados foi um clone do isolado LBT 6824, isolado de *Didelphis albiventris* da Caatinga, apresentando um genoma mitocondrial similar aos isolados da Amazônia. A direção hipotética do evento de introgressão (acceptor e doador) é detalhado no mapa desta figura. A inclusão de perfis microssatélites nucleares de isolados de outros países ao longo das Américas na Fig. 4 foi útil como referência para a diversidade apresentada pelos isolados brasileiros deste estudo. A disversidade apresentada por nossos isolados TcI foi amplamente maior e incluiu as toda a diversidade de TcI conhecida na América do Sul abaixo da linha do Equador. Mais notavelmente, isolados pertencentes ao agrupamento 11 na Fig. 3 forma um novo clado e que difere toda diversidade de TcI conhecida nas Américas. Este grupo foi homogêneo em termos genéticos e foi composto por 19 isolados de três biomas, Mata Atlântica (13 isolados), Cerrado (4 isolados) e Pantanal (2 isolados). Os isolados da Mata Atlântica foram originados de 3 *Rhodnius prolixus*, 5 *Leontopithecus rosalia*, 1 *Didelphis aurita* e 4 *Philander opossum*. O Pantanal foi representado neste clado por isolados do roedor *Oecomys* sp. O Cerrado foi representado exclusivamente por isolados de quirópteros de 4 espécies diferentes. Devido ao caráter distinto deste clado e a presença de isolados de quirópteros o sequenciamento do gene GPI de todos os isolados do foi realizado para confirmá-los como pertencentes a DTU I. As sequências do gene GPI deste grupo o confirma como TcI sem nenhuma afinidade com TcBAT (dados não demonstrados).

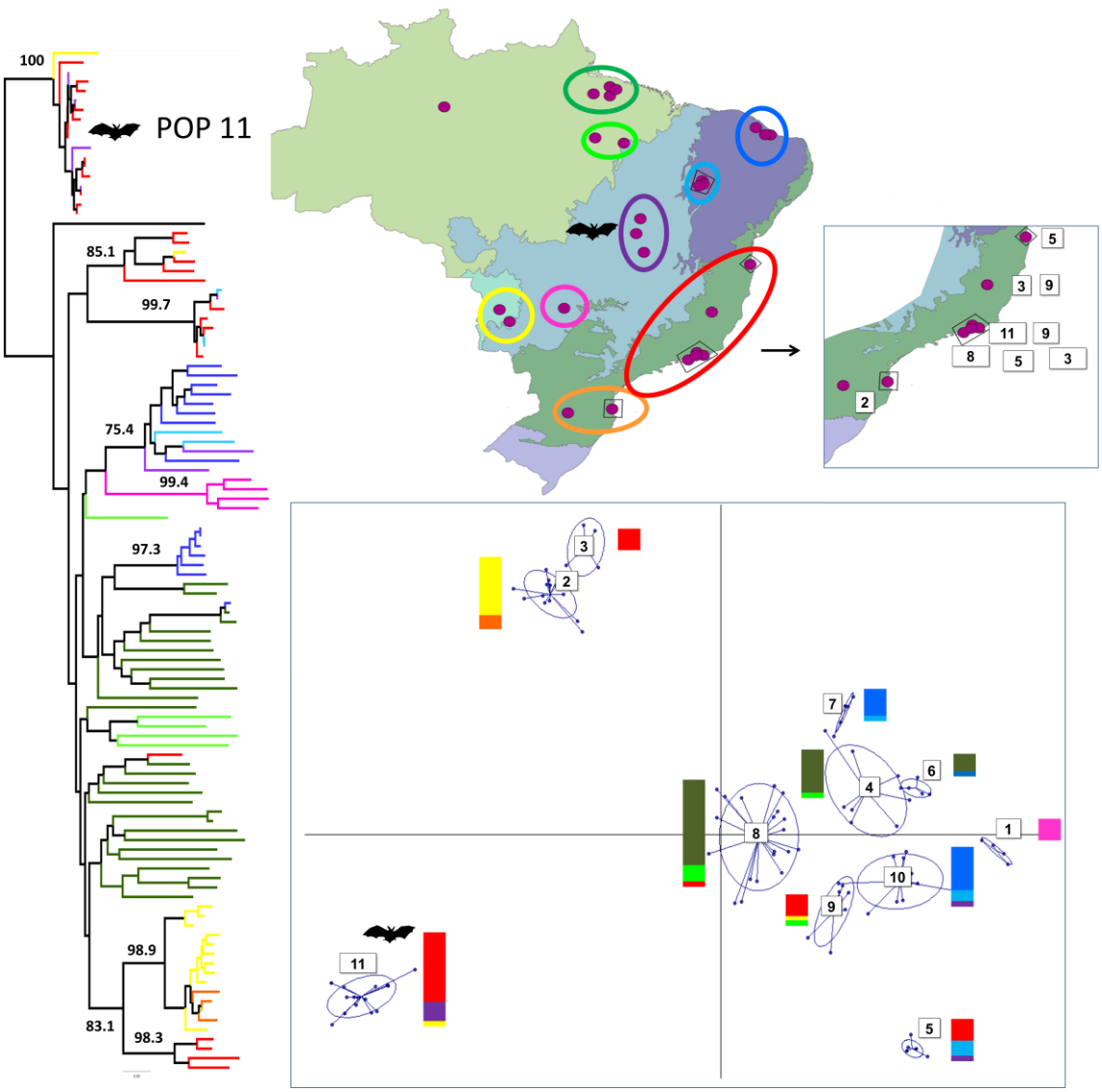

Figura 3. Mapa, dendrograma e plotagem multidimensional retratando o agrupamento dos 107 isolados de *Trypanosoma cruzi* I por bioma e área geográfica**.**

Esquerda: árvore neighbour joining não enraizada da distância par-a-par baseado na medida inversa de alelos compartilhados (D_{AS}) entre os isolados. Ramos coloridos correspondem a origem geográfica dos isolados como mostrado no mapa. Valores de bootstrap indicam porcentagem da estabilidade topológica mostrada a partir de 1000 replicatas. Direita ao alto: mapa mostrando a distribuição de isolados pelos biomas brasileiros. Abaixo à direita mostra a distribuição de isolados e identidade genética entre isolados da Mata Atlântica. Números na figura correspondem a populações definidas na plotagem multidimensional mostrada abaixo. Direita abaixo: plotagem multidimensional mostrando os resultados de uma análise discriminante de principais componentes. Onze grupos foram definidos via *K*-medidas agrupando (10^9 repetições, 17 PCs (80% de variação) no valor ótimo para a curva do critério de informação Bayesiano (não demonstrado). Elipses azuis representam grupos de isolados. Barras verticais próximas a cada agrupamento indicam a origem geográfica dos isolados que eles contêm e corresponde ao agrupamento do isolado no mapa. Comprimento das barras é proporcional ao número de isolados.

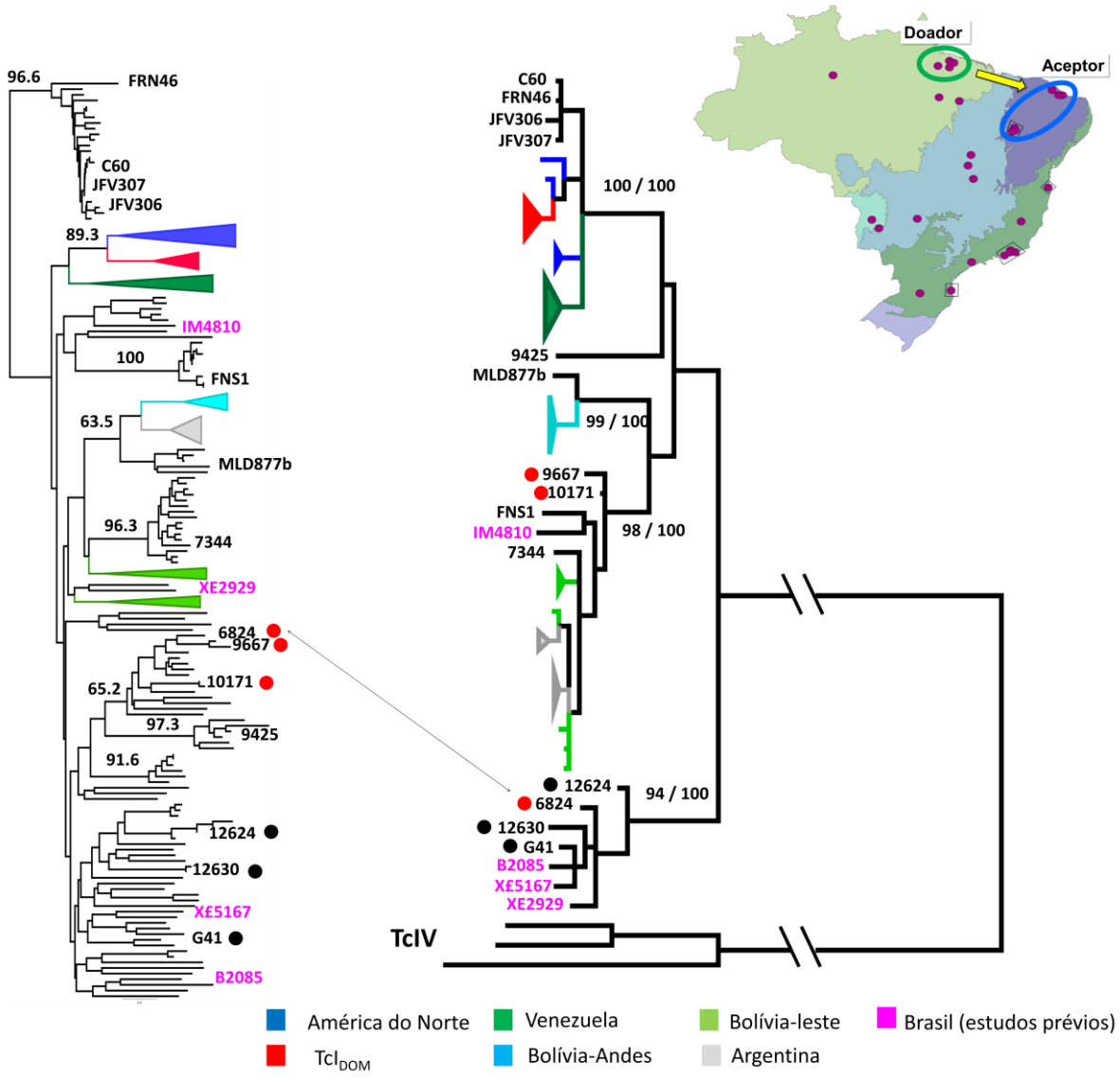

Figura 4. Comparação do agrupamento das filogenias nuclear e mitocondrial entre isolados *Trypanasoma cruzi* I do Brasil revelando troca genética.

Filogenia nuclear (esquerda) é baseada na distância genética par-a-par (D_{AS}) calculada a partir de 19 microsatellite loci agrupados via neighbour-joining. Bootstraps indicam estabilidade topológica baseada em 10.000 replicatas. Ramos coloridos indicam origem dos isolados. Ramos pretos correspondem aos isolados do Brasil. Códigos roxos indicam isolados do estado do Pará analisados em um estudo prévio (Llewellyn *et al.*, 2009). Filogenia mitocondrial (direita), consenso da topologia Bayesiana é exibida. Análise de probabilidade Bayesiana posterior (Bayesian posterior probability analysis-BPP) foi realizada usando MrBAYES v3.1. Cinco analyses independentes foram processadas usando um árvore inicial aleatória com três cadeias quentes e uma cadeia fria sobre 10 milhões de gerações com amostragem a cada 10 simulações (25% burn-in). Valores decimais (segundo número) nos nós indicados de probabilidades Bayesiana para agrupar. Primeiros numerous indicam porcentagem de bootstrap da Máxima Verossimilhança (ML) suportada pela topologia dos clados, que foi estimado a partir dos dados de 1000 replicatas. Ramos coloridos indicam origem dos isolados. Isolados que demonstram clara incongruência entre genótipo nuclear e mitocondrial são marcados. O evento de introgressão nesse conjunto de dados é indicado por setas pretas, em que 6824 é um clone recombinante. A origem geográfica do doador e do aceptor da mitocôndria é mostrado no mapa, acima à direita.

Tabela 4. Parâmetros de genética populacional das nove populações de *Trypanosoma cruzi* I dos cinco biomas brasileiros.

População	N	A_r ± SE	D_{AS} ± SD	% PL H_E[c]	% PL Hd[d]	F_{IS} ± SE[e]
Ceará	14	1.746 ± 0.121	0.290 ± 0.131	0	0	0.020 ± 0.012
Goáis	4	1.734 ± 0.101	0.136 ± 0.067	0	0	-0.526 ± 0.032
Pará Norte	28	2.134 ± 0.143	0.445 ± 0.082	0	19.2	0.147 ± 0.008
Pará Sul	5	2.027 ± 0.152	0.416 ± 0.053	0	0	0.250 ± 0.019
Pantanal	13	1.698 ± 0.121	0.219 ± 0.197	26.3	5.2	0.068 ± 0.029
Piauí	6	1.930 ± 0.140	0.357 ± 0.188	0	0	0.080 ± 0.023
Mata Atlântica t	27	2.010 ± 0.133	0.369 ± 0.199	33.3	33.3	0.077 ± 0.015
Santa Catarina	3	1.412 ± 0.098	0.057 ± 0.020	0	0	-0.740 ± 0.033
Tocantins	7	1.959 ± 0.133	0.362 ± 0.221	14.2	0	0.180 ± 0.025

N = Número de isolados na população.

A_r = Riqueza alélica como uma média dos loci ± erro padrão, calculado em FSTAT

D_{AS} – Média pareada inversa de alelos compartilhados entre amostras ± desvio padrão, calculado em MICROSAT

[c]Proporção de loci demonstrando excesso de heterozigosidade significante depois de correção sequencial de Bonferroni. Calculado em ARLEQUIN v3.1

[d]Proporção de loci demonstrando um significativodeficit em heterozigosidade depois de correção sequencial de Bonferroni. Calculado em ARLEQUIN v3.1

[e]Média FIS sobre os loci ± erro padrão, calculado em FSTAT

Objetivo específico 2-Estudar a diversidade genética e estrutura populacional de isolados de *T. cruzi* TcII oriundos de primatas de dois fragmentos de Mata Atlântica (sudeste e nordeste), e de outros mamíferos da Caatinga e Cerrado também pela abordagem de MLMT.

Composição de subpopulações

Sendo o *T. cruzi* um parasita diplóide, cada gene, ou região gênica são representados nos dois cromossomos homólogos. Esses genes ou regiões gênicas podem ser idênticos, no caso de um locus homozigoto, ou com polimorfismo entre as cópias caracterizando um locus heterozigoto. Os fragmentos amplificados de microssatélites após o sequenciamento são visualizados como picos, e quando estes picos são únicos se caracteriza um locus homozigoto, ou seja os dois alelos são idênticos. A presença de dois picos pode indicar que o locus é heterozigoto ou que se trata de duas subpopulações homozigotas no mesmo isolado, pois utilizamos isolados originais e não clones. A presença de mais que dois picos indica necessariamente que dado isolado é composto por mais que uma subpopulação. Dos 60 isolados analisados, 36 apresentaram mais que dois fragmentos em até 7 loci e 24 apresentaram até 2 picos. Portanto a maioria dos isolados TcII analisados são compostos de mais que uma população. Esse parâmetro de pluralidade de população foi analisado confrontando com a origem dos isolados e localidade, e não houve associação entre os dois perfis de composição populacional, mista ou clonal, com hospedeiros ou localidades.

Análise filogenética

No cladograma da Figura 5 observa-se que os isolados TcII são diversos geneticamente, e cada isolado exibiu um genótipo TcII distinto (60 genótipos em 60 isolados). O grau de distância genética foi variável entre os componentes do mesmo clado. De acordo com o cladograma, foram identificados 9 clados ou grupos de genótipos que compartilham mais alelos entre si comparado com os isolados de outros clados. Esses clados foram a base das análises populacionais.

Os isolados da Reserva Biológica de Poço das Antas, no Rio de Janeiro, marcados com a cor vermelha, se gruparam em 4 subpopulações diferentes denomionadas, Poço1, Poço 2, Poço 3 e Poço 4, os isolados do Piauí se dividiram em duas subpopulações, Piauí 1 e Piauí 2, os isolados da Reserva Biológica de Una na Bahia, se subdiviram em 2 subpopulações, Una 1 e Una 2, e por fim a subpopulação de Santa Catarina compondo o nono grupo. O clado Piauí

2 englobou o único isolado (em azul escuro) do Cerrado no estado do Tocantins, além de um isolado do Rio de Janeiro de *Philander opossum* (Fig. 5). O número de isolados de cada população ou clado está sumarizado na Tab. 6.

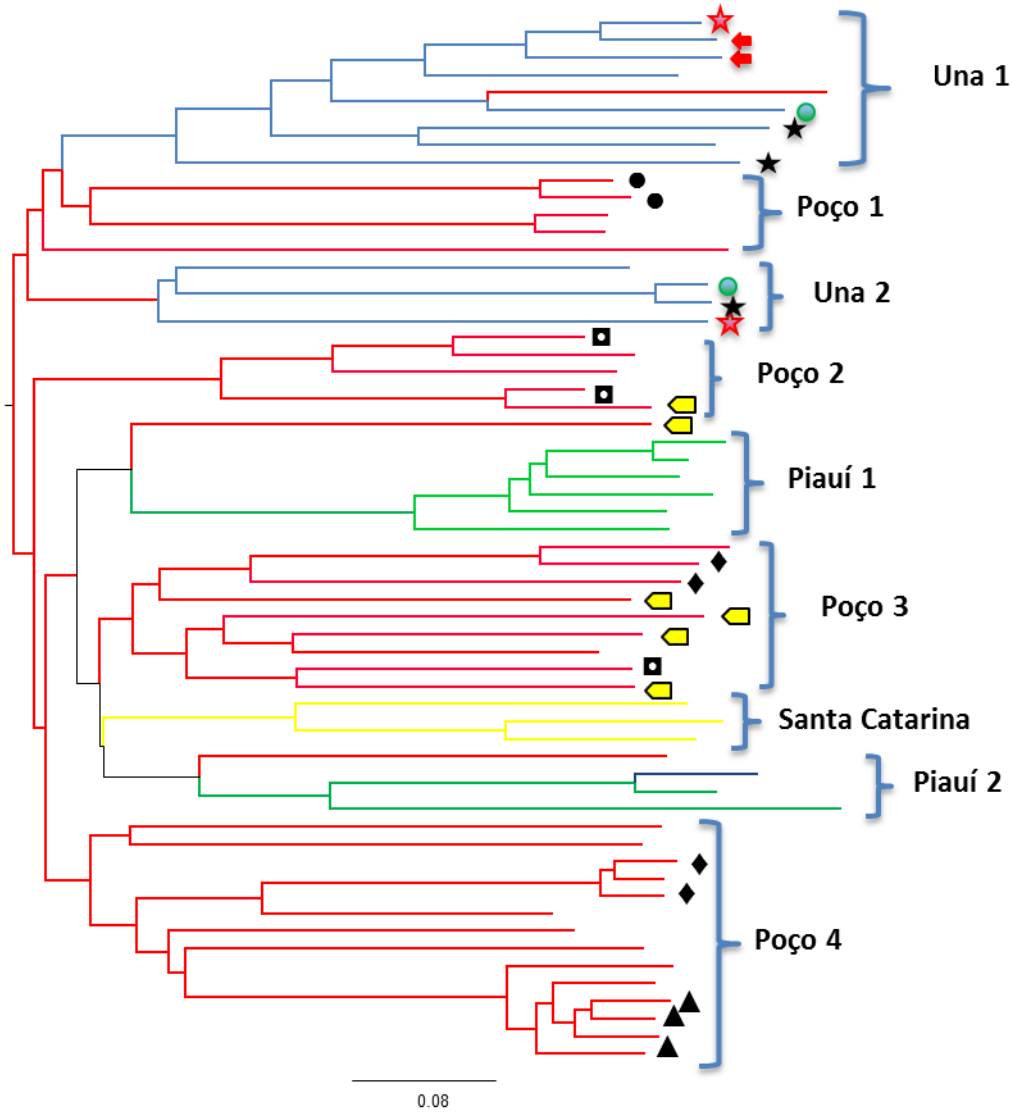

Figura 5. Reconstrução filogenética baseada na distância genética entre os 60 isolados TcII na abordagem de análise multilocus de DNA microssatélite.

Árvore Neighbour-Joining do índice de distância genética DAS. Ramos vermelhos representam isolados do Rio de Janeiro; ramos azuis representam isolados da Bahia; ramos verdes representam isolados do Piauí; ramos amarelos representam isolados de Santa Catarina e ramo azul-escuro representa isolado de Tocantins.

Tabela 5. Parâmetros de genética populacional das nove populações silvestres de TcII

População	N	Genótipos	Ar ± SE*	$H_O^{\#}$	$H_E^{\#}$	$F_{ST}^{\#\#}$
Poço das Antas 1	4	4	2,260 ± 0,146	0,655	0,592	0,184
Poço das Antas 2	6	6	2,505 ± 0,160	0,660	0,635	0,125
Poço das Antas 3	9	9	2,823 ± 0,260	0,577	0,630	0,131
Poço das Antas 4	15	15	2,760 ± 0,231	0,613	0,602	0,169
Una 1	9	9	2,147 ± 0,175	0,439	0,495	0,231
Una 2	4	4	2,464 ± 0,225	0,636	0,633	0,127
Piauí 1	6	6	1,826 ± 0,099	0,770	0,532	0,266
Piauí 2	4	4	2,300 ± 0,168	0,583	0,618	0,148
Santa Catarina	3	3	1,850 ± 0,117	0,558	0,564	0,221

N: número de isolados;

*Riqueza alélica, corrigida pelo método de rarefação de Hurlbert e erro padrão (SE);

#Média de heterozigosidade observada (Ho) e esperada (He) por todos os loci;

índice de fixação, medida de diferenciação (estrutura) populacional.

Tabela 6. Medidas de diferenciação FST par a par entre as subpopulações silvestres de TcII da Caatinga e Mata Atlântica.

Método de distância (FST) par a par:									
	Poço 4	Una 2	Piauí 1	Poço 3	Poço 1	Piauí 2	Santa Catarina	Una 1	Poço 2
Poço 4	0.00000								
Una 2	0.20965	0.00000							
Piauí 1	0.26256	0.29582	0.00000						
Poço 3	0.15447	0.20467	0.24564	0.00000					
Poço 1	0.13445	0.15387	0.34190	0.18810	0.00000				
Piauí 2	0.23149	0.26586	0.28578	0.14736	0.27092	0.00000			
Santa Catarina	0.23531	0.25284	0.33230	0.16308	0.28604	0.25093	0.00000		
Una 1	0.31443	0.25801	0.36235	0.23818	0.25740	0.29906	0.27829	0.00000	
Poço 2	0.16259	0.17181	0.26362	0.13617	0.19619	0.23243	0.22020	0.30231	0.0000

O comportamento social em grupos das espécies *L. rosalia* e *L. chrysomelas* foram avaliadas quanto a composição de genótipos ou a dispersão dos mesmos. Na Fig. 5 são plotados símbolos representando os diferentes grupos sociais aos quais pertencem esses primatas. Como o objetivo foi analisar a correlação entre grupo social e proximidade genética, só foram representados os grupos sociais com mais de um representante (isolado) nessa amostragem. Em relação aos micos de Una, podemos observar que dos quatro grupos sociais plotados, três apresentaram genótipos pertencentes aos dois clados de Una, em apenas um grupo social houve congruência entre convivência dos hospedeiros e similaridade genética dos seus isolados. O mesmo fenômeno foi verificado nas populações de Poço das Antas, em que dos quatro grupos sociais discriminados, apenas um teve genótipo exclusivo de um mesmo clado.

Análises populacionais

Índice de diversidade de isolados de *T. cruzi* DTU II

A riqueza de alelos foi a medida de diversidade genética utilizada neste trabalho e os maiores níveis foram observados no conjunto de populações de *T cruzi* TcII de Poço das Antas (n=4) (Tab. 6). Considerando a média das subpopulações, Una foi a segunda localidade com os maiores índices de riqueza alélica (2,305), seguida do Piauí (2,063). As subpopulações de *T. cruzi* DTU II com menores índices de diversidade alélica foram obtidas de triatomíneos,

Triatoma brasiliensis na subpopulação Piauí 1 (0,1826) e *T. tibiamaculata* da subpopulação Santa Catarina (0,1850) (Tab. 6).

Estrutura populacional de isolados silvestres de *T. cruzi* II

O índice de diferenciação populacional (FST) reflete a estruturação populacional e todas as subpopulações analisadas de TcII estiveram na faixa entre moderada e alta estruturação populacional. As quatro subpopulações de Poço das Antas apresentaram índices de moderada estruturação, entre 0,125 e 0,184. No entanto, as populações do Piauí (Piauí 1 e 2) e de Una (Una 1 e 2), apresentaram-se com distintos padrões de estruturação. Piauí 1 apresentou índice de 0,266, o mais alto dentre todas as subpopulações, ou seja, é altamente estruturada, em contrapartida, Piauí 2 apresentou índice moderado de estruturação (0,148) Em Una os valores de FST das duas subpopulações foram 0,231 em Una 1 e 0,148 em Una 2 (Tab. 6).

O índice de fixação (FST) par a par entre as subpopulações utilizado como um indicador de associação entre distância genética e distância geográfica, foi concordante entre as subpopulações do Rio de Janeiro (Poço1 a 4) e do Sul (Santa Catarina). No entanto não houve correspondência entre distância geográfica e distância genética entre subpopulações do Piauí e da Bahia, pois observou-se índices mais altos de distância genética em subpopulações da mesma localidade comparado aos índices entre subpopulações de regiões diferentes (nordeste/sudeste) (Tab. 7).

Objetivo específico 3 - Avaliar a dispersão das DTU de *T. cruzi* II e híbridas (V/VI) na Amazônia, bioma brasileiro ainda sem registro de sua ocorrência

T. cruzi II e híbrido (V ou VI) na região amazônica

A genotipagem das amostras de *T. cruzi* deste estudo é mostrada nas Tab. 8 e Fig. 6, demonstrando a ocorrência dos genótipos TcII e híbrido (TcV ou TcVI) na região amazônica, respectivamente em dois exemplares de *Rhodnius pictipes* (LBT 1458 e LBT 1814) e um cão (Fig. 6C, 6D e Tab. 4). Este é o primeiro registro destes genótipos no bioma Amazônico. Essas três amostras ocorreram em infecção mista com TcI como mostrou o ensaio de miniexon (Fig. 6). Entre a inerente dificuldade em detectar e caracterizar infecções mistas está à pressão seletiva exercida pelos meios de isolamento e cultivo em laboratório. Este fato foi observado no isolado LBT 1458 que em uma primeira amplificação do gene de miniexon apresentou perfil misto TcI e Tc2 (dados não demonstrados) e em subsequentes amplificações de uma nova extração de DNA desse isolados, este passou a exibir um perfil único Tc2 (Fig 6B). No entanto, clones TcI (dados não demonstrados) além de clones TcII (Fig. 6C) foram obtidos após clonagem biológica desse isolado.

A identificação da DTU TcII no isolado misto LBT 1814 foi possível usando o protocolo de RFLP do gene Histona 3 com a enzima AluI (Fig. 6D).

A análise da sequência nucleotídica do fragmento de 250 pb do gene de miniexon da amostra de DNA de soro do cão LBT 1822 submetida ao BLAST no site NCBI mostrou 100% de cobertura e 99% de identidade com três cepas híbridas de referência (TcV- SC43 e MN; TcVI - CL Brener). Somente de 20 a 40% de cobertura com 100% de similaridade foi observada com cinco cepas TcII. Contudo uma única cepa TcII (Tu18) apresentou a mesma cobertura e identidade das três cepas híbridas. O perfil exibido pelo protocolo de RFLP do gene H3 com a enzima AluI confirmou o genótipo híbrido (TcV ou TcVI) na amostra LBT 1822 (dados não demonstrados).

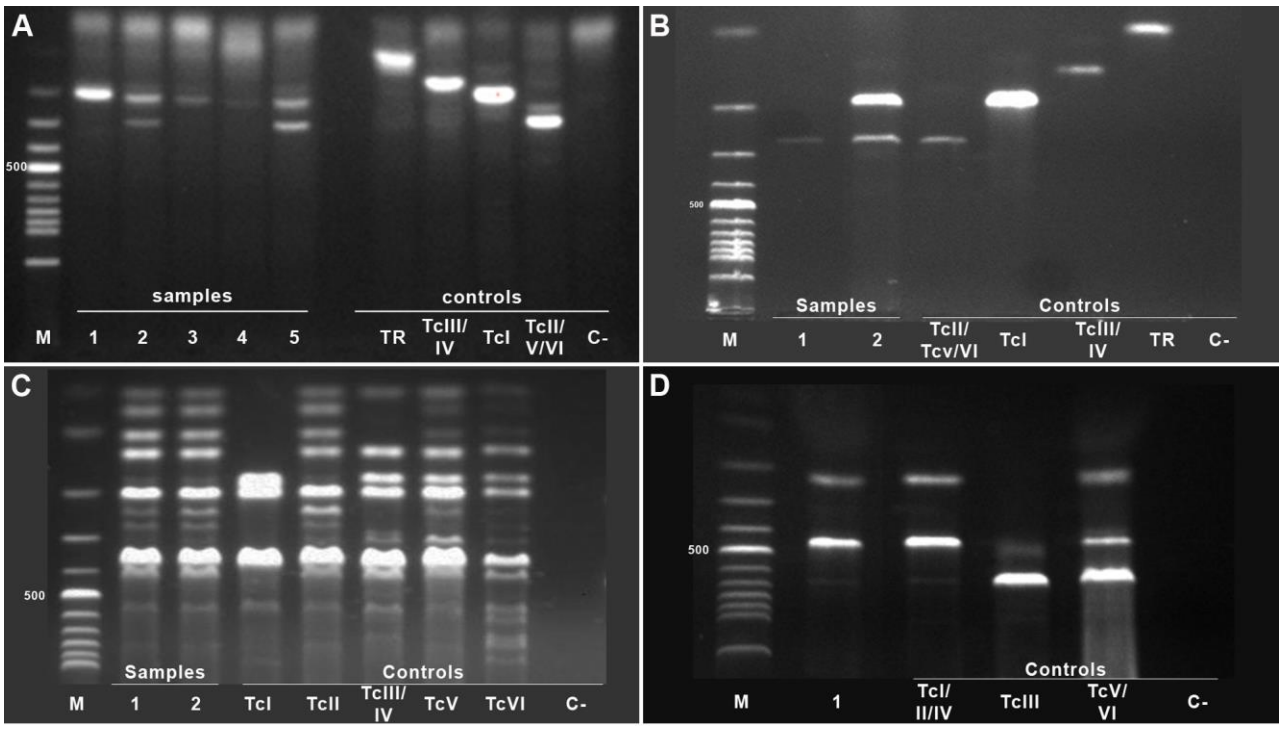

Figura 6. Genotipagem de isolados de *Trypanosoma cruzi* e DNA de *T. cruzi* de amostras de soro do bioma Amazônico.

(A) Ensaio de miniexon, PCR da região intergênica não transcrita do gene de miniexon (SL-RNA) de DNA de *T. cruzi* a partir de soro de cães do município de Monte Alegre, amostras: 1- cão LBT 1818, 2- cão LBT 1819, 3- cão LBT 1820, 4- cão LBT 1821, 5- cão LBT 1822 (B) Ensaio de miniexon de isolados de *Rhodnius pictipes*, amostras: 1- LBT 1458, 2- LBT 1814 (C) Genotipagem de *T. cruzi* por PCR-RFLP do gene 1f8 gene/ enzima de restrição Alw21I dos clones 5 e 7 do isolado LBT 1458, amostras: 1- LBT 1458 clone 5, 2- LBT 1458 clone 7 (D) Genotipagem de *T. cruzi* por PCR-RFLP do gene Histona 3/enzima de restrição AluI do isolado LBT 1814, amostra1- LBT 1814 . As DTUs de *T. cruzi*, *T. rangeli* e controles negativos são diretamente indicados na figura. Gel de agarose a 3%, corado com brometo de etídio.

Diversidade do locus gp72

O locus gp 72 se mostrou polimórfico entre os isolados TcII testados. Os 12 isolados exibiram 8 diferentes sequências. A topologia da árvore neighbor-joining gerada mostra a falta de correlação entre os isolados TcII, considerando as distâncias geográficas e genéticas (Tab. 4, Fig. 7). Os 5 isolados de Reserva Biológica de Poço das Antas-RBPA (prefixo MLD) exibiram 4 diferentes sequências. O inverso ocorreu entre os isolados de diferentes biomas, pois sequências idênticas foram observadas entre os isolados de cães do Cerrado (CD 621 e CD 640) e os 2 clones do isolados LBT1458 da Amazônia (Fig. 7). Os dois distintos perfis no RFLP da gp72 foram devido a um SNP (single nucleotide polymorphism) em um dos sítios de restrição pela enzima TaqI. Isolados com mutação de uma timina para uma guanina não sofreram digestão em um dos sítios exibindo assim um perfil distinto dos demais isolados.

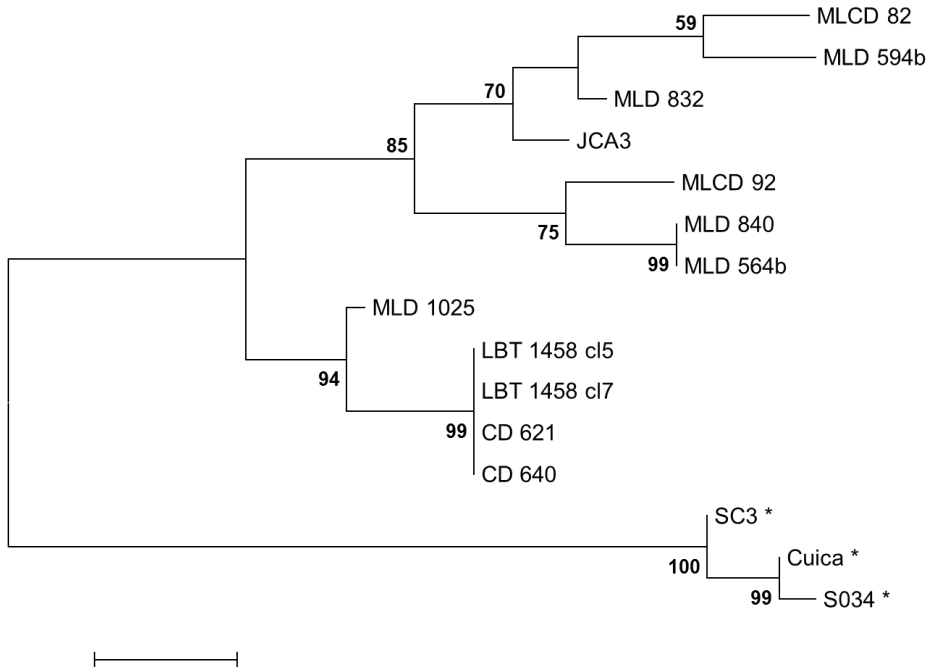

Figura 7. Relações filogenéticas a partir do gene gp 72 entre 12 isolados silvestres de *Trypanosoma cruzi* e clones TcII.

A árvore foi construída sob método neighbour-joining baseado em Kimura-2-parâmetros. Valores de bootstrap são demonstrados acima dos maiores clados. As amostras com prefix MLD são de *Leontopithecus rosalia* da Reserva Biológica de Poço das Antas na Mata Atlântica do estado do Rio de Janeiro, as amostras com prefix MLCD são de *Leontopithecus chrysomelas* da Reserva Biológica de Una na Mata Atlântica do estado da Bahia, as amostras com prefixo CD são de cães do Cerrado no estado de Minas Gerais, os isolados com prefixo LBT são de *Rhodnius pictipes* da Amazônia e o isolado JCA3 é de *Triatoma brasiliensis* do estado do Piauí na Caatinga.
*sequências de cepas TcI do GenBank publicadas por Flores-Lópes *et al.*, 2011.

Tabela 7. Caracterização molecular de *Trypanosoma cruzi* de hospedeiros naturalmente infectados do estado do Pará, Brasil.

Amostras de *T. cruzi* (material biológico)	Espécie de hospedeiro	Município	Ensaio de Mini-exon [b]	Outros protocolos de genotipagem	Caracterização de *T. cruzi*
LBT 1458 (isolado)	*Rhodnius pictipes*	Abaetetuba	TcI e TcII/V/VI	-	TcI e TcII
LBT 1458 (clone 5)		Abaetetuba	TcII-V-VI	Gene 1f8[c]	TcII
LBT 1458 (clone 7)		Abaetetuba	TcII-V-VI	Gene 1f8[c]	TcII
LBT 1814 (isolate)	*Rhodnius pictipes*	Belém	TcI e TcII/V/VI	Gene Histona 3[d]	TcI e TcII
LBT 1818[a](DNA)	*Canis familiaris*	Monte Alegre	TcI	-	TcI
LBT 1819[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI e TcII/V/VI	-	TcI e TcII/TcV/TcVI
LBT 1820[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI	-	TcI
LBT 182[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI	-	TcI
LBT 1822[a] (DNA)	*Canis familiaris*	Monte Alegre	TcI e TcII/V/VI	Gene Histona 3 [d] e sequenciamento do gene de miniexon [e]	TcI e TcV/TcVI

(-) não realizado;

[a] Amostras de soro positiva para K-DNA de *Trypanosoma cruzi* (Xavier *et al*. 2012);

[b] Ensaio de miniexon de acordo com protocol de Fernandes *et al* (2001);

[c] PCR-RFLP do gene 1f8 de acordo com protocol de Rozas *et al* (2007);

[d] PCR-RFLP do gene histona 3 de acordo com protocolo de Westenberger *et al*. (2005);

[e] Sequência do fragment de 250bp do gene de miniexon. Número de acesso no GenBank KJ402456.

Discussão

A diversidade genética do genótipo TcI no Brasil é claramente maior além de incluir toda a diversidade conhecida do genótipo em outros países da América do Sul, América Central e do Norte como demonstrado na Fig 4. Além da mais ampla diversidade observada nesse painel de isolados TcI brasileiros, um novo clado correspondendo a população 11 se destaca de toda diversidade de TcI até então conhecida Fig. 3. Este novo clado é composto de 19 isolados entre eles, quatro isolados de morcegos, o que nos levou a suspeitar que poderia pertencer a TcBat, uma DTU recentemente descrita com afinidades com TcI e originariamente isolada da ordem Chiroptera no estado de São Paulo, mas agora reconhecida como mais dispersa (Marcili *et al.*, 2009; Ramirez *et al.*, 2013). Contudo, a comparação de sequências do gene GPI desses isolados e de isolados Tcbat, rejeitou essa hipótese (dados não demonstrados). É interessante destacar que esse novo clado é composto de isolados originados de oito espécies diferentes de hospedeiros e uma de vetor, de três diferentes biomas, Mata Atlântica, Cerrado e Pantanal, o que indica que este é um grupo de genótipos tem uma ampla distribuição no meio silvestre. Portanto, a diversidade de TcI observada neste estudo é provavelmente consequência da maior diversidade de espécies hospedeiras silvestres e ambientes analisados em relação aos estudos anteriores (Llewellyn *et al.*, 2009b; Ocaña-Mayorga *et al*., 2010; Segovia *et al*., 2013). No entanto, mesmo nosso estudo tendo amostrado um grande número de ambientes, a amostragem é pontual e provavelmente não inclui o universo de diversidade de TcI.

Todos os demais isolados brasileiros analisados mantêm-se agrupados em um grande clado diversificado, que inclui isolados da Bolívia e Argentina, mas que se mantêm distinto de isolados ao Norte da bacia Amazônica (Venezuela, América do Norte e Central) (Fig. 4). Essa distribuição coincide com a origem geográfica desses isolados acima ou abaixo da Equador. Os dados disponíveis na literatura sugerem que troca genética é um fenômeno comum entre isolados TcI na meio silvestre (Carrasco *et al*., 1996; Ramírez *et al.*, 2012; Ocaña-Mayorga *et al.*, 2010), e também possível em laboratório (Gaunt *et al.*, 2003). Uma consistente característica em eventos de trocas genética é a herança uniparental de DNA mitocondrial. Ao nível populacional, bem como entre DTU's, esses eventos conduzem a claros exemplos de introgressão mitocondrial (Ramírez *et al.*, 2012). Deste modo um par de isolados pode ser altamente similar a nível nuclear, mas pode não apresentar similaridade entre seus genomas mitocondriais. Nós identificamos um desses híbridos entre os clones analisados do isolado LBCE 6824 (Fig. 4). Em uma recente revisão, foi proposto que diferentes pressões evolutivas

e relógios moleculares entre regiões nucleares não codificantes como DNA microssatélites e DNA mitocondrial codificante, ao invés de troca genética, poderiam explicar tais sinais de introgressão (Tibayrenc & Ayala 2013). Contudo, tal hipótese requer uma situação em que dois ou mais genótipos nucleares idênticos ou quase idênticos (por exemplo LBCE 6824 e LBCE 9667) sofram diferentes pressões evolutivas radicais em seus genomas mitocondriais, o que não foi observado. O genoma mitocondrial dos demais clones acompanhou o agrupamento do genoma nuclear com a exceção do clone LBCE 6824 mencionado. Um outro fato é que esse padrão de introgressão foi o mesmo obesrvado com os híbridos em laboratório (Gaunt *et al.*, 2003), e entre DTUs no meio silvestre (TcI/TcIV) (Roellig *et al.*, 2013), portanto, recombinação é a única razoável explicação para o fenômeno observado.

De particular interesse em nosso estudo foi a distribuição e estrutura da diversidade genética dos isolados dentro e entre biomas. Os isolados da Amazônia no nordeste do estado do Pará apresentaram alta diversidade e se mantiveram agrupados entre si, não havendo dispersão de seus genótipos em outros clados (Fig 3). Os demais biomas apresentaram isolados que agruparam com isolados de outros biomas. Esse fato pode estar indicando a capacidade dispersora de genótipos de TcI através de seus hospedeiros mamíferos ou vetores por esses biomas. Comparativamente, a Amazônia é o bioma mais preservado, e esse fato pode estar relacionado com o grau de diversidade apresentado por seus isolados e à manutenção de seus genótipos nativos. Em destaque a Mata Atlântica foi o bioma cujos isolados mais se dispersaram através dos clados de todos os outros biomas. Apesar da área de Mata Atlântica amostrada neste estudo ser mais extensa do que os outros biomas, a maioria dos isolados é oriunda do estado do Rio de Janeiro (23/30) (Tab.1), portanto essa fragmentação provavelmente não é devida à distância entre as áreas. O gráfico multidimensional na Fig 1 ilustra com mais detalhe a diversidade genética entre os isolados da Mata Atlântica. Isolados do extremo norte desse bioma têm afinidade com isolados da Caatinga (clado 5), esperado devido a proximidade geográfica. Contudo, a similaridade genética de isolados da Mata Atlântica com outras populações, especialmente da Amazônia e do Pantanal, é menos esperada, pois essas regiões distam milhares de quilômetros da Mata Atlântica. O impacto da fragmentação da Mata Atlântica na abundância de espécies e em sua diversidade é bem documentada (Gonçalves-Souza & Matallana *et al.*, 2008; Ogrzewalska *et al.*, 2011; Santos *et al.*, 2010). A maioria dos estudos mostram a perda de diversidade alfa inversamente relacionada com tamanho de fragmentos dentro bem como entre espécies (Gonçalves-Souza & Matallana 2008; Haag *et al.*, 2010). Em contraste, o índice de riqueza alélica em nosso estudo sugere substancial diversidade genética de *T. cruzi* na Mata Atlântica

(Tab. 5). No entanto, a invasão de espécies de outros biomas já foi documentado na Mata Atlântica (Oliveira & Viveiros-Grelle 2012), e provavelmente uma longa história de introduções pode ser uma das explicações a diversidade genética de TcI nessa região. Uma outra questão envolvida nesta proposição seria a competência das espécies hospedeiras da Mata Atlântica em manter esses genótipos introduzidos.

Uma das vias de dispersão de populações de *T. cruzi* de longo alcance pode ter sido promovida por morcegos. De fato, a presença do clado *T. cruzi* de tripanosomas na África pode ser explicado pela rápida dispersão aérea (Hamilton *et al.*, 2012). O clado 11 contém isolados de quirópteros, o que pode explicar a diversidade geográfica de isolados neste clado, com origens da Mata Atlântica, Pantanal e Cerrado, bem como sua homogeneidade genética. No entanto, outros agrupamentos de isolados de diferentes áreas geográficas contendo isolados da Mata Atlântica não têm morcegos na sua composição. Há uma circunstancial ligação entre a perda de 88% da extensão da Mata Atlântica (Galindo-Leal 2003), diversidade de população humana e mistura genética de TcI nesta região. Deste modo, pode ser possível que muitas das introduções de populações TcI nesta região se devam possivelmente à imigração humana acompanhada de seus animais domésticos e/ou sinantrópicos e transporte passivo de vetores infectados.

De fato, o estudo da diversidade de *T. cruzi* como um possível indicador da preservação ambiental deve ser analisado de forma completa, observando-se também a fragmentação dessa diversidade. No caso da Amazônia, houve correlação entre diversidade genética e estruturação populacional com o maior grau de preservação desta região. No entanto a Mata Atlântica também apresentou alta riqueza alélica porém apresentando fragmentação em sua diversidade, que discutimos como tendo provável relação com sua fragmentação ambiental.

Com a ressalva da maior frequência em isolamentos da DTU I, não há exclusividade de ocorrência na maioria dos biomas brasileiros dos dois genótipos mais isolados no meio silvestre no Brasil, TcI e TcII. Portanto a avaliação da estrutura populacional do segundo genótipo mais isolado no Brasil no ambiente silvestre foi nosso segundo assunto para melhor entender a diversidade de *T. cruzi* no Brasil. Esta etapa do estudo que avalia a estrutura da diversidade genética de populações TcII com a abordagem de MLMT representa um primeiro passo no entendimento da diversidade genética e estrutura populacional e no ambiente silvestre de um genótipo (TcII) historicamente associado à infecção e doença humana no Brasil.

O estudo mostra um alto grau de diversidade genética entre esses isolados (Fig 5). Considerando que diferentes espécies de hospedeiros funcionam como filtros biológicos com diferentes graus de estringência a diferentes subpopulações, a maior diversidade genética observada nas subpopulações TcII da Mata Atlântica do sudeste e do nordeste (Rio de Janeiro e Bahia, respectivamente) pode estar refletindo a maior diversidade de fauna encontrada neste bioma comparado com a Caatinga (Paglia *et al.*, 2012). Paralelo a isso, essas duas espécies do gênero *Leontopithecus* sp apesar de homogêneas geneticamente, foram capazes de manter a ampla diversidade encontrada nestas localidades. O que contraria uma linha de pensamento que a cada espécie de hospedeiro, com seu características genéticas, seria um filtro seletor para subpopulações do *T. cruzi* (Andrade et al., 2002; Macedo *et al*., 2004).

Concordância entre distância genética e geográfica foi observada na maioria das subpopulações, as únicas exceções ocorreram nos grupos de isolados de Una e do Piauí (Una 2 e Piauí 2) que tiveram mais proximidade com subpopulações de Poço das Antas no estado do Rio de Janeiro, o que pode estar relacionado ao potencial de dispersão desse genótipo. Devido a maior estruturação e diversidade observadas das subpopulações do Rio de Janeiro em relação a essas duas subpopulações do nordeste (Tab. 6), é provável ter havido um aporte de genótipos da Mata Atlântica do Rio de Janeiro para essas regiões. No entanto, um cenário oposto também deve ser considerado, que é a dispersão para a Mata Atlântica de genótipos de outras regiões como se observa em TcI. E uma vez estabelecidos, esses genótipos foram se diversificando ao mesmo tempo que as populações doadoras devem ter sofrido processo de extinção de genótipos de TcII possivelmente como consequência, em grande parte, da retração dos ciclos de transmissão por este genótipo no nordeste.

As diferentes subpopulações de TcII exibiram diferenças quanto aos parâmetros populacionais (Tab. 6), indicando um ecletismo moldado provavelmente pelas características ecológica/epidemiológica relacionadas aos isolados. As diferenças na estruturação populacional entre os isolados TcII das duas espécies irmãs de *Leontopithecus* sp, endêmicas das regiões estudadas, respectivamente, pode estar refletindo essas diferenças ecológicas locais. A uniformidade tanto em estruturação quanto em diversidade nas quatro subpopulações de *L. rosalia* da Reserva Biológica de Poço das Antas no Rio de Janeiro, pode estar sinalizando um dinamismo na dispersão dos genótipos na área da Reserva ou um contínuo aporte de novos genótipos das áreas no entorno da Reserva, oriundas de outros hospedeiros silvestres. Em contrapartida, apesar do número reduzido de isolados em Una (n=13), uma de suas duas subpopulações (Una1), apresentou alto grau de estruturação e baixa

diversidade, o que pode indicar um ciclo de transmissão menos sujeito ao aporte do ambiente entorno.

A maioria dos nossos isolados TcII (36/60) apresentou mais que uma subpopulação, e analisamos a eventual aptidão entre mamíferos (*L. rosalia* e *L. chrysomelas*) e vetores (*T. tibiamaculata* e *T. brasiliensis*) em manter mais que uma subpopulação de *T. cruzi*, no entanto, nenhuma correlação foi observada. Mesmo pertencentes a grupos geneticamente tão distintos (vertebrado e invertebrado), os dois taxa parecem ter a mesma competência na manutenção de diferentes populações de TcII.

Geralmente há associação direta entre diversidade genética e tamanho populacional (De Meeûs *et al.*, 2006; Birky 1996), portanto, o alto grau de diversidade genética em TcII observado neste estudo, refletido no igual número de isolados testados e de genótipos encontrados e dados de riqueza alélica, podem ser indícios de uma maior prevalência desse genótipo na natureza do que reflete a prevalência de isolamento em laboratório.

Devido à grande extensão de ocorrência do *T. cruzi* e às dificuldades envolvendo captura, transporte de material biológico e isolamento de parasita de mamíferos e vetores silvestres, os dados sobre a distribuição de genótipos de *T. cruzi* são agregados e não contemplam todos os habitats de ocorreência do parasita. Em adição, peculiaridades de interação de diferentes hospedeiros com cada DTU representa distintas pressões seletivas além da pressão seletiva exercida pelos métodos de isolamento. Esses viéses podem resultar em interpretação incorreta sobre a ecologia e a biologia deste parasita. A região Amazônica, em especial, foi o bioma que atraiu nossa atenção nos últimos anos devido ao aumento no número de casos de doença de Chagas por surtos por via de transmissão oral (Miles *et al.*, 2009; Zingales *et al.*, 2012; Coura *et al.*, 2002). A aparente diferença na prevalência de DTUs de *T. cruzi* na natureza é intrigante e é provavelmente determinada pelas diferenças nas estratégias de transmissão exibidas por cada DTU de *T. cruzi*. Sendo verdadeira essa proposição, essas diferentes estratégias ainda não definidas, devem ser bem sucedidas, uma vez que esses genótipos são mantidos na natureza há milhões de anos. Provavelmente cada DTU estabelece um peculiar padrão de interação com seus hospedeiros. De fato, em relação ao genótipo TcII, o acompanhamento da infecção em algumas espécies de mamíferos, como cão doméstico, *Didelphis aurita*, *Philander frenatus*, *L. rosalia* e *L. chrysomelas* e *Nasua nasua* indicam diferenças no curso de sua infecção dependendo da espécie e provavelmente da subpopulação desse genótipo (Rocha *et al*., 2013; Pinho *et al*., 2000; Lisboa *et al*., 2004; Herrera *et al*., 2008). Em cães do Cerrado, foi observado que depois de um curto período de alta parasitemia detectável por hemocultura, cães mantiveram-se sorologicamente positivos

porém não foi possível recuperar parasitas por hemocultivo. Isto sugere que a infectividade nesses animais foi restrito a um curto período de aproximadamente dois meses (Rocha *et al.*, 2013). Estudos experimentais com *Didelphis aurita* mostram que esses animais controlam algumas cepas TcII (cepas Y e FL) em uma diferente forma, onde esses animais foram hábeis para controlar parasitemia mesmo ainda muito jovens, dependentes do marsúpio (Jansen *et al.*, 1991). Contudo, apesar de pouco frequente, *Didelphis* spp é encontrado naturalmente infectados por *T. cruzi* II. Em contraste, outras espécies hospedeiras se mostraram competentes em manter subpopulações de TcII. *Philander frenata* foi competente para manter infecção por *T. cruzi* com cepa Y com alta prevalência de hemoculturas positivas e por longo tempo (Pinho *et al.*, 2000). Um ciclo de transmissão de longa duração por TcII no meio silvestre foi descrito em duas espécies de primatas *L rosalia* and *L. chrysomelas*, de dois fragmentos de Mata Atlântica (Lisboa *et al.*, 2004). Também, os carnívoros *Nasua nasua* no Pantanal demonstraram ser um hábil hospedeiro para TcII no meio silvestre (Herrera *et al.*, 2008).

Neste estudo nós demonstramos pela primeira vez a presença das DTUs TcII e híbrido (TcV/TcVI) no estado do Pará, na Amazônia brasileira. Esse dados mostram que o genótipo TcII está presente nos ciclos de transmissão silvestres de todos os biomas brasileiros estudados (Figs. 2 e 6), com exceção dos pampas onde não há estudos, e não somente restrito às áreas abaixo da bacia Amazônica como tem sido frequentemente assumido na literatura (Miles *et al.*, 2009; Zingales *et al.*, 2012). Adicionalmente, a presença de triatomíneos infectados e cães domésticos por respectivamente, TcII e Tc híbrido, implica que outros mamíferos e triatomíneos na área de estudo ou no seu entorno deve ser também infectado e envolvidos nos ciclos de transmissão dessas DTUs no estado do Pará. Vale a pena mencionar que antes desse estudo, Tc híbrido foi registrado no meio silvestre no Brasil somente uma vez, em roedores silvestres, *Thrichomys a. laurentius* na região nordeste do país, no bioma Caatinga (Araújo *et al.*, 2009) demonstrando que nós estamos longe de entender a distribuição dos genótipos híbridos e seus hospedeiros. Neste trabalho o genótipo Tc híbrido (TcV/TcVI) encontrado em um cão, um mamíferos doméstico, pode ser considerado como derivado do meio silvestre devido às características da área de estudo além do fato que no bioma Amazônico não há nenhuma clara distinção entre ambiente doméstico e silvestre.

O isolamento de TcII de *Rhodnius pictipes* demonstra a habilidade dessa espécie em manter este genótipo. Estudos com esse gênero de triatomíneos têm demonstrado sua inabilidade em manter infecção por *T. cruzi* TcII (Mello *et al.*, 1996; Azambuja *et al.*, 2004; Mejía-Jaramillo *et al.*, 2009), em estudos experimentais baseados no comportamento

biológico de três cepas TcII (Y, AF-1, Tu18) as quais provavelmente não representam a diversidade desse genótipo no meio silvestre. A infecção natural por *T. cruzi* no gênero *Rhodnius* sp no bioma Amazônico foi intensamente estudado nas décadas passadas, no entanto, esses estudos não abordaram a genotipagem de *T. cruzi* (Coura *et al.*, 2002). À época de alguns destes estudos, ferramentas metodológicas para genotipagem do parasita não eram disponíveis. Estudos da infecção natural por *T.cruzi* em *R. pictipes* na região Amazônica até o momento tem reportado a infecção por TcI, TcIV e *T. rangeli* (Valente *et al.*, 2009; Marcili *et al.*, 2009). O maior desafio no nosso entendimento da estrutura populacional dessas DTUs é sua aparente distribuição focal na natureza. O insuficiente conhecimento do comportamento e dinâmica de infecções de TcII e Tc híbridos em mamíferos e vetores é provavelmente, como mencionado acima consequência de pressões seletivas nos processos de isolamento e manutenção e diferentes sensibilidades em métodos diagnósticos, além de viés de coleta no meio silvestre. Isso foi claramente demonstrado no diagnóstico por PCR de uma infecção TcII em amostras de soro de três espécimes de *Didelphis aurita* da área de um surto no estado de Santa Catarina State (Maldonado *et al.*, unpublished data) cujos parasitas isolados por hemocultura foram caracterizados como TcI (Roque *et al.*, 2008). O mesmo evento foi observado neste estudo com o isolado LBT 1458 caracterizado como mistura TcI/Tc2(TcII/TcV/TcVI) em um primeiro ensaio de miniexon e após uma segunda extração de DNA, esse isolado demonstrou somente padrão de banda Tc2 (Fig. 6B). Contudo a presença de TcI neste isolado foi demonstrada pela clonagem biológica. Esses fatores contribuem pra a interpretação errônea sobre a prevalência de genótipos de *T. cruzi* na natureza. Um outro fator que pode contribuir para a subestimação da prevalência de genótipos é o grande número de mamíferos silvestres com sorologia positiva que não resulta em recuperação de parasitas por hemocultura (em torno de 70%) (comunicação pessoal, Jansen AM).

O protocolo de RFLP do gene gp72 com a enzima de restrição TaqI usado em isolados TcII brasileiros discriminou dois grupos nesta DTU, um exibindo perfil como descrito pelos autores (Rozas *et al.*, 2007) e outro com perfil distinto (dados não demonstrados). O sequenciamento deste locus apontou para uma única SNP como responsável por esses dois perfis, contudo a reconstrução filogenética demonstrou que a presença da mesma SNP não implicou em mais alta relação filogenética entre os isolados. No entanto, essa reconstrução filogenética demonstrou um aspect ainda pouco conhecido que é a diversidade em TcII. A diversidade genética entre os doze isolados silvestres e dois clones de quatro biomas (Caatinga, Cerrado, Mata Atlântica e Amazônia) (Fig 7) a partir de um gene constitutivo

permite-nos sugerir que TcII tem uma maior prevalência do que é reportado, uma vez que diversidade genética em geral, é diretamente relacionada ao tamanho populacional (De Meeûs *et al.*, 2006; Birky 1996). A ausência de correlação entre área geográfica e tipo de sequência de TcII sugere que variantes de TcII provavelmente não são limitados às espécies de primatas na Mata Atlântica mas incluem outras espécies de hospedeiros em outros biomas. Esses dados corroboram os dados obtidos de estrutura da diversidade genética de TcII no Brasil por MLMT e junto podem sugerir uma maior prevalência de TcII na natureza do que é realmente reportado. Uma rota alternativa de estudo para verificar essa hipótese seria a pesquisa de genótipos de *T. cruzi* a partir de soro de mamíferos silvestres.

Conclusões

-A análise por multilocus microssatélites revelou mais alta diversidade genética entre os isolados TcI brasileiros comparado ao restante das Américas.

-Um clado divergente de toda diversidade de TcI nas Américas conteve isolados de diferentes biomas brasileiros, mostrando que o universo de TcI ainda não é conhecido.

-Diversidade genética de TcI variou entre populações sendo as populações da Amazônia as mais diversas.

-Com exceção da Amazônia todos os biomas apresentaram algum grau de mistura de genótipos de outras regiões, o maior grau foi observado na Mata Atlântica. Mostrando que, em geral, não há associação estrita entre subpopulações TcI e biomas.

-Foi evidenciado evento de troca genética (introgressão mitocondrial) entre isolados TcI da Amazônia e da Caatinga.

-A análise por multilocus microssatélites revelou alta diversidade genética entre os isolados TcII silvestres brasileiros e esta diversidade não foi associada à diversidade de hospedeiros e biomas.

-As populações TcII de Poço das Antas (RJ) foram as mais diversas geneticamente.

-Populações TcII apresentaram diferentes graus de estruturação genética, variando entre moderada estruturação e alta estruturação.

-A organização social em grupos dos hospedeiros *Leontopithecus* sp não coincidiu com a composição de genótipos TcII.

-Os genótipos TcII e híbrido (V ou VI) estão presentes na Amazônia brasileira.

-Ao contrário do que é classicamente admitido sobre o gênero *Rhodnius* sp, *Rhodnius pictipes* é capaz de manter infecção pelo genótipo TcII.

-A resolução de composição de genótipos em infecções mistas TcI/TcII ou TcI/Tc híbrido é possível através do protocolo de RFLP do gene Histona 3/AluI.

-A diversidade genética do genótipo TcII foi expressiva tanto nas análises de regiões genéticas de evolução rápida (MLMT) quanto na análise do gene constitutivo gp72.

-A genotipagem de *T. cruzi* a partir de soro é uma opção na ausência de isolamento do parasita em animais sorologicamente positivos. Outra função dessa técnica é a diminuição do impacto de pressões seletivas nos meios de cultivo, aproximando a pesquisa por genótipos nas infecções o mais próximo do real.

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ANEXO 1

Lima *et al. Parasites & Vectors* 2014, **7**:263
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Parasites & Vectors

RESEARCH Open Access

Wild *Trypanosoma cruzi* I genetic diversity in Brazil suggests admixture and disturbance in parasite populations from the Atlantic Forest region

Valdirene S Lima[1], Ana M Jansen[1], Louisa A Messenger[2], Michael A Miles[2] and Martin S Llewellyn[2,3*]

Abstract

Background: *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae) infection is an ancient and widespread zoonosis distributed throughout the Americas. Ecologically, Brazil comprises several distinct biomes: Amazonia, Cerrado, Caatinga, Pantanal and the Atlantic Forest. Sylvatic *T. cruzi* transmission is known to occur throughout these biomes, with multiple hosts and vectors involved. Parasite species-level genetic diversity can be a useful marker for ecosystem health. Our aims were to: investigate sylvatic *T. cruzi* genetic diversity across different biomes, detect instances of genetic exchange, and explore the possible impact of ecological disturbance on parasite diversity at an intra-species level.

Methods: We characterised 107 isolates of *T. cruzi* I (TcI; discrete typing unit, DTU I) from different major Brazilian biomes with twenty-seven nuclear microsatellite loci. A representative subset of biologically cloned isolates was further characterised using ten mitochondrial gene loci. We compared these data generated from Brazilian TcI isolates from around America.

Results: Genetic diversity was remarkably high, including one divergent cluster that branched outside the known genetic diversity of TcI in the Americas. We detected evidence for mitochondrial introgression and genetic exchange between the eastern Amazon and Caatinga. Finally, we found strong signatures of admixture among isolates from the Atlantic Forest region by comparison to parasites from other study sites.

Conclusions: Atlantic Forest sylvatic TcI populations are highly fragmented and admixed by comparison to others around Brazil. We speculate on: the possible causes of Atlantic Forest admixture; the role of *T. cruzi* as a sentinel for ecosystem health, and the impact disrupted sylvatic transmission cycles might have on accurate source attribution in oral outbreaks.

Background

Trypanosoma cruzi (Kinetoplastida, Trypanosomatidae) infection is an ancient and widespread zoonosis distributed throughout the Americas south of 33˚ latitude, where it infects approximately 8 million people [1,2]. *T. cruzi* is eclectic in terms of its mammalian hosts and haematophagous triatomine vectors. Several hundred species of mammal and many of the 140 extant triatomine species maintain transmission of *T. cruzi* in wild (sylvatic) transmission cycles [2-4]. Transmission to the host occurs usually via contamination of the mucosae or abraded skin with infected vector faeces. Oral transmission to humans via contaminated foods, especially fruit juices and sugar cane, is increasingly reported, and suspected to occur widely among sylvatic mammals through opportunistic insectivory of triatomines [5].

T. cruzi population genetic diversity is well described at a species level. Six discrete typing units (DTUs) are now accepted by international consensus [6]. Dates for the origin of *T. cruzi* in the Americas range between 5 and 1 MYA (calibrated biogeographically at 100 MYA) [7-9]. Estimates for the MRCA of TcI strains, arguably the most widely dispersed and abundant of all the DTUs, are younger: 1.3-0.2 MYA [7]. Nonetheless, the

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age of TcI in the Americas has been sufficient to see this genotype expand throughout multiple ecological settings, from Amazonian forests [10] to highland Andean puna [11]. Furthermore, the last 1.3-0.2 MYA in Latin America have seen intense climatic fluctuations, including at least two glaciations [12]. The impact of Pleistocene cycles of warming and cooling on the biomic, ecological and species diversity of Latin America, in particular in Brazil and the Brazilian Amazon, are a matter of long debate [13]. Nonetheless, there is evidence that historical cycles of forest expansion, contraction and fragmentation have impacted on the current ecology of Brazil, including small mammal distribution and diversity [14].

Today the terrestrial ecology of Brazil is summarized by several distinct biomes or 'ecoregions' [15]. The largest of these is the Amazon basin to the north, bordered by the dryer Cerrado and seasonally flooded Pantanal to the south. North-eastern Brazil is dominated by the xeric scrubland of the Caatinga. Along the Atlantic coast of Brazil south of Recife, a tropical forest ecosystem, the Atlantic Forest, predominates. The diversity of wild TcI hosts across this ecological mosaic is striking: caviomorph rodents in the Caatinga [16]; lion tamarins in the Atlantic forest [17]; coatis, peccaries and felid carnivores in the Pantanal [18-20]; and multiple species of primates, marsupials and rodents in Amazonia [2]. Some important genera are widespread – especially Didelphid opossums. Human Chagas disease was once widespread in Brazil, especially in central and southern parts of the country [21]. Indeed, Chagas disease has probably been endemic in human populations in Brazil since the earliest human settlements more than 10,000 years ago. It is important not to overlook the impact that humans, an abundant and mobile *T. cruzi* host species, present throughout all Brazilian ecoregions, may have had on contemporary parasite diversity.

Parasite alpha diversity at a species level is recognised as a marker for ecosystem persistence, productivity, organization and resilience [22]. Put simply, those ecosystems in which host organisms are parasitized by an array of different parasite species, fairly evenly distributed among hosts and host species, are considered to be healthy. Furthermore, parasites, with their short life-cycles and rapid mutational turnover with respect to their hosts, can facilitate fine-scale analyses of host population dispersal and differentiation [23]. However, close association between host and parasite species is a prerequisite for the use of parasite genetic diversity to track host populations. Multi-host parasite lineages like TcI are therefore unsuitable for such applications. Nonetheless, there is some evidence that habitat fragmentation impacts on both *T. cruzi* diversity and prevalence of infection [24-26]. Thus, alpha diversity in a multi-host parasite like *T. cruzi* might be a useful proxy for parasite diversity as a whole, and thus for ecosystem health.

Multilocus microsatellite typing (MLMT) is now a widely established means of defining genetic diversity among TcI isolates and clones [27]. Simultaneous analysis of multilocus sequence data from the mitochondrial (maxicircle) genome (mMLST) provides a proven means of detecting genetic exchange among clones [25,28]. Here we undertook a comparison of representative TcI isolates from across the ecological diversity of Brazil, examining the relationship between biomes and diversity within biomes. We found considerable genetic diversity among several populations, and multiple instances of genetic admixture, especially in the Atlantic Forest region. We consider these data, and the potential affect of human-mediated habitat fragmentation on the diversity of wild TcI in Brazil.

Methods

Parasite strains and biological cloning

One hundred and seven strains, the great majority sampled from mammalian reservoir hosts captured at sylvatic foci throughout Brazil, were assembled for analysis and their genotype confirmed as TcI via sequencing of a short fragment of the glucose-6-phosphate isomerase (GPI) gene [29]. Details of strain origin are given in Additional file 1: Table S1 and geographic distribution in Figure 1. A total of fourteen strains were selected from across all biomes and biologically cloned using the plate cloning technique described by Yeo *et al.* [30].

Microsatellite analysis

Twenty-seven microsatellite loci, distributed across eight putative chromosomes, were amplified following previously described protocols across 107 strains [27]. A reduced subset of 19 microsatellites was employed to evaluate diversity among a larger panel of 161 samples including the original strains, derived clones and thirty-three previously published multilocus microsatellite profiles [28]. Population genetic diversity parameters were first calculated from sample groupings based on geography and biome for the full 27 locus dataset (Table 1). There were nine such groupings, as identified in Figure 1 and listed in Additional file 1: Table S1. Population-level genetic diversity was assessed first using sample size corrected allelic richness (A_r) in FSTAT 2.9.3.2 [31]. Secondly, to provide a better measure on intra-population sub-clustering, mean pairwise D_{AS} and associated standard deviation was also evaluated per population. F_{IS}, a measure of the distribution of heterozygosity within and between individuals, was estimated per locus per population in FSTAT 2.9.3.2 [31]. Tests for population specific departures from Hardy Weinberg Equilibrium at specific loci were calculated in ARLEQUIN v3.1 and associated

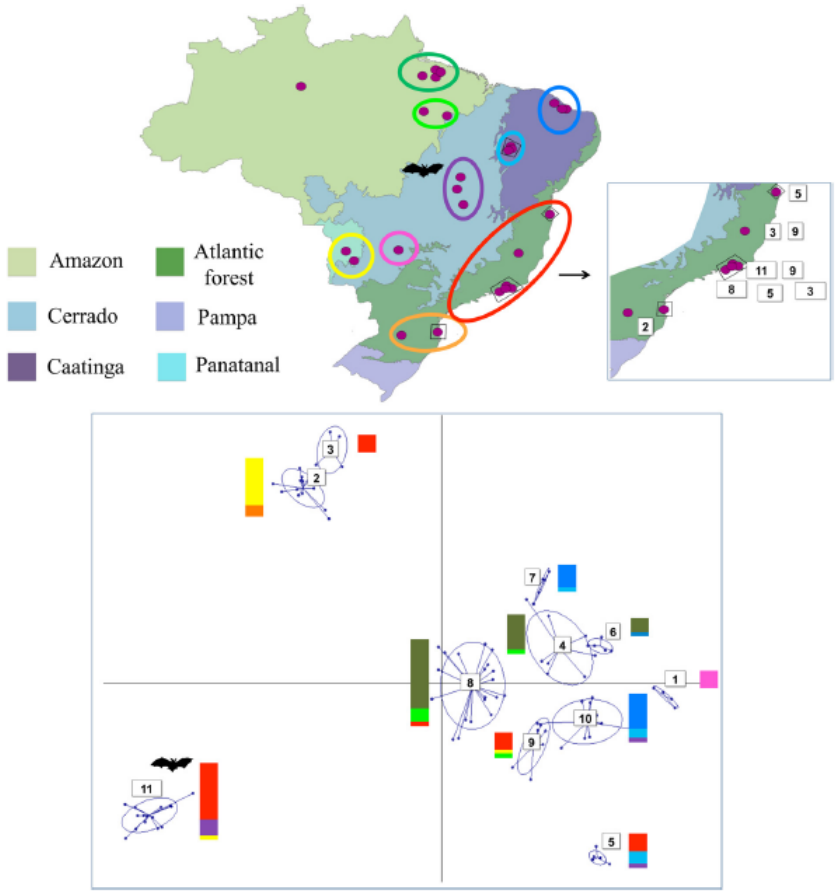

Figure 1 Composite map and multidimensional scaling plot depicting sample clustering by biome and geography among 107 *Trypanosoma cruzi* I isolates.

Table 1 Population genetic parameters across nine *Trypanosoma cruzi* I populations sampled from five biomes in Brazil

Population	N	$A_r \pm SE$	$D_{AS} \pm SD$	% PL H_E^a	% PL Hd[b]	$F_{IS} \pm SE$[c]
Ceara	14	1.746 ± 0.121	0.290 ± 0.131	0	0	0.020 ± 0.012
Goais	4	1.734 ± 0.101	0.136 ± 0.067	0	0	-0.526 ± 0.032
$PARA_{NORTH}$	28	2.134 ± 0.143	0.445 ± 0.082	0	19.2	0.147 ± 0.008
$PARA_{SOUTH}$	5	2.027 ± 0.152	0.416 ± 0.053	0	0	0.250 ± 0.019
Pantanal	13	1.698 ± 0.121	0.219 ± 0.197	26.3	5.2	0.068 ± 0.029
Piaui	6	1.930 ± 0.140	0.357 ± 0.188	0	0	0.080 ± 0.023
Atlantic Forest	27	2.010 ± 0.133	0.369 ± 0.199	33.3	33.3	0.077 ± 0.015
Santa Catarina	3	1.412 ± 0.098	0.057 ± 0.020	0	0	-0.740 ± 0.033
Tocantins	7	1.959 ± 0.133	0.362 ± 0.221	14.2	0	0.180 ± 0.025

N number of isolates in population.
A_r allelic richness as a mean over loci ± standard error, calculated in FSTAT.
D_{AS} mean pair-wise inverse allele sharing between samples ± standard deviation calculated in MICROSAT.
[a]Proportion of loci showing significant excess heterozygosity after a sequential Bonferroni correction. Calculated in ARLEQUIN v3.1.
[b]Proportion of loci showing a significant deficit in heterozygosity after a sequential Bonferroni correction. Calculated in ARLEQUIN v3.1.
[c]Mean FIS over loci ± standard error, calculated in FSTAT.

significance levels for p values derived after sequential Bonferroni correction to minimise the likelihood of Type 1 errors [32].

For the 19 locus dataset, individual level sample clustering was defined via a neighbour-joining tree based on pairwise distances between multilocus genotypes MLGs [evaluated using D_{AS} (1 – proportion of shared alleles at all loci/n)] calculated in MICROSAT [33] (Figure 2). For the 27 locus dataset we defined genetic composition via a *K*-means clustering algorithm, implemented in adegenet [34], with which the optimal number of populations is defined by reference to the Bayesian Information Criterion. These groupings were subsequently submitted to a discriminant analysis of principal components (DAPC) [35], and the resulting plot is found in Figure 1.

Maxicircle analysis

Ten maxicircle sequence fragments were amplified and sequenced from fourteen *T. cruzi* clones (see Additional file 1: Table S1 for clone identity) following previously described protocols [28]. Sequence fragments were then concatenated in each sample and aligned against previously published sequences prior to analysis [28]. Phylogenies were inferred using Maximum-Likelihood (ML)

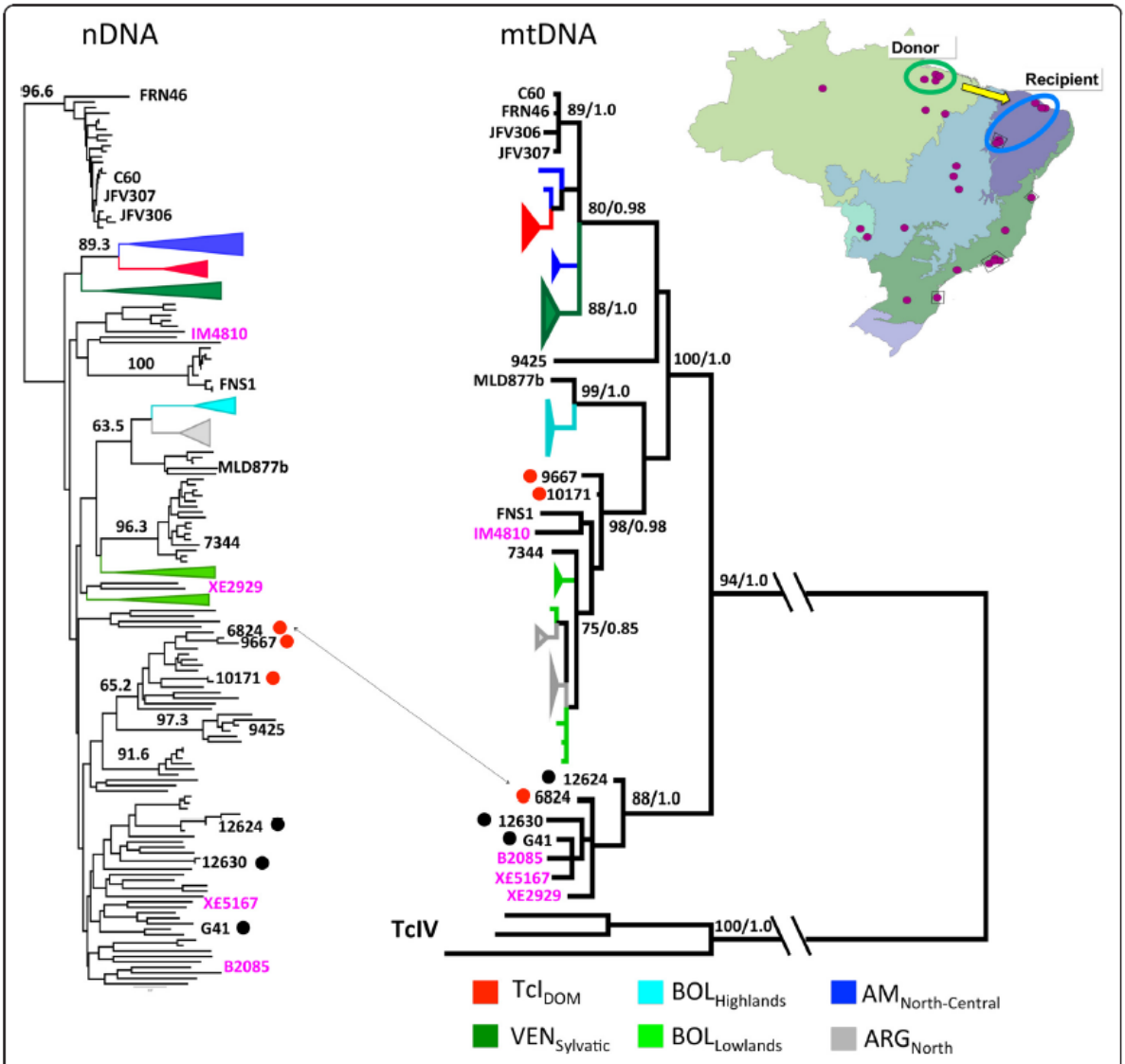

Figure 2 Comparison of phylogenetic clustering between nuclear and mitochondrial phylogenies among *Trypanasoma cruzi* I isolates from Brazil and beyond reveals genetic exchange.

implemented in PhyML (4 substitution rate categories) [36]. The best-fit model of nucleotide substitution was selected from 88 models and its significance evaluated according to the Akaike Information Criterion (AIC) in jMODELTEST 1.0 [37]. The best model selected for this dataset was GTR + I + G. Bootstrap support for clade topologies was estimated following the generation of 1000 pseudo-replicate datasets. Bayesian phylogenetic analysis was performed using Mr BAYES v3.1 [38] (settings according to jMODELTEST 1.0). Five independent analyses were run using a random starting tree with three heated chains and one cold chain over 10 million generations with sampling every 10 simulations (25% burn-in).

Results

Nuclear microsatellite loci demonstrated considerable genetic diversity among the 107 strains studied. For comparative purposes isolates were grouped *a priori* according to both geography and biome of origin (Figure 1). As such nine populations were defined. Sample assignment to these populations is presented in Additional file 1: Table S1 and population genetic parameters associated with them in Table 1. Of primary interest are sample size corrected values for allelic richness (A_r). A_r is highest among $PARA_{NORTH}$ and $PARA_{SOUTH}$ samples in the Eastern Amazon ($A_r = 2.027$ & 2.134), as well as in the Atlantic Forest ($A_r = 2.010$) and Tocantins, in the Cerrado (A_r 1.959). While A_r is a useful measure of overall sample size corrected genetic diversity, structured diversity within a population may be overlooked. We thus also calculated mean pairwise allele sharing (D_{AS}) between multilocus genotypes (MLGs) in each population – Table 1. The standard deviations associated with mean D_{AS} values are particularly informative. Diverse populations with elevated standard deviations (e.g. Atlantic Forest – 0.369 ± 0.199, Tocantins - 0.362 ± 0.221) are likely to possess intra-population sub-clusters. By contrast genetic diversity is uniformly distributed among samples within populations with low standard deviations about the mean D_{AS} ($PARA_{NORTH}$ - 0.445 ± 0.082, $PARA_{SOUTH}$ - 0.416 ± 0.053). Observed heterozygosity varied considerably across populations. However, where population sizes ($N > 10$) are likely to facilitate meaningful interpretation, positive values for F_{IS} prevailed, and by inference heterozygous deficit compared to Hardy-Weinberg expectations (Table 1).

Sample clustering based on pair-wise nuclear genetic distances provides insight into the idiosyncratic patterns of genetic diversity noted across populations. As such, considerable admixture is present between multiple populations. This phenomenon is best represented by the composite bars adjacent to the clusters in the multidimensional scaling plot displayed in Figure 1. Samples recovered from the Atlantic Forest and Tocantins cluster among multiple, divergent groups. Meanwhile TcI from $PARA_{NORTH}$, $PARA_{SOUTH}$ and Ceara occur among the same or closely related clusters. Remaining clusters represent intermediates between these two extremes. In summary, genetic diversity among some populations looks considerably more fragmented than among others. Mean pair-wise values for D_{AS} and their associated SD seem to reflect this (Table 1).

Given the intense degree of admixture and substructure in several populations we decided not to calculate population specific linkage disequilibrium indices. Substructure is known to inflate such measures and increase the likelihood of a type 1 error [39]. Instead we chose to evaluate congruence between nuclear and mitochondrial genome clustering as evidence for rare genetic exchange events. To make such a comparison we incorporated previously published nuclear and mtDNA data into our dataset [28]. Figure 2 shows the resulting trees and the single recombinant we were able to detect mong the 14 clones assayed – 6824, isolated from *Didelphis albiventris* in the Caatinga, possesses a mitochondrial genome of Amazonian origin. The hypothetical direction of the introgression event (recipient and donor) is detailed in the map inset.

The inclusion of nuclear reference microsatellite profiles from throughout the Americas in Figure 2 provides insight into the wider affinities of the Brazilian isolates. Most notably, isolates belonging to cluster 11 in Figure 1 form a homogenous group that cluster basally, well outside global TcI diversity. *GPI* sequences for this group nonetheless confirmed this group as TcI and no affinities with Tcbat were apparent based on the same target (data not shown).

Discussion

TcI diversity in Brazil is clearly considerable by comparison to that in the rest of South, Central and North America. Figure 2 shows a comparison of isolates evaluated in this study with those analysed previously [27]. Nuclear genetic data (left hand tree) indicate a clade (corresponding to population 11 in Figure 1) that lies outside the known diversity of TcI in the Americas. The presence of a bat trypanosome among this group led us to suspect that this cluster may be Tcbat, a novel DTU with affiliations to TcI originally isolated from chiroptera in Sao Paolo state, but now recognised as more widespread [40,41]. However, sequence comparison of this clade and Tcbat at the *GPI* gene rejected this hypothesis (data not shown). In contrast, all remaining TcI isolates from Brazil fall alongside their congeners, including isolates from Bolivia and Argentina, but distinct from isolates north of the Amazon basin (Venezuela, North and Central America).

The available data suggest that genetic exchange is a fairly common phenomenon among TcI isolates [25,42],

which is also capable of genetic recombination in the laboratory [43]. A consistent feature of genetic exchange events is the uniparental inheritance of mtDNA. At a population level, as well as between DTUs, these events lead to clear instances of mitochondrial introgression [25]. Thus a pair of isolates maybe highly genetically similar on a nuclear level, but lack any affinity between mitochondrial genomes. We identified one such hybrid among those clones we assayed - 6824. In a recent review, it was proposed that 'different evolutionary pressures and molecular clocks' between non-coding nuclear microsatellite and coding mtDNA, rather than genetic exchange, might account for such signals of introgression [44]. However, such a theory requires a situation in which two (or more) near identical nuclear genotypes (e.g. 6824 and 9667) experience radically different evolutionary pressures on their mitochondrial genomes, which end up closely resembling the mitochondrial genotype of nearby or sympatric clones, in this case from the same host (*Didelphis albiventris*). Given that this pattern of introgression fits precisely with that observed in hybrids in the laboratory [43], and between DTUs in the field (TcI/TcIV) [45], recombination is the only reasonable explanation.

Of particular interest in our study was the distribution and structure of genetic diversity within and between ecoregions. Admixture was most common in the Atlantic forest region, and largely absent from the Amazon region in Pará state (Figure 1). As such, samples from the Atlantic Forest region have strong affinity with those from around Brazil and are thus distributed across multiple genetic clusters in Figure 1. The inset in Figure 1 provides fine details of parasite genetic diversity in the Atlantic Forest region. Isolates at the northern extreme of this region have predictable affinity with samples from the Caatinga (cluster 5). However, admixture into Atlantic forest from other populations is far less predictable, especially from Amazonia, and the Pantanal, which lie thousands of kilometres from the Atlantic forest. The impact of Atlantic forest fragmentation on species abundance and diversity is well documented (e.g. [46-48]). Most studies report loss of alpha diversity correlating inversely with forest fragment size, within as well as between species [46,49]. In contrast, allelic richness indices in our study suggested substantial *T. cruzi* genetic diversity within the Atlantic Forest (Table 1). However, invasive species introductions are common in the Atlantic Forest region (e.g. [50]), and it seems that several long range introductions from distant populations may also explain the high genetic diversity of TcI in the region. Thus, unlike TcI populations from Amazonia and Caatinga, which generally exhibit high genetic diversity but little admixture, high genetic diversity in the Atlantic Forest region is explained by these introductions and associated admixture. Long-range sylvatic dispersal of *T. cruzi* can be achieved by bats. Indeed, the presence of *T. cruzi* clade trypanosomes in Africa can be explained by rapid aerial dispersal [51]. Cluster 11 contains several isolates from bats, which could explain the geographic diversity of isolates in this clade (Atlantic Forest, Pantanal, Cerrado), as well as its genetic homogeneity. However, other geographically diverse isolate groupings containing Atlantic Forest isolates have no link to volant mammals.

There is a circumstantial link between Atlantic Forest loss (88% of its former extent [52]), human population density, and TcI genetic admixture in the region. *T. cruzi* infection is commonly termed a 'zoonosis', which implies unidirectional dispersion from sylvatic transmission cycles to man. Until the successful triatomine eradication campaigns of the 1970s and 1980s, domestic *T. cruzi* infection was endemic throughout much (although not all) of the Atlantic Forest region [21]. It is thus possible that many of these long-range introductions into the Atlantic are 'enzooses', i.e. TcI strains imported via immigrant human populations, which subsequently escaped in the local sylvatic environment.

Conclusions

Rather like primary rainforest, 'pristine' sylvatic *T. cruzi* diversity may be now relatively rare in South America, especially where human population densities and infections rates have been historically high. The presence of disturbed and admixed sylvatic *T. cruzi* populations in populous areas has major implications for the effective source attribution and thus future prevention of oral outbreaks [5]. Many such outbreaks have occurred in Brazil in recent years [53]. As such, the discrimination of the source of oral outbreak strains as being from either the local wild population or from another region via the importation of foodstuffs becomes complex. This is because the local wild strains themselves may represent long-range introductions. Nonetheless, admixture among sylvatic parasite populations has a possible role as a proxy for environmental disturbance. Future approaches could involve high-resolution genotyping and focused sampling of Atlantic forest fragments, including co-variates like mammalian and insect biodiversity, to further explore the use of *T. cruzi* as a sentinel species for ecosystem health.

Additional file

Additional file 1: Table S1. *Trypanosoma cruzi* I isolates evaluated in this study.

Competing interests
The authors declare they have no competing interests. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions
VL and ML generated the data. VL, ML and LM analysed the data. VL, MM and ML wrote manuscript. AJ and ML designed the study. All authors read and approved the final version of the manuscript.

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ANEXO 2. Expanding the knowledge of geographic distribution of *Trypanosoma cruzi* genotypes: TcII and TcV/TcVI in the Brazilian Amazon. *Submetido na Plos One em 24/04/2014.*

Expanding the knowledge of geographic distribution of *Trypanosoma cruzi* genotypes: TcII and TcV/TcVI in the Brazilian Amazon

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Abstract

Background: *Trypanosoma cruzi* infection is a complex sylvatic enzootia involving a wide range of animal species. Six Discrete Typing Units (DTU's) of *T. cruzi* are recognized, named TcI to TcVI. The distribution pattern of TcII and hybrid DTUs in nature is one of the numerous gaps in the knowledge of the epidemiology of *T. cruzi* including their virtual absence in the Brazilian Amazonian, the current endemic area of Chagas disease in Brazil.

Methodology/Principal Findings: The enzooty of *T. cruzi* in the Pará State, Amazon region, was analyzed in consecutive campaigns. In this study we performed *T. cruzi* genotyping using two markers, 1f8 and Histone 3 genes, in mixed infection from triatomine isolates and dog serum samples. Herein, we reported for the first time the presence of TcII and hybrid DTUs in the Amazon region. Further, the sequencing of the constitutive gene, gp72, demonstrated diversity in TcII even in one same forest fragment. The characterization of *T. cruzi* DNA directly from the serum of one naturally infected *Didelphis* was able to show mixed TcI and TcII infection that the previous performed blood cultures did not detect. This fact underlines the importance of laboratory methods as selective forces and therefore as inductors of misinterpretations.

Conclusion/Significance: TcII is dispersed in the five principal Brazilian biomes and probably is more prevalent than currently described. Our data suggest that there is no biological or ecological barrier to the transmission and establishment of any DTU in any biome from Brazil. We are probably facing *T. cruzi* DTUs displaying distinct strategies of dispersion, still little known, which may hide the real prevalence and distribution of *T. cruzi* DTUs.

Keywords: Amazon Basin; *Trypanosoma cruzi*; TcII; DTU; epidemiology; Brazil

Author Summary

American trypanosomiasis by *Trypanosoma cruzi* is primarily a sylvatic enzootia involving a wide range of mammalian and triatomine vectors species. Six Discrete Typing Units (DTU's), named TcI to TcVI are recognized in *T. cruzi*. Among the numerous gaps in knowledge of the epidemiology of *T. cruzi* DTU's, we highlight the DTU TcII distribution pattern: its "apparent" lower overall prevalence in nature and virtual absence of Amazonian. Amazon biome is the largest and more diverse Brazilian biome and the current endemic area to Chagas disease. This purpose led us to study the enzootic transmission cycle of *T. cruzi* in the Amazon by use of a more sensitive parasitological method, the search for parasite DNA in serum. In this study we show for the first time the presence of TcII and hybrid DTU's in the Amazon. Also, we discuss data as genetic diversity and recovery of TcII DNA from serum of mammals with positive TcI hemoculture solely. Our data suggest that there is no biological or ecological barrier to the transmission and establishment of any DTU in any biome. Besides we suggest to be TcII more prevalence than is described up to now.

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 included in the transmission cycle probably as soon as they arrived in the Americas around 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, have been observed and discussed [2]. The currently employed molecular tools allowed the recognition of six Discrete Typing Units (DTU's), named TcI to TcVI [3]. Nevertheless, the complexity of *T. cruzi* is far from being resolved considering, for example, the recent discovery of an additional *T. cruzi* genotype exclusive of bats [4] and the recognition of heterogeneity within TcI [5].

T. cruzi is considered as presenting mainly a clonal population structure, nevertheless, numerous evidences point to the importance of hybridization events as the cause of the extensive heterogeneity of the taxon [6]. The two more divergent lineages are TcI and TcII, which separation time is still under debate as it ranges from 3 to 88 million years bp [7,8]. The more recent diverged DTU's are respectively TcV and TcVI that resulted from at least one hybridization event that is estimated to have occurred 0.9 million years ago (MYA) [9].

The epidemiology of the *T. cruzi* DTUs is still a challenging issue. The great gaps in the knowledge of the distribution of the distinct DTUs are due mainly to the difficulties in obtaining representative samples of a so widely distributed enzootic taxon as it is the case of *T. cruzi*. Up to now, TcI is reported as being more frequently isolated of all mammalian taxa throughout the geographic range of the occurrence of the parasite in an ample diversity of biomes and habitats [3]. Comparatively, the DTU's III and IV are isolated less frequently, although also widely distributed [3,10,11,12]. Very little is

77 known about the wild hosts of the hybrid DTU's V and VI, that, up to now, have been
78 mainly isolated from humans or from domiciliated triatomines [3,13,14]. In Brazil, there
79 is one single report of TcV infecting a wild host, a caviomorph rodent species,
80 *Thrichomys a. laurentius* [15].

81 The TcII sylvatic hosts and distribution in the wild also need to be clarified. This
82 parental and ancient *T. cruzi* DTU, previously mainly associated to human infection, is
83 reported to occur in the central belt of South America, covering the countries Brazil,
84 Chile, Colombia, Bolivia, Uruguay and Paraguay [3,13,14]. Above the Amazon region,
85 TcII was found infecting *Triatoma dimidiata* in Guatemala [16]. In Brazil this DTU has
86 been detected infecting a broad range of mammal species, respectively in the Atlantic
87 Forest, Caatinga, Pantanal and Savannah biomes [11,17,18,19]. In spite of being able
88 to infect a high variety of wild host species, TcII was isolated from a reduced number of
89 animals and therefore it was proposed that this DTU occurs in more focal cycles [19].
90 In the last decades, *T. cruzi* isolates from the Amazon region has been subjected to
91 several studies involving molecular characterization, however, only DTUs TcI, TcIII and
92 TcIV have been reported in this biome [20,21,22]. All together, moreover the recurrent
93 emergence of Chagas disease in the Amazon region, as yet without control, and the
94 overall sparse knowledge about this biome have led us to study the enzootic
95 transmission cycle of *T. cruzi* since 2006 in this region [23,24,25]. Herein, we extended
96 these studies including new data on the distribution of Tc hybrids and TcII.

97 **Material and Methods**

98 ***Study area***

99 This study was conducted in three municipalities/localities in the Pará State:
100 Abaetetuba/Ajuaí (01º43'24'' S; 48º52'54'' W) and Belém/Val-de-Cans (01º27'21''S;
101 48º30'16''W) are located in the northeastern mesoregion of the State of Pará and
102 Monte Alegre/Setor 11 (01°38'20"S; 54°14'32"W) is located in the lower Amazon

mesoregion of the Pará State [24,25]. 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 the river banks. (Figure 1).

Biological samples

We characterized *T. cruzi* isolates derived from two *Rhodnius pictipes* and *T. cruzi* DNA samples obtained from sera of five naturally infected dogs. The two *T. cruzi* isolates, respectively, LBT 1458 and LBT 1814 were obtained from *R. pictipes* captured in palm trees (*Attalea pharelata*), respectively from the Rio Ajuaí and Val de Cans localities (Table 1). *T. cruzi* isolates were achieved by culture of the triatomine intestinal content 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 submitted to DNA extraction using the phenol-chloroform protocol and deposited in the "*Trypanosoma* from Sylvatic and Domestic Mammals and Vectors Collection", Oswaldo Cruz Foundation – COLTRYP.

The owners of the dogs from that we obtained the serum samples were from a rural locality (Sector 11) in the Monte Alegre municipality, Pará State (Table 1). The scarse population of this area lives of subsistence agriculture and uses dogs for hunting. It is worth mentioning that the dogs were autochthonous and never moved to other sites. The dog's infection was detected by fresh blood smear examination and confirmed as *T. cruzi* by PCR of the variable region of the kDNA [25]. The DNA of the five dogs serum samples, respectively, LBT 1818, LBT 1819, LBT 1820, LBT 1821 and LBT 1822 was extracted using the phenol-chloroform protocol, as used for extraction from parasite cultures, but excluding SDS in pre-treatment [25].

Mini-exon assay

All *T. cruzi* samples were initially submitted to multiplex PCR of the mini-exon gene performed according to conditions described by Fernandes *et al.*[26], in order 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).

Biological cloning of a T. cruzi isolate

As the isolate LBT 1458 presented controversial results in the mini-exon assay (mixed TcI/Tc2 in a first characterization and only Tc2 when typed again by the same method in the same conditions), it was submitted to biological cloning in order to assess the composition of subpopulations and so genotyping them separately. For this purpose we used the technique of solid phase medium in a Petri plate [27]. We typed ten of the obtained biological clones, observing that three of them were TcI and seven were Tc2, from which we selected two.

Molecular cloning of the mini-exon gene of the serum samples

PCR products from dog serum samples (LBT 1819 and LBT 1822) with mixed *T. cruzi* DTU's 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 DTU's. The colonies (clones) were collected randomly, and submitted to PCR of the mini-exon gene [26] in order to select the clones with fragments of 250bp, 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) in order characterize LBT1458 clones, we performed the restriction fragment length polymorphism (RFLP) analysis of the nuclear 1f8 gene after digestion by the Alw21I enzyme that distinguishes DTU's TcII from TcV and TcVI [28] and (b) PCR-RFLP of the gp72gene and analysis of fragments digested by TaqI enzyme was performed as a confirmatory marker that discriminates TcII and to distinguish TcV from TcVI [28]. Nevertheless, this protocol did not allow the characterization of LBT 1458 clones 5 and 7.

For genotyping the original *T. cruzi* isolate (LBT 1814) and the dog serum sampleLBT 1822 we used PCR-RFLP of H3/AluI since this marker distinguishes TcII from TcV and TcVI without overlapping of fragments. (TcV and TcVI) [19,29]. Each reaction included negative controls and positive controls from representative DTU's. The results of PCR and RFLP were visualized in agarose 3% gels stained with ethydium bromide under UV illumination.

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

Mini-exon gene. In order to compare with other sequences in GenBank and to identify the *T. cruzi* DTU we sequenced the fragment of 250 bp of the mini-exon assay of sample LBT 1822. This fragment was obtained after molecular cloning of this gene. The primers used were Tc2 and Exon, the same used for amplification of this fragment in the multiplex assay.

Glicoprotein 72 gene (gp72 gene). To clarify the odd profile exhibited by the RFLP protocol of gp72 in LBT 1458 cl 5 and cl 7 we extended the study testing this protocol in twenty isolates from ColTryp previously identified as TcII by a combination of PCR-RFLP of HSP60, GPI loci and 24S αDNA AFLP markers [30] and GPI sequencing [31]. We then sequenced these two clones besides five TcII isolates with patterns as described by Rozas et al [28] and five isolates exhibiting a c5 and c7 similar pattern (table 2). The analysis of this locus also allowed access to the possible

179 phylogenetic significance of this difference. The amplicons (1290 bp) were purified
180 using the illustra GFX PCR DNA and Gel Band purification kit (GE Healthcare Life
181 Sciences,Little Chalfont, Buckinghamshire, UK) and subjected to cycle sequencing
182 reaction with the Big Dye Terminator v 3.1 commercial kit (Applied Biosystems, Foster
183 City, California, USA). The products were sequenced in an automatic sequencer 3100
184 (Applied Biosystems) using the same primers as for amplification.

185

186 ***Sequence analysis***

187 The sequence edition, alignment and phylogenetic tree construction was
188 performed using Chromas v. 1.45 (School of Health Sciences, Griffith University,
189 Queensland, Australia) and the Mega v 5.1 free program [32]. The parameters and
190 statistical method for phylogenetic reconstruction used were the Kimura-2-parameter
191 model and Neighbor-joining. The bootstrap was acquired from 1,000 replicate trees.
192 We included in this phylogenetic reconstruction analysis six sequences from GenBank,
193 three TcI as outgroup (Table 2). The command BLAST (Basic Local Alignment Search
194 Tool) option Nucleotide was used to compare sequences acquired from the serum of
195 the dog with *T. cruzi* sequences of mini-exon from GenBank.

196

197 **Results**

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

199 *T. cruzi* characterization of the samples is summarized in Table 1 and Figure 2,
200 that show the occurrence of TcII and Tc hybrid (TcV or TcVI) in the Amazon region,
201 respectively in two *Rhodnius pictipes* (LBT 1458 and LBT 1814) and one dog (Figure
202 2C, D and Table 3). This is the first report of these genotypes in the Amazonian region.

These three samples were in mixed infection with DTU I as demonstrated by mini-exon assay. Among the inherent difficulty in to detect and to characterize mixed infections, one is the selective pressure exercised by axenic medium. This fact was observed in our isolate LBT 1458 that in the first amplification of mini-exon gene presented mixed infection TcI/Tc2 (data not shown) and in subsequent amplifications from a new extraction, this isolate exhibited a profile of Tc2 only (Figure 2B). However, TcI clones (data not shown) besides TcII clones (Figure 2C) were obtained from biological cloning of this isolate.

The identification of TcII in mixed isolate LBT 1814 was possible using the H3/AluI RFLP protocol (Figure 2D).

We obtained the sequence referent to the 250bp fragment (referent to mini-exon Tc2 group) from the LBT 1822 dog sample and then submitted it to BLAST analyses hosted at NCBI. The sequence obtained showed 100% of coverage and 99% of identify with three hybrid strains (TcV- SC43 and MN; and TcVI - CL Brener). Solely 20 to 40% of coverage and 100% of similarity was observed with the five TcII sequences, nevertheless a single TcII strain (Tu18) presented 100% of coverage and 99% of identify. The profile exhibited by RFLP of the H3 gene using AluI as restriction enzyme confirmed the hybrid genotype (TcV/TcVI) in dog LBT 1822 although the intensity of bands was very weak (data not shown).

Gp72 locus diversity

Our TcII isolates were genetically diverse as revealed by sequencing of gp72 (Figure 3). The topology of the generated neighbor-joining tree shows the lack of correlation between TcII isolates, considering both geographic and genetic distances (Table 2, Figure 3). The sequencing analysis showed even more diversity in TcII than was previously observed in the two patterns of RFLP gp 72 analysis. The two distinct

229 patterns in RFLP analysis of the gp72 locus are due to a SNP (single nucleotide
230 polymorphism) in one of the restriction sites for enzyme TaqI. Isolates with mutation of
231 a thymine to a guanine are not cut by the enzyme.

232

233 **Discussion**

234 Trypanosomiasis by *T. cruzi* is a zoonosis widely dispersed in nature in the
235 Americas. Due to the large extension of occurrence of this parasite, and the difficulties
236 involving capture, transport of biological material and parasite isolation from wild
237 mammals and vectors, the data about distribution of *T. cruzi* genotypes are aggregated
238 and do not contemplate all the habitats of occurrence of this parasite. In addition,
239 peculiarities of interaction of different hosts with each DTU represents distinct selective
240 pressures besides the selective pressure exercised by isolation methods. These bias
241 may result in misinterpretation about the ecology and biology of this Trypanosomatid.
242 The Amazon region, in special, was the biome that attracted our attention in recent
243 years due to the increase of cases of Chagas disease by oral route [2,3,20]. The
244 apparent difference in the prevalence of the *T. cruzi* DTU's in nature is intriguing and is
245 probably determined by the different transmission strategies selected by each *T. cruzi*
246 DTU. These differences are not yet clarified but all of them were demonstrated to be
247 successful, since they allow their maintenance in nature. Indeed, very probably, each
248 DTU establishes a peculiar interaction pattern with their several host species. In this
249 sense, concerning TcII, it was observed that after a short period of high parasitemia,
250 detectable by hemocultures, dogs remained serologically positive but it was no longer
251 possible to recover the parasites by hemoculture [19]. This suggests that the
252 transmission strength of these animals was restricted to a short period of approximately
253 two months [19]. Opossums (*Didelphis aurita*) handle TcII in a different way; thus, in
254 experimental infections with Y and FL strains, both TcII, these mammals were able to

255 control parasitemia even when very young, still dependent of the marsupium [33].
256 Nevertheless, naturally infected *Didelphis* spp show that they are able to maintain TcII
257 in the wild. In contrast, the four eyed opossum (*Philander frenata*) was able to maintain
258 long lasting infections with high prevalence of positive hemocultures when
259 experimentally infected with the Y strain (TcII) [34]. A long lasting TcII transmission
260 cycle in the wild was described in two tamarin species, *Leontopithecus rosalia* and *L.*
261 *chrysomelas*, in two fragments of the Atlantic Forest Biome [35]. Also, the carnivore
262 *Nasua nasua* in the Pantanal biome demonstrated to be a suitable wild TcII host [11].

263 In this study we show for the first time the presence of *T. cruzi* DTU's TcII and a
264 hybrid DTU (TcV or TcVI) in the Pará State of the Brazilian Amazon. These data
265 highlight that TcII is present in wild transmission cycles in all Brazilian biomes (Figure
266 1) and is not restricted to areas below the Amazonian Basin as has always been
267 assumed [2,3]. Additionally, the presence of infected triatomine bugs and domestic
268 dogs by TcII and Tc hybrid, imply that other surrounding mammals and triatomine bugs
269 should be also infected and involved in the transmission cycle of these DTUs in the
270 Pará State. It is worth mentioning that before this study, Tc hybrid was reported in
271 Brazil only once, in a sylvatic rodent species, *Thrichomys a. laurentius* in the
272 northeastern region of the country [15] showing that we are far from understanding the
273 distribution and hosts of hybrid DTUs. In this report the Tc hybrid from dog, a domestic
274 mammal, may be considered as derived from a sylvatic environment because in the
275 Amazon Biome there is no distinction between domestic and sylvatic environments and
276 these animals use wild areas. Nevertheless, this report of Tc hybrid in the Amazon
277 casts more questions than it does clarify the epidemiology of these DTU's, mainly due
278 to the huge gap in reports of these DTU in Brazil, as most of the isolates obtained are
279 from the Southern part of the country, and solely one isolate was reported in a wild
280 rodent from the northeastern caatinga [15].

The isolation of TcII from *Rhodnius pictipes* shows the ability of this species to maintain this genotype. This genus has been described as unsuitable to maintain *T. cruzi* TcII [36,37,38], even though these experimental studies were based on the observations of only three TcII strains (Y, AF-1, Tu18) which do not represent the diversity of this genotype in the wild. The natural infection by *T. cruzi* in genus *Rhodnius* sp in the Amazonian biome was intensely studied in past decades, nevertheless, the majority of these studies lacked employment of molecular genotyping tools [20]. Studies of natural infection by *T.cruzi* in *R. pictipes* in the Amazon region up to now have reported the infection by TcI, TcIV and *T. rangeli* [22,39].

The greater issue that challenges our understanding of the populational structure of these DTU's is their apparent focal distribution in nature. The insufficient knowledge of behavior and dynamics of infection of TcII and Tc hybrids in mammals and vector species is probably, as mentioned above, the consequence of selective pressures in the process of isolation and maintenance and different sensitivity in diagnostics methods, besides 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 of Santa Catarina State (Maldonado et al., unpublished data) whose hemoculture was characterized as TcI [23]. Another example is a *T. cruzi* isolate obtained from a *Didelphis albiventris* from the caatinga biome that after several years maintenance in the cryobank of our Laboratory, always characterized as single TcI infection by different typing techniques [26,30] demonstrated to include also TcII after biological cloning was performed (Lima et al., manuscript in preparation). The same event was observed in this study with isolate LBT 1458 characterized as mixed TcI/Tc2 in the first mini-exon assays and, in a second DNA extraction, demonstrated only the Tc2 band pattern (Figure 2B). Moreover, the presence of TcI in this isolate was subsequently demonstrated by biological cloning. These factors contribute to misinterpretation about the prevalence of

T. cruzi DTU's in nature. Another fact that may contribute to underestimating prevalence is the large number of sylvatic mammals with positive serology which do not result in recovery of parasites by hemoculture (70%) (Jansen AM, personal communication).

The protocol of RFLP of gp72/TaqI used in Brazilian TcII isolates discriminated two groups of this DTU, one with a profile as described by other authors and another with an odd profile (data not shown). The sequencing of this locus points to a SNP as responsible for these two profiles, nevertheless, the phylogenetic reconstruction showed that the presence of the same SNP does not imply in higher phylogenetic relatedness. However the sequencing showed an aspect still poorly known that is the diversity in the TcII DTU. The genetic diversity among twelve sylvatic isolates and clones from Caatinga, Cerrado, Atlantic Forest and Amazon (Figure 3) in a constitutive gene allow us to suggest that TcII has a greater prevalence than is reported since diversity is directly related to the populational size [40,41]. The absence of correlation between geographic area and sequence type of TcII suggest that variants of TcII are probably not limited to tamarin species in the Atlantic Forest but includes other mammal host species in other biomes [31]. Studies based on molecular targets with greater discriminatory power should assess more accurately the populational structure and diversity of wild TcII.

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465

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Figure Legends

Figure 1. Distribution of *Trypanosoma cruzi* TcII and hybrid genotypes in the Amazon and in Brazil. Red circles indicate location of TcII isolates, blue circles indicate localization of hybrid isolates, black symbols represent the mammalian and vector host species. The upper right figure shows the localization of TcII and hybrid DTUs of this study in the Pará State.

Footnotes: Atlantic Forest biome [17,34,35]; Caatinga biome [15,18]; Cerrado biome [19]; Pantanal biome [11].

Figure 2. *Trypanosoma cruzi* genotyping from dogs and triatomines naturally infected from the Amazon Biome. (A) PCR product size polymorphism of the non-transcribed intergenic region of the SL -RNA mini-exon (mini-exon assay) of *T. cruzi* DNA of dog sera from Monte Alegre in the Pará State, samples: 1- dog LBT 1818, 2- dog LBT 1819, 3- dog LBT 1820, 4- dog LBT 1821, 5- dog LBT 1822 (B) Mini-exon assay of *Rhodnius pictipes* isolates from Abaetetuba (LBT 1458) and Belém (LBT 1814) in the Pará State, samples: 1- LBT 1458, 2- LBT 1814 (C) *T. cruzi* genotyping profiles for PCR-RFLP of the 1f8 gene/Alw21I restriction enzyme of LBT 1458 clones 5 and 7, samples: 1- LBT 1458 clone 5, 2- LBT 1458 clone 7 (D) *T. cruzi* genotyping profiles for PCR-RFLP of Histone 3/AluI restriction enzyme for the LBT 1814 isolate, sample 1- LBT 1814 isolate. The *T. cruzi* DTU's, *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; VI – CL Brener. Agarose gel 3%, stained by ethidium bromide.

Figure 3. Phylogenetic relationships between *Trypanosoma cruzi* sylvatic isolates and TcII clones assessed by gp 72 gene. The tree was constructed under neighbour-joining using 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 Rio de Janeiro State, MLCD: *L. chrysomelas* from the Una Biologic Reserve in the Atlantic Forest of Bahia State, CD: dogs of the Cerrado in Minas Gerais State, LBT: *Rhodnius pictipes* from the Amazon Biome and the isolate JCA3 is from a *Triatoma brasiliensis* of Piauí State in the Caatinga Biome. *GenBank sequences strain TcI first published by Flores-López et al., 2011.

511 **Table 1. *Trypanosoma cruzi* molecular characterization in naturally infected**

512 **hosts from Pará State, 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 182[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

513

514 Footnotes:

515 (-) Not available;

516 [a] *Trypanosoma cruzi* kDNA positive serum samples from Samanta et al. (2012);

517 [b] Mini-exon assay according to Fernandes et al (2001);

518 [c] PCR-RFLP assay of the 1f8 gene according to Rozas et al (2007);

519 [d] PCR-RFLP assay of the histone 3 gene according to Westenberger et al. (2005);

520 [e] Sequence of 250bp fragment of the Mini-exon gene. GenBank accession number

521 KJ402456.

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526 **Table 2. *Trypanosoma cruzi* II isolates submitted 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

527

528 Footnotes:

529 [a] RJ - Rio de Janeiro; BA - Bahia; PI - Piauí; PA - Pará; MG - Minas Gerais;

530 [b] A - according to profile described by Rozas et al (2007); B - distinct profile than

531 described by Rozas et al. (2007).

532

Figure 1
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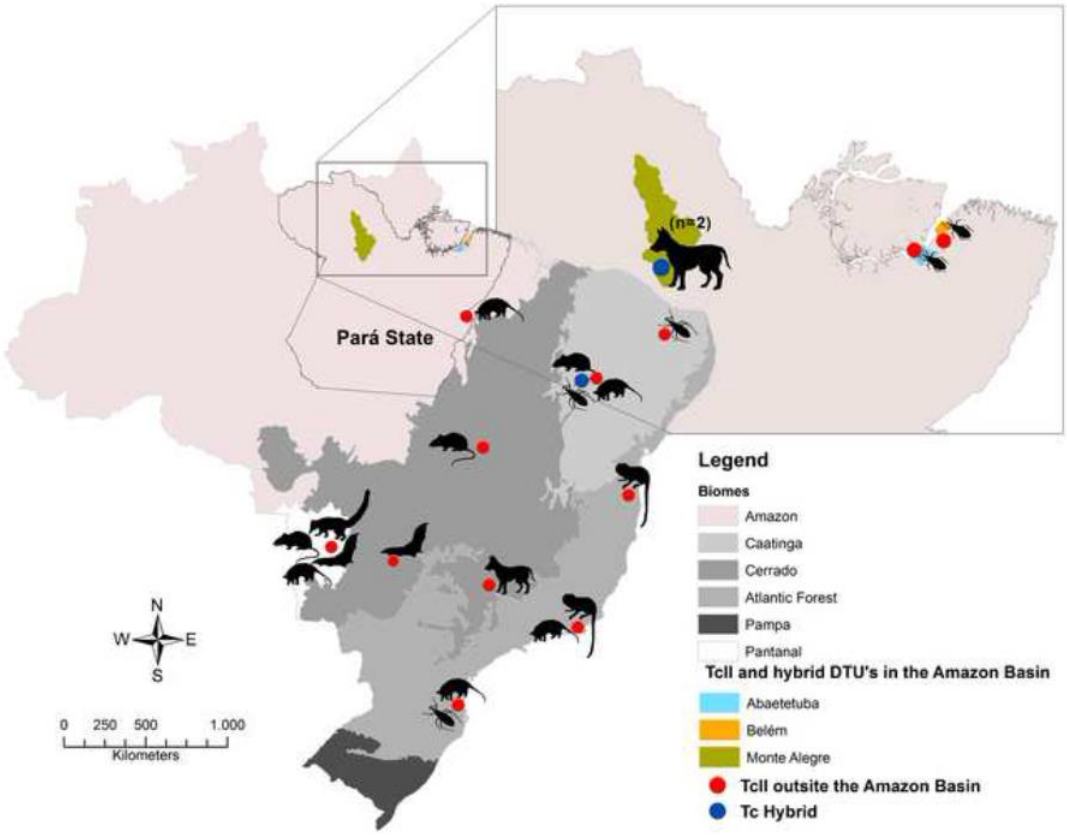

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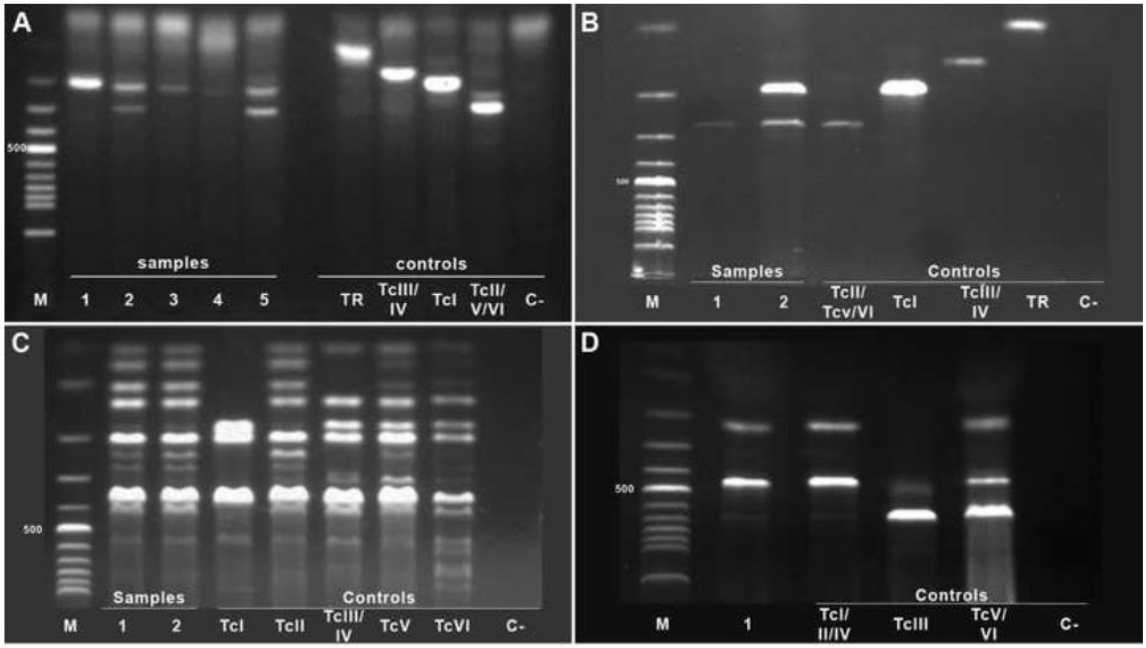

Figure 3
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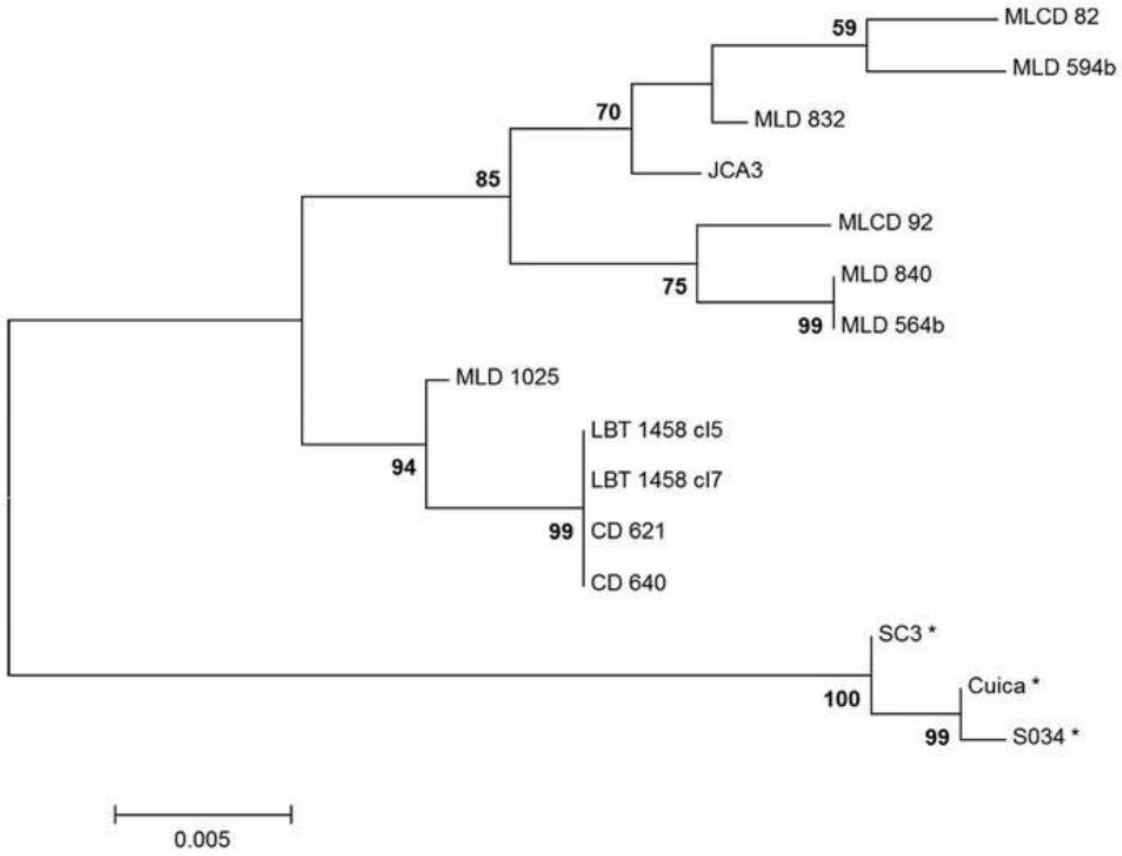

ANEXO 3. Artigo: Lower Richness of Small Wild Mammal Species and Chagas Disease (2012)Plos Neglected Tropical Diseases, PLoS Negl Trop Dis.;6(5):e1647.

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PLoS NEGLECTED TROPICAL DISEASES

Lower Richness of Small Wild Mammal Species and Chagas Disease Risk

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Abstract

A new epidemiological scenario involving the oral transmission of Chagas disease, mainly in the Amazon basin, requires innovative control measures. Geospatial analyses of the *Trypanosoma cruzi* transmission cycle in the wild mammals have been scarce. We applied interpolation and map algebra methods to evaluate mammalian fauna variables related to small wild mammals and the *T. cruzi* infection pattern in dogs to identify hotspot areas of transmission. We also evaluated the use of dogs as sentinels of epidemiological risk of Chagas disease. Dogs (n = 649) were examined by two parasitological and three distinct serological assays. kDNA amplification was performed in patent infections, although the infection was mainly sub-patent in dogs. The distribution of *T. cruzi* infection in dogs was not homogeneous, ranging from 11–89% in different localities. The interpolation method and map algebra were employed to test the associations between the lower richness in mammal species and the risk of exposure of dogs to *T. cruzi* infection. Geospatial analysis indicated that the reduction of the mammal fauna (richness and abundance) was associated with higher parasitemia in small wild mammals and higher exposure of dogs to infection. A Generalized Linear Model (GLM) demonstrated that species richness and positive hemocultures in wild mammals were associated with *T. cruzi* infection in dogs. Domestic canine infection rates differed significantly between areas with and without Chagas disease outbreaks (Chi-squared test). Geospatial analysis by interpolation and map algebra methods proved to be a powerful tool in the evaluation of areas of *T. cruzi* transmission. Dog infection was shown to not only be an efficient indicator of reduction of wild mammalian fauna richness but to also act as a signal for the presence of small wild mammals with high parasitemia. The lower richness of small mammal species is discussed as a risk factor for the re-emergence of Chagas disease.

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Introduction

The causative agent of Chagas disease, *Trypanosoma cruzi* (Chagas, 1909), is a multi-host parasite capable of infecting almost all tissues of more than one hundred mammal species [1]. Dozens of species of insects from the Triatominae subfamily can act as its vector. Except for the epimastigote form, all other *T. cruzi* evolutive forms can infect mammals by oral and congenital pathways as well as by contamination of the mucosae and skin abrasions by infected triatomine feces. The biological plasticity of *T. cruzi* results in transmission cycles that are characterized by being multivariate and complex on unique temporal and spatial scales [1,2].

Classically, Chagas disease was characterized as prevalent in rural populations, where houses were heavily infested by domiciliated triatomine species, mainly *Triatoma infestans* (Klug, 1834). The campaigns launched by the "Cone Sul" Intergovernmental Commission to eliminate the domiciliary vectors succeeded in that Brazil and other countries in South America are currently considered free from domestic transmission of Chagas via *Triatoma infestans* [3]. However, extradomiciliary vectorial transmission, domiciliary or peridomestic transmission by non-domiciliated vectors and oral transmission by ingestion of food contaminated by feces from infected insects (the principal method of current transmission), pose new challenges. In fact, mainly in the northern part of the Brazil, the number of Chagas disease outbreaks due to the ingestion of food contaminated by infected triatomine feces are increasing [4–6]. This is currently considered a new epidemiological scenario, demanding systematic surveillance methods that consider all components of the transmission cycle as well as the landscape and ambient conditions in which transmission is occurring.

In several reports, the maintenance of biodiversity has been pointed to as a strong buffering system and regulator in the dispersal of parasites; this has been named the "Dilution Effect". Such a dilution effect has already been demonstrated to be of importance in the transmission of West Nile encephalitis, Hantavirus, Lyme disease and Schistosomiasis [7,8]. Despite the

Author Summary

The classical methodology of mapping works with discrete units and sharp boundaries does not consider gradient transition areas. Spatial analysis by the interpolation method, followed by map algebra, is able to model the spatial distribution of biological phenomena and their distribution and eventual association with other parameters or variables, with a focus on enhancing the decision power of responsible authorities. Acute Chagas Disease outbreaks are increasing in the Amazon Basin as result of oral transmission. This scenario requires a new approach to identify hotspot transmission areas and implement control measures. We applied a geospatial approach using interpolation and map algebra methods to evaluate mammalian fauna variables related to these outbreaks. We constructed maps with mammalian fauna variables including the infection rates by *Trypanosoma cruzi*, in dogs and small wild mammals. The results obtained by visual examination of the maps were validated by statistical analysis. We observed that high prevalence of *T. cruzi* infection in dogs and small wild mammals was associated with mammal lower richness. Monitoring of *T. cruzi* infection in dogs may be a valuable tool for detecting the fauna lower richness of small wild mammals and elucidating the transmission cycle of *T. cruzi* in the wild.

demonstration of this effect, studies on the impact of biodiversity variation on the *T. cruzi* transmission cycle in the wild mammals using a Geographical Information System (GIS) have been scarce up to now [9–12]. The destruction of an ecosystem imposes important area and food restrictions onto wild mammal populations and may promote their greater contact with humans. The consequence of this process is the increased opportunity for contact among humans, domestic animals and wildlife [2]. In this scenario, the transmission of *T. cruzi* may be increased due to the following: (i) positive selection of generalist species with high transmissibility competence such as *Didelphids* and some caviomorph rodents that undoubtedly adapt and survive in degraded habitats, (ii) the consequent amplification of the parasite's transmission cycle due to higher abundance of competent reservoir species and (iii) the increased prevalence of infected bugs. In addition, the scarcity of food sources for triatomines (i.e., loss of wildlife due to destruction of the environment) led them to invade human dwellings and annexes [13]. Also, the quantitative and spatial patterns of the landscape and artificial lighting in human dwellings play a fundamental role in domiciliary invasion [14]. Human residences acting as light-traps for insects has significant epidemiological importance, as species with high rates of *T. cruzi* infection are drawn to human dwellings [15]. In this scenario, peridomestic mammals are more frequently exposed. Thus, their infection usually precedes the human infection. Hence, dogs have been proposed as being suitable sentinel hosts for *T. cruzi* transmission in areas at risk for human infection [16,17].

Dogs can be important reservoirs of this parasite. They display both a high prevalence of infection and high parasitemia as evidenced in Panama [17], Argentina [18], Venezuela [19], Mexico [20], and the United States [21]. In contrast, in Brazil, a high serum prevalence in dogs has also been described in several areas, but the importance of dogs as a reservoir species has been described as negligible because no high patent parasitemia has been observed in these animals [16,22].

Geospatial analysis based on the fundamental concepts of landscape epidemiology [23] is a powerful tool in the study of the association between landscape- and vector-borne diseases such as Chagas disease, Schistosomiasis and American Visceral Leishmaniasis [9,10,24]. Geospatial analysis allows for the identification of disease risk areas and disease interactions with the environment [10,24].

The classical methodology of mapping works with discrete units and sharp boundaries, and does not consider transition areas. Nevertheless, environmental and biological phenomena are typically continuous and exhibit a gradual transition from one characteristic to another. Unlike the classical methodology, spatial analysis by the interpolation method, followed by map algebra, is able to model the spatial distribution of the continuous biological phenomena, representing the distribution and association of these phenomena in a more realistic way. This modeling can enhance and facilitate decision making [25].

The present paper evaluates and compares *T. cruzi* infection rates of dogs from three Brazilian biomes, including areas where orally transmitted Chagas disease outbreaks were reported and areas where Chagas disease is endemic. Our objectives were to (i) assess the impacts of lower richness of small wild mammals on the prevalence of *T. cruzi* infection in dogs, (ii) discuss the role of dogs in the transmission cycle of *T. cruzi* and their putative role as sentinels and (iii) to assess the interpolation and map algebra method as a tool for the construction of potential Chagas disease risk area maps.

Materials and Methods

Study area

Dogs (n = 649) were sampled in 3 geographic Brazilian biomes: the Amazon, Caatinga and Pantanal, from 5 states and 13 municipalities (28 localities) (Figure 1 and Table 1).

Among these municipalities, samples were taken immediately after an outbreak of Acute Chagas Disease (ACD) from Redenção, Cachoeiro do Arari, Belém, Curralinho and Axixá do Tocantins. The other monitored areas were Abaetetuba, Monte Alegre, Augustinópolis, Esperantina, Jaguaruana, Russas and João Costa, while Corumbá (Midwest Brazil) was used as a control area. The areas included in our study reflect the locations where our laboratory has been developing research and reference services over the past few years.

Biome Caatinga

The states of Piauí and Ceará display similar patterns: a high density of naturally *T. cruzi* infected Triatominae, which are the main vectors of disease in both regions. Despite that, no new vectorial transmission Chagas disease cases have been observed in the last decade and, as far as we know, only one outbreak has occurred due to oral transmission [2,26].

The municipalities of Jaguaruana, Redenção and Russas, which are endemic for Chagas disease, are located in the mesoregion of lower Jaguaribe, in the northeastern state of Ceará. In Jaguaruana, the average annual temperature ranges from 23°C to 33°C. The collection area consists of clay and sandy soil plains, which are characterized as Caatinga, and include the typical vegetation of semi-arid areas. Redenção (ACD outbreak in 2006) is located in the Baturité mountain range region. The climate is semi-arid, and the average annual temperature ranges from 24°C to 35°C. The collection area, originally part of a tropical semi-humid forest, is currently characterized by secondary vegetation consisting of small trees (up to 6 m), rocky formations, and remnant patches of semi-humid forest near deforested areas occupied by monoculture plantations or unplanned households (slums). The municipality of Russas is located in the state of Ceará. The climate is semi-arid with average temperatures ranging from 18.8°C to 35.4°C. The

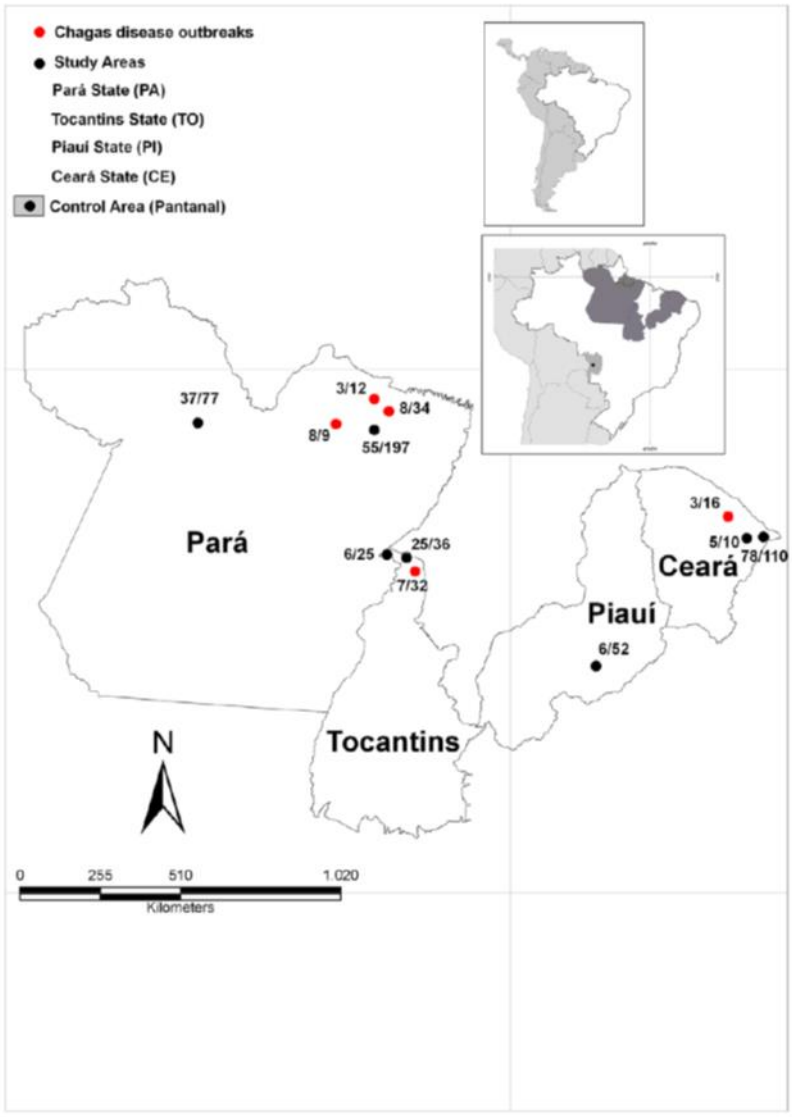

Figure 1. Geographical location of study area. Study areas are located in the 3 geographic Brazilian biomes: Amazon (Pará and Tocantins states), Caatinga (Piauí and Ceará states) and Pantanal (Mato Grosso do Sul state, control area). **Red markers** indicate areas investigated immediately after the occurrence of Chagas disease outbreaks; **Black markers** indicate non-outbreak areas.
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vegetation comprise open scrub and savanna, with deciduous thorny forest areas. The municipality of João Costa is located in the southeast of the state of Piauí and is characterized as a megathermic semi-arid region. The average annual temperature ranges from 12°C to 39°C. The vegetation in this area displays the typical Caatinga features and residual semi-deciduous forest patches.

Amazon biome

T. cruzi oral transmission in the Brazilian Amazon region has been reported since 1968 [5,27], although this region was considered an endemic area for many years. Just after 2005, when the prevalence of Chagas cases in other parts of the country decreased and surveillance in the Amazonian region was improved, microepidemics of ACD began to appear regularly and frequently, mainly associated with the consumption of the palm-tree fruit açaí and other foods [4–6].

The municipalities of Abatetetuba and Belém (ACD outbreak in 2009) are located in the northeastern mesoregion of the state of Pará. Cachoeiro do Arari (ACD outbreak in 2006) and Curralinho (ACD outbreak in 2009) are located in the mesoregion of Marajó,

Table 1. Seroprevalence of *Trypanosoma cruzi* infection in dogs from three biomes: Caatinga, Amazon Forest and Pantanal.

Biome/State	Municipalities	Localities	Coordinates		Serological+/total (%)
Caatinga	Jaguaruana	Caatinguinha	04°48′28″S	37°47′09″W	11/26 (42)
Ceará		Córrego das Melancias	04°50′10″S	37°47′20″W	22/23 (96)
		Dió	04°50′23″S	37°46′19″W	19/29 (65)
		Perímetro Irrigado	04°49′18″S	37°50′22″W	18/23 (78)
		Figueiredo do Bruno	04°47′45″S	37°49′19″W	8/9 (89)
					78/110 (71)
	Redenção*[2]	Salobro	04°11′48″S	38°42′56″W	1/2 (50)
		Alto Cassiano	04°13′18″S	38°43′56″W	1/9 (11)
		Manoel Dias	04°10′54″S	38°43′45″W	1/2 (50)
		Sítio Outeiro	04°12′21″S	38°44′09″W	0/3
					3/16 (19)
	Russas	Cipó	04°57′47″S	38°09′18″W	**5/10 (50)**
Piauí	João Costa[1]	Urban Area	08°33′24″S	42°26′12″W	**6/52 (11)**
Amazon Forest					
Tocantins	Augustinópolis	2000	05°24′11″S	48°01′09″W	11/12 (92)
		São Roque	05°30′26″S	48°02′46″W	14/24 (58)
					25/36 (69)
	Esperantina	São Francisco	05°23′32″S	48°28′46″W	**6/25 (24)**
	Axixá do Tocantins*	Lagoa de São Salvador	05°38′37″S	47°44′19″W	4/10 (40)
		Piquizeiro	05°42′09″S	47°44′29″W	3/22 (14)
					7/32 (22)
Pará	Abaetetuba	Ajuaí**	01°45′29″S	49°03′25″W	6/18 (33)
		Genipauba	01°45′29″S	48°54′01″W	0/4
		Panacaueira	01°48′18″S	49°06′15″W	11/26 (42)
		Urban Area	01°42′58″S	48°51′30″W	38/149 (25)
					55/197 (28)
	Belém*	Jurunas	01°28′14″S	48°30′10″W	2/15 (13)
		Val de Cans	01°23′12″S	48°28′17″W	6/19 (32)
					8/34 (23)
	Cachoeira do Arari*[2]	Aranaí	01°05′42″S	48°39′39″W	2/4 (50)
		Furinho	01°05′14″S	48°39′07″W	0/2
		Mata Fome	01°04′15″S	48°37′50″W	1/5 (20)
		Sede Furo Grande	01°05′31″S	48°39′14″W	0/1
					3/12 (25)
	Curralinho*	São José da Povoação**	01°40′28″S	50°08′41″W	**8/9 (89)**
	Monte Alegre	Setor 11***	01°38′20″S	54°14′32″W	**37/77 (48)**
Pantanal (Control Area)					
Mato Grosso do Sul	Corumbá	Farms	19°34′29″S	56°14′44″W	**0/39**
					241/649 (37)

Footnotes
*Chagas disease outbreaks.
**Positive hemoculture.
***Trypomastigote forms in fresh blood preparations.
[1]-Data published in [22].
[2]-Data published in [2].
doi:10.1371/journal.pntd.0001647.t001

while Monte Alegre is located in the lower Amazon mesoregion of Pará. The common climate is characterized as tropical humid, with regular rainfall and winds, and temperatures between 27°C and 36°C. The area is known as varzean, which is a freshwater swamp forest. 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 the river banks. The municipalities of Augustinópolis, Axixá do Tocantins (ACD outbreak in 2009) and Esperantina are located in the northwestern mesoregion of the Tocantins state,

almost at the border of Pará. The climate of these cities is tropical subhumid, with maximum temperatures occurring during the dry season that reach 39°C.

Pantanal biome (control area)

This region presents an enzootic cycle of *T. cruzi* transmission; however, it is not considered an endemic area for Chagas disease, as human cases have never been recorded in the region. This region comprises a large natural environment with a multiplicity of habitats and a wide variety of biodiversity. Farms encompass an area located in the core of a biodiversity corridor in the Pantanal of Mato Grosso do Sul, Brazil.

Wild mammals

The capture of small wild and synanthropic mammals was performed as follows: live traps were arranged in linear transects, and the capture points were established with Tomahawk (Tomahawk Live Traps, Tomahawk, WI) and Sherman (H. B. Sherman Traps, Tallahassee, FL) traps. The traps were baited with a mixture of peanut butter, banana, oat and bacon and set at 20-m intervals in all types of vegetation formations and habitats. The trapped animals were taken to a field laboratory ≤2 km from the capture point, where the remaining procedures were performed. The trapped animals were examined for the prevalence and pattern of *T. cruzi* infection, as previously described [2,9,28,29]. Some data (from the municipalities of João Costa, Cachoeira do Arari, Redenção) used in this meta-analysis comprise both already published studies [2,9,22] and some unpublished data (from the municipalities of Jaguaruana, Russas, Abaetetuba, Belém, Monte Alegre, Curralinho, Axixá do Tocantins, Augustinópolis and Esperantina) were collected by our laboratory. The sampling efforts to capture mammals were similar in all 28 localities (820-1.100 traps/night), with 4 or 5 nights of capture each season, and the captures were performed in every season of the year (Table S1 and Figure S1).

Selection of dogs

The active search for dogs was conducted in the houses neighboring the linear transects where the small wild and synanthropic mammals were captured and in the houses where oral outbreaks of Chagas disease had occurred. Blood samples were collected from 649 dogs living in houses located in twelve municipalities from four Brazilian states. Dog blood was collected in three biomes; collections from the Caatinga (n = 188) and Amazon (n = 422) biomes were compared to collections from the Pantanal biome (n = 39) (Table 1).

Parasitological and serological surveys

Herein, we considered that each dog represents one single event, even when related to the same house. This is due to: (i) dogs are separated individuals, differing each other in age, behavior, activities, etc… This fact is reflected in their different degrees of *T. cruzi* exposition; (ii) dogs have no pack behavior; and (iii) dogs are not confined in the intradomociliar area and have different and multiple opportunities to be infected during their activities. The interpretation of our results was based on different patterns of infection of the mammals. Fresh blood smears and hemoculture, when positive (especially the first due to its lower sensibility), show a high parasite load in the peripheral blood of the animals, which means a high chance of transmission to the vector, reflecting transmissibility. These tests are very specific but less sensitive – their importance lies in detecting infected animals that may represent a source of infection for the vector. Serological assays indicate infection of the animal. Therefore, serological positive and parasitological negative tests for a given animal demonstrates its infection with a low rate of parasite, this mammal is a host of the parasite, but is not involved in the amplification of parasite populations, i.e., its transmission potential to the vector is low.

Blood was collected from dogs in heparinized vacutainer tubes by puncture of the cephalic vein. To evaluate *T. cruzi* infection, four tests were conducted. Two of these tests were parasitological assays including (i) the examination of fresh blood smears (microscopic analysis) and (ii) a hemoculture assay, in which 0.2–0.4 mL of blood was cultured in two tubes containing Novy-Mc Neal-Nicole medium [NNN] with a liver infusion tryptose medium [LIT] overlay. When those tests were positive, the parasites were amplified for cryopreservation and DNA extraction for molecular characterization and two serologic diagnostic assays were performed: (iii) the Indirect Immunofluorescence Antibody Test (IFAT) as previously described [30] and (iv) the Enzyme-Linked Immunosorbent Assay (ELISA, Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil). Disease diagnosis was based on serology by the ELISA (Cut-off: optical absorbance ≥0.200, mean ± 3 SD) and IFAT (Cut-off: titer of 1/40). Each microtiter polystyrene plate had 2 positive and 2 negative control sera. Animals were defined as seropositive when samples were reactive in both the IFAT and ELISA. Seropositive animals that displayed negative results in the parasitological assays were considered to have sub-patent infections. To evaluate possible cross-reactions and/or mixed infection by *T. cruzi* and *Leishmania* spp., dog sera were also assayed for *Leishmania infantum* using IFAT and the Rapid Test for Diagnosis of Canine Visceral Leishmaniasis (CVL) (TR DPP®, Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil). The IFAT cut-off value adopted for *T. cruzi* infection was 1/40 when the IFAT result for *L. infantum* was lower than 1/40 and the DPP results were negative. When infected by *L. infantum*, dogs were considered positive for *T. cruzi* infection only when the IFAT titer was 1/80 or higher. For *L. infantum* infection, the adopted IFAT cut-off value was 1/40 when the infection was also confirmed by DPP and 1/80 when the DPP assay was negative. The interpretation of these tests in assemblage indicates the role played by the tested mammals in the transmission cycle.

PCR amplification

DNA was extracted from logarithmic phase cultures and serum samples of dogs with patent parasitemia (positive blood slide smears) in the absence of hemocultivated parasites, using a phenol chloroform protocol [31]. PCR was performed using the primer pair S35 (5′-AAATAATGTACGGGGGAGATGCATGA-3′) and S36 (5′-GGGTTCGATTGGGGTTGGTGT-3′) [31]. Cyclic amplifications were performed with an initial denaturation of five minutes at 94°C, followed by 35 amplification cycles (94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds) and a final ten-minute elongation step at 72°C. Each 25 µL total reaction volume contained 25 ng total DNA, 10 ρmol of each primer, 0.4 mM dNTPs, 2 mM $MgCl_2$, and 2.5 U Taq polymerase (AmpliTaq®Gold, Applied Biosystems). PCR products were visualized on a 2% agarose gel after ethidium bromide staining. PCR resulted in a 330-bp amplicon for *T. cruzi* and a 760-bp amplicon and heterogeneous set of fragments ranging in size from 300 to 450 bp for *T. rangeli*.

Geospatial analysis

The base map was acquired from the IBGE (Brazilian Institute of Geography and Statistics). The coordinates of all biological data were captured using a hand-held GPS (Global Positioning System) receiver (Garmim III GPS, Garmin International, Olathe, KS,

USA) and recorded in the WGS 84 Datum (World Geodetic System 1984) geodetic coordinate system.

Maps representing the spatial distribution of *T. cruzi* infected dogs (response variable) and species richness, abundance and parasitological and serological prevalence of wild small mammals (covariables) were generated using the interpolation method of Inverse Distance Weighted (IDW) with the 12 nearest sampled data points selected. However, for map analysis, only the polygon of the studied municipality, (*i.e.*, only a local analysis) was used. A variable radius was applied specifying the number of nearest input sample points (n = 12) to perform interpolation. After that, we used map algebra to find evidence of spatial correlation of the response variable with each covariable by the use of arithmetic operators (subtraction). The algebraic analysis of maps (spatial variables, response variable and covariables), represented by pixels, results a new map where the values in each geographical position was the result of subtraction (in our case) of the values of the variables associated with the geographical position.

The term "map algebra" was established by Dana Tomlin in the early 1980s [32] with the development of the "Map Analysis Package GIS". Map algebra provides tools to perform spatial analysis operations and is based on a matrix algebra, which refers to the algebraic manipulation of matrices (as maps in raster data structures). Spatial data were analyzed in a GIS platform using ArcGis 9.3 software (Environmental Systems Research Institute, Redlands, CA, USA).

Statistical analysis

For the analysis of the proposed hypothesis (small wild mammal lower richness is associated with the increase of prevalence of *T. cruzi* infected dogs), we used the GLMs (Generalized Linear Models) with a Poisson link function [33]. For the response variable we used the infection of the dogs (based on serological assays – IFAT and ELISA, as described above). The following covariables were included: (1) Species richness of small wild mammals collected (DS): The richness was calculated as being the number of species captured in each area; (2) Abundance of small wild mammals collected (NM): The abundance for each localities was based on: n = total number of mammals captured. In the present model, aiming to evaluate the influence of both parameters (normally associated in ecological studies), these two covariables (DS*NM) were considered together and estimated by the "manual" selection method; (3) Prevalence of small wild mammals with positive *T. cruzi* parasitological assays (THC): That included (i) the finding of flagellates with typical *T. cruzi* morphology in fresh blood examination and (ii) the isolation and characterization of *T. cruzi* from blood in axenic medium – hemoculture; (4) Prevalence of small wild mammals with positive *T. cruzi* serological assay: based on the detection of specific anti-*T. cruzi* antibodies in the IFAT. The criterion of comparison between the models was based on the Akaike Information Criterion (AIC) and residual deviance [34,35] to determine which model fits best considering the level of significance ($p<0.05$). For the analysis of normality, the Shapiro-Wilk normality test was performed. Each model is specified as a combination of covariables that can influence the probabilities of dogs becoming infected. The comparison between dogs from Chagas disease-outbreak and non-outbreak areas were calculated using 2×2 contingency tables along with a Chi-squared test. Each dog was considered as one independent event, even when living in the same house. Both analyses were performed using the software R (Version 2.11.1) [36].

Owner consent and protocol of ethical treatment of animals

All wild animal manipulation procedures were performed in accordance with the 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 field workers who manipulated animals directly were adequately dressed with protective equipment, following protocols previously approved by the CEUA-FIOCRUZ Committees of Biosafety and of Bioethics (licenses: P0007-99; P0179-03; L0015-07; P0292/06). The wild animal captures were licensed by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) licenses 068-2005 and 225-2006 (IBAMA/CGFAU/LIC). In all cases, consent from the dog owners was obtained. In addition, the owners also helped handle the animals during sampling to avoid incidents. A canine standard questionnaire was applied. For each dog, the questionnaire included name, sex, age, size, color and main phenotypic features, birthplace, age at which the pet entered the house, the dog's main function and movement areas.

Results

Parasitological and molecular characterization

Trypanosoma cruzi infection in dogs was sub-patent in the majority of the cases. Only five dogs from Monte Alegre displayed trypomastigote forms in fresh blood preparations (Table 1). These dogs with patent parasitemia displayed severe clinical symptoms (fever, pale mucous membranes, generalized edema, rigid abdomen and splenomegaly), and the disease was fatal for two of them. Hemocultures performed in the three dogs surviving two months after the first blood collection were negative; these dogs produced Chagas negative hemocultures 3 months after the first blood collection. Positive hemocultures were detected only in two dogs, one from Abaetetuba and one from Curralinho. From one of these two dogs, it was possible to isolate the *T. cruzi* on two occasions after an interval of seven months (Abaetetuba). Molecular characterization was performed on these isolates and on five serum samples of dogs from Monte Alegre. *T. cruzi* k-DNA was amplified from these six dogs, from both serum and culture samples, with the exception of one dog from Curralinho with *Trypanosoma rangeli* (Figure 2 and Table 1).

Serological data

Overall, the distribution of *T. cruzi* infection in dogs was not homogeneous among houses, localities, municipalities and/or biomes (Figure 3), as shown by the high variation in *T. cruzi* prevalence in dogs in the different municipalities (11–89%, Table 1). Within the same biome, municipalities with high and low *T. cruzi* seroprevalence in dogs were observed. In the Jaguaruana and João Costa municipalities located in the Caatinga biome, dogs displayed 71% and 11% seropositivity, respectively. Similar differences in dog seroprevalence were also observed in distinct localities in the Amazon biome (22–89%). Even within the same municipality, seroprevalence in dogs was not homogeneous. In Abaetetuba (Amazonia biome), the seroprevalence in dogs ranged from 0 to 42% according to the locality. In the Pantanal, where Chagas disease is not reported, all dogs serologically examined were negative for *T. cruzi* infection (n = 39) (Table 1). Each dog was considered as one independent event, even when living in the same house due do the fact that different seroprevalence rates could be observed in dogs of one same house. The not homogeneous distribution of *T. cruzi* seropreva-

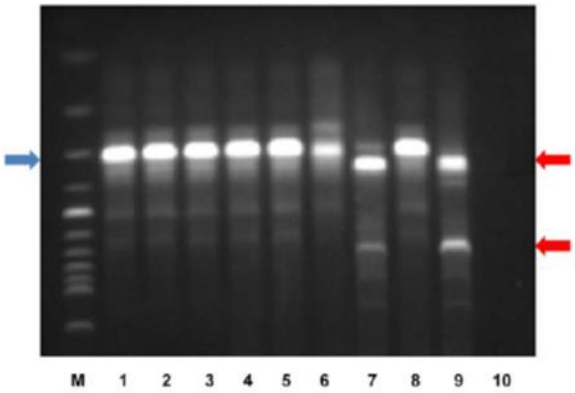

Figure 2. *Trypanosoma* **sp. characterization in blood samples from dogs from Pará State.** Parasite identification targeting the variable region of minicircle kDNA, **Lanes 1–5** DNA extracted from serum samples of dogs designated LBT1818, LBT1819, LBT1820, LBT1821 and LBT1822, respectively; **lane 6**: FNS258; **lane 7** LBT1831; **lane 8** *T. cruzi* positive control; **lane 9** *T. rangeli* positive control; **lane 10** negative control; **lane M**: 100-bp DNA ladder. **Red arrows**: 760 bp fragment and fragments varying in size from 300 to 450 bp derived from *T. rangeli* minicircles; **Blue arrow**: 330-bp fragment derived from *T. cruzi* minicircles.
doi:10.1371/journal.pntd.0001647.g002

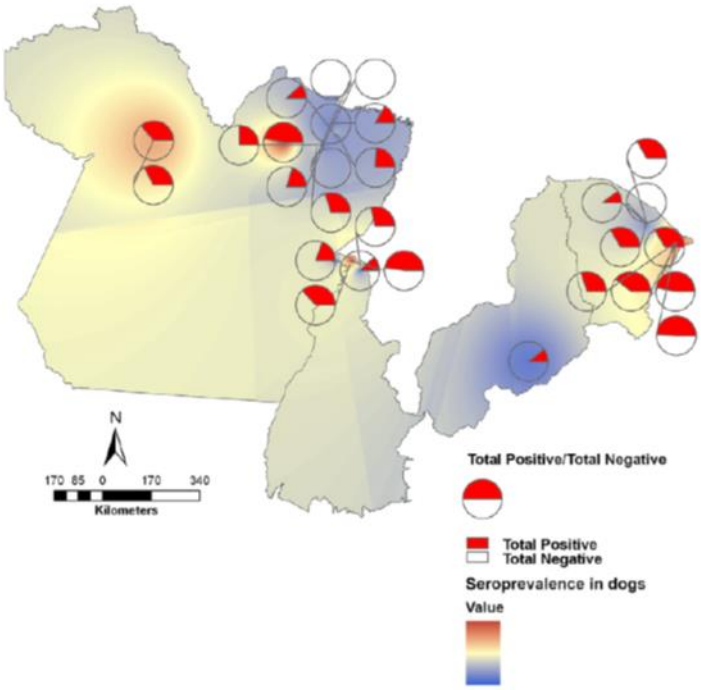

Figure 3. Mapping of distribution of *Trypanosoma cruzi* **infection in dogs (municipalities).** Distribution of seroprevalence in dogs by the interpolation maps method (Inverse Distance Weighted - IDW): Total positive/Total tested.
doi:10.1371/journal.pntd.0001647.g003

lence in dogs of one single house reflect that these dogs have not been equally exposed to *T. cruzi* infection.

Co-infection with *Leishmania infantum*

Co-infection was observed in 17% and 16% of sera from dogs examined in the Caatinga and Amazon biomes, respectively, as evaluated by the IFAT and/or Quick Test for Diagnosis of Visceral Leishmaniasis (CVL) (TR DPP®, Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil), indicating co-infection by both parasite species.

Geospatial analysis

An analysis of the maps generated using the interpolation method indicated an inverse distribution correlation among *T. cruzi* infection in dogs and a decrease in the richness and abundance of small wild mammal species. This spatial correlation evidence was confirmed by map algebra and demonstrated that among the response variable and covariables there is also an inverse correlation, which indicated that in areas with greater richness and abundance of small mammal species, dogs were less prone to be infected with *T. cruzi*. Since the resulting map algebra was subtracted from inverse correlation with two variables our result shows a distribution that is not homogeneous. A more indirect indication was given by the rates of parasitological and serological *T. cruzi* prevalence in dogs (Figure 4A–D).

Statistical analysis

Statistical analysis confirmed the generated maps and demonstrated that the covariables DS (Species Richness) and THC (parasitological prevalence) influence the average response variable (*T. cruzi* infection in dogs), polygons of the studied municipalities. The estimated DS (−0.095596) indicates that in areas that present greater mammal biodiversity, dogs are less prone to infection by *T. cruzi*. The estimated TCH (0.009066) indicated that in areas that present higher parasitological prevalence of infection in the small wild mammals, dogs are more exposed to the *T. cruzi* infection. The estimated rate of *T. cruzi* infection in dogs was 0.909 ($CI_{95\%}$ 0.870–0.949) for DS and 1.009 ($CI_{95\%}$ 1.004–1.014) for TCH. The analysis of residuals versus fitted values indicated that the behavior of the variance of residuals and homoscedasticity presented random residuals. For confirmation of the normality of the data, the Shapiro-Wilk test was performed (W = 0.9814), $P = 0.8714$ at 5%. We found a significant difference in the *T. cruzi* infection rate between dogs sampled from areas that suffered a

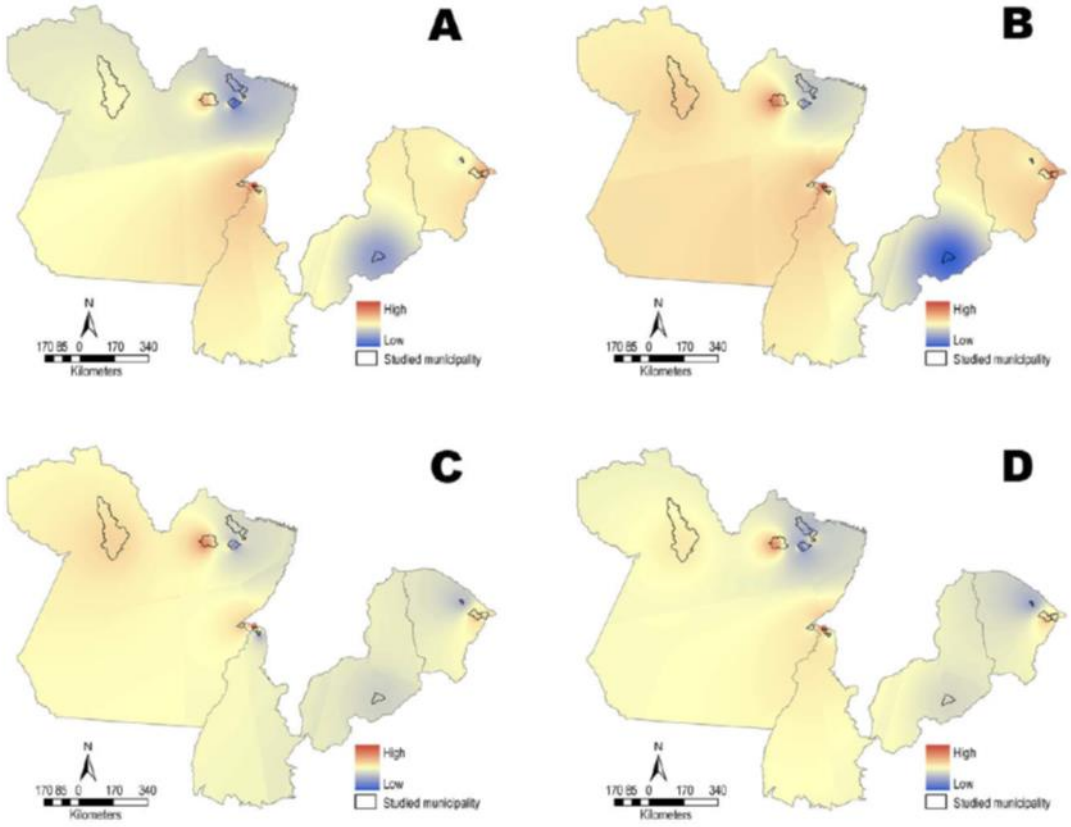

Figure 4. Mapping of lower richness of wild mammal species. A geospatial analysis by the map algebra of the association of *T. cruzi* infection in dogs (response variable) with covariables: (**A**) species richness (DS) of small wild mammals; (**B**) abundance (NM) of small wild mammals; (**C**) serological prevalence (IFAT) of small wild mammals; (**D**) parasitological prevalence (THC) of small wild mammals.
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Chagas disease outbreak compared to dogs from non-outbreak areas (28/103 versus 217/546, Chi-squared 5.81, $P=0.01$). In other words, this probability would rise 0.5 points in areas with Chagas disease outbreaks.

Discussion

The sustainability of successful control of Chagas disease requires a more accurate knowledge of the environmental factors that underlie the transmission cycle of this parasite in the wild, mainly, if there are still unknown and undetermined aspects of the current epidemiology of this trypanosomiasis. This demands multidisciplinary and complex studies, as *Trypanosoma cruzi* is a multihost parasite that displays a huge intraspecific heterogeneity and a complex transmission cycle that may exhibit local peculiarities. Oral transmission of *T. cruzi* to humans was reported as sporadic until 2004, but in the following years, this epidemiological profile of transmission became increasingly important in the epidemiology of Chagas disease, particularly in the Amazon region [4–6]. Outside this region, oral transmission has also been responsible for recent outbreaks of ACD in several Brazilian states, mainly in the North [2,3]. Such outbreaks have also been reported in other Latin American countries [37,38].

Our results indicate that infection by *T. cruzi* in dogs is not homogeneous but focal, as demonstrated by the differences in seroprevalence among close localities; these differences may be due to landscape features. The seroprevalence observed in dogs could be associated with their proximity to forest and rural areas and with the loss of richness and abundance and rates of infection of the small wild mammal fauna. One aspect that distinguishes the present and previous data of our group from other studies is the scarcity of the number of dogs that displayed positive hemocultures [17–19]. In fact, dogs in Brazil are apparently only rarely involved in the amplification of *T. cruzi* and seem to play a minor role in the dispersion of the parasite. Even the dogs from Monte Alegre/PA seem not be of epidemiological importance because hemocultures were negative 3 months after the detection of *T. cruzi* in their blood smears.

The importance of a host species as a reservoir of a vector-borne parasite mainly depends on its prevalence of infection, capacity to infect the vectors, and the rate of host-vector contact [39]. A possible consequence of a local simplification of the small wild mammal fauna, where generalist mammals are favored at the expense of specialist species, is an increase in the rate of infection among the remnant mammalian fauna when the selected species are competent reservoirs of *T. cruzi*. As a result, the parasite population increases in the area, favoring vector infection and exposure of dogs to parasites, as reflected by their seroprevalence. This scenario suggests that the assessment of potential disease risk factors requires detailed knowledge of local, site-specific conditions. The small wild mammalian fauna diversity plays an important role in the profile of the enzootic infection patterns in a given area, as shown by the high transmission focus described in a previous study [22].

Overall, despite many remaining questions, the current evidence indicates that preserving intact ecosystems and their endemic biodiversity should generally reduce the prevalence of infectious diseases [7,8].

The determination of the spatial distribution of the elements that compose the epidemiological chain of a parasitic disease is of pivotal importance for the determination of trends and risk evaluation. Moreover, it is worth mentioning that the attempts to control a given multihost parasite based on the control of one single vector or host species will always be insufficient because parasite transmission very rarely relies on a single system. The simplification of the mammalian host diversity, associated with an increase in the abundance of competent reservoir host species as described here is certainly one of the risk factors involved in the reemergence of Chagas disease [9]. Reduced disease risk with increasing host diversity is especially likely when pathogen transmission is frequency-dependent, and when pathogen transmission is greater within a species than between species, particularly when the most competent hosts are also relatively abundant and widespread [7].

Piauí and Ceará display similar patterns regarding the presence of a high density of naturally *T. cruzi* infected Triatominae, which are the main vectors in both regions. Our results demonstrate a high prevalence of *T. cruzi* infection in dogs from the Caatinga, as described in previous studies from our group [9,22]. Despite this high prevalence, no new Chagas disease cases of vectorial transmission have been observed there in the last decade [13,26]. This may reflect the effectiveness of the already long-lasting epidemiological surveillance campaigns exerted in these areas despite their lack of regularity. Local people are aware of the risk of disease and adopt local measures to avoid infection risk. Further, although dogs were exposed to the *T. cruzi* transmission cycle and are hosts of the parasite, they do not display high parasitemia (i.e., had negative hemocultures) and are therefore not involved in the amplification of parasite populations, so consequently, the potential for transmission from these dogs to the vector is low.

The high prevalence of seropositive dogs in the Amazon region can be attributed to the elevated rate of contact among these domestic animals and the wild environment and because the houses are practically located inside wild forest areas. In these areas, it is difficult to delimit the of peridomestic and wild areas, and many local inhabitants and dogs are involved in hunting activities. Empirical evidence indicates that habitat fragmentation can increase or decrease disease prevalence (and also *T. cruzi* infection among wild small mammals) within a host species, depending on the specific biology of the host–parasite relationship [28,40]. Another important factor that should be taken into account is the importance of the definition of risk area based on the characteristics of the micro-regional management of domestic animals that are sometimes reared in semi-extensive ways. In this case, these animals are more exposed to the wild cycle of transmission and this is reflected by a high prevalence of infection. The presence of seropositive dogs in strictly domiciled habitats, as observed in a previous study in Navegantes, in the state of Santa Catarina, indicates, for example, the presence of a transmission cycle very close to the animal's home [2]. Moreover, the high prevalence of infection in domestic mammals reared in a semi-extensive way (such as pigs from Cachoeira do Arari/PA) indicates that transmission is occurring farther from homes but within the areas of interface between the peridomestic and wild environments [16].

Surveillance for canine Chagas disease should be a useful tool for the design of suitable epidemiological control programs in areas where sylvatic triatomines are responsible for human infection, as in many rural endemic areas [17].

The geospatial analysis approach involving interpolation and the map algebra method are a powerful tool in the study of the association between lower richness and areas with high transmission rates in small wild mammals and the risk of exposure of dogs to *T. cruzi* infection. Dogs are important sentinels and efficient indicators of areas at risk for Chagas disease outbreaks, lower richness in wild mammalian fauna diversity and selection of suitable *T. cruzi* reservoir hosts.

Therefore, the monitoring of domestic animals can and should be used as a first measure in the diagnosis of areas with elevated risk of *T. cruzi* transmission. Dogs, in particular, are easy to handle and have a generally accessible traceability. The collection of blood from these hosts and serologic testing (the sending of material to a central diagnostic institute) does not require great cost and infrastructure. Moreover, blood samples can be easily obtained in areas where dogs are routinely collected and tested for *Leishmania* sp. Or the anti-rabies vaccination campaigns can be used to collect blood from a representative sample of dogs in a given area. The presence of seropositive dogs reflects exposure to *T. cruzi* and points to the transmission of the parasite in areas where these animals roam. Once this measure is implemented, we should have an efficient indicator of areas at risk for human Chagas disease that require particular epidemiological investigation, implementation of control measures and health education.

Supporting Information

Figure S1 Mapping of distribution of parasitological prevalence, richness and abundance of small wild mammals. Geospatial analysis showed that the lower richness of the mammal fauna (richness and abundance) was associated with higher parasitemia in small wild mammals and higher exposition of dogs to infection.
(TIF)

Table S1 Prevalence of infection by *Trypanosoma cruzi* in small wild mammals. Caatinga, Amazon Forest and Pantanal. Richness indicates the number of species captured in each area; Prevalence of small wild mammals with positive *T. cruzi* parasitological assays includes mammals that displayed: flagellates with typical *T. cruzi* morphology in fresh blood examination and/or positive hemoculture, *i.e.*, isolation and characterization of *T. cruzi* from blood in axenic medium; Prevalence of small wild mammals with positive *T. cruzi* serological assay was based on the detection of specific anti-*T. cruzi* antibodies in the IFAT.
(DOC)

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Author Contributions

Conceived and designed the experiments: SCCX LFCFS AMJ. Performed the experiments: SCCX VSL KJLM. Analyzed the data: SCCX ALRR VSL JCRO LFCFS AMJ. Contributed reagents/materials/analysis tools: LFCFS AMJ. Wrote the paper: SCCX ALRR AMJ. Participated in data collection: ALRR AMJ SCCX.

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ANEXO 4 – Artigo: *Trypanosoma cruzi* TcI and TcII transmission among wild carnivores, small mammals and dogs in a conservation unit and surrounding areas, Brazil. *Parasitology (2013), 140: 160–170.*

Trypanosoma cruzi TcI and TcII transmission among wild carnivores, small mammals and dogs in a conservation unit and surrounding areas, Brazil

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SUMMARY

Aiming to better understand the ecological aspects of *Trypanosoma cruzi* transmission cycles, wild carnivores, small mammals and dogs were examined for *T. cruzi* infection in the Serra da Canastra National Park region, Brazil. Isolates were genotyped using mini-exon gene and PCR-RFLP (1f8 and H3) genomic targets. *Trypanosoma cruzi* transmission was well established in the area and occurred in both wild and peridomestic environments. Dog seroprevalence was 29·4% (63/214) and TcI and TcII genotypes, besides mixed infections were observed. Only TcI was detected in wild mammals. Marsupials displayed lower relative abundance, but a high prevalence of positive haemocultures (4/22), whereas rodents displayed positive haemocultures (9/113) mainly in the abundant *Akodon montensis* and *Cerradomys subflavus* species. The felid *Leopardus pardalis* was the only carnivore to display positive haemoculture and was captured in the same region where the small mammal prevalence of *T. cruzi* infection was high. Two canid species, *Chrysocyon brachyurus* and *Cerdocyon thous*, were serologically positive for *T. cruzi* infection (4/8 and 8/39, respectively), probably related to their capacity to exploit different ecological niches. Herein, dog infection not only signals *T. cruzi* transmission but also the genotypes present. Distinct transmission strategies of the *T. cruzi* genotypes are discussed.

Key words: transmission cycles, trophic network, reservoir, Discrete Typing Units, Chagas disease, Serra da Canastra National Park, Brazil.

INTRODUCTION

The aetiological agent of Chagas disease, *Trypanosoma cruzi,* is a multi-host parasite found in more than 100 mammalian species and capable of infecting almost all cell types (Noireau *et al*. 2009). Human infections have been generally associated with contact with the contaminated feces of infected triatomine bugs, besides blood transfusion, organ transplantations, congenital transmission and oral transmission. Indeed, this latter has been responsible for the most recent outbreaks in Brazil and is probably the most ancient route of infection among wild animals (Noireau *et al*. 2009, Shikanai-Yasuda and Carvalho, 2012). The continually new human cases demonstrate that numerous aspects of Chagas disease epidemiology still remain unclear, probably because the transmission cycles of the parasite are maintained in intricate transmission networks that embrace several mammalian and vector species, resulting in unique epidemiological scenarios.

Trypanosoma cruzi is a highly diverse complex of genetic lineages. The current nomenclatural consensus recognizes 6 major genotypes or 'Discrete Typing Units' (DTUs) within the taxon, *T. cruzi* I (TcI) to *T. cruzi* VI (TcVI) (Zingales *et al*. 2009). To date, all of them occur in Brazil, although with different geographical distribution patterns and ecological characteristics. TcI is described to be an ubiquitous lineage in view of the diversity of its hosts, vectors and habitats. The TcII lineage is reported to have a more restricted geographical distribution and to

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occur in focal transmission cycles. However, it has been described in several mammalian taxa and biomes (Lisboa *et al*. 2006, 2008; Herrera *et al*. 2008), suggesting that it may be much more widespread than is currently acknowledged. TcIII is found mainly in the Amazonia biome, although sparsely reported throughout the country. TcIV has been recorded in northern and northeastern Brazil, whereas TcVI has been found in the middlewestern and southern regions (Zingales *et al*. 2012). TcV is described to occur in Argentina, Bolivia, Paraguay and in northeastern Brazil (Araujo *et al*. 2011). This broad distribution in distinct mammalian host species suggests that we are far from understanding the dispersion strategies of each lineage and its consequence for the epidemiology of *T. cruzi* infection.

The importance of each mammalian species in the maintenance and dispersion of a multi-host parasite like *T. cruzi* will rely mainly on the ability of the parasite to persist in the mammalian host and be transmitted to the vector, besides the host's relative abundance. In this sense, we consider as reservoir a species or community responsible for the long term survival of a parasite in a given area (Ashford, 1996), in which the role of each host species should be interpreted at intervals of time and space as well as accounting for the community composition and environmental characteristics. Thus, the importance of domestic dogs as reservoirs of *T. cruzi* varies throughout Latin America. In northwestern Argentina, dogs displaying high parasitaemias and infectiousness to vectors for long periods have been recorded (Gurtler *et al*. 2007), whereas in most countries, including Brazil, dogs display high seroprevalence but rarely present high parasitaemia levels (Roque *et al*. 2008; Pineda *et al*. 2011; Xavier *et al*. 2012). With regard to small mammals, a considerable number of marsupial and rodent species have been found naturally infected (World Health Organization, 2002). From these, species of the opossum genus *Didelphis* have been generally pointed out as the main reservoir, mostly due to the fact that studies focused mainly on this synanthropic genus, thus discounting other mammal species that may compose the *T. cruzi* reservoir system within a given area.

Little is known about the role of carnivores in the *T. cruzi* transmission network, probably because the examination of free-ranging carnivores requires long-term and technically challenging studies. Data are available for 2 Procyonidae species, the raccoon (*Procyon lotor*) and the ring-tailed coati (*Nasua nasua*) (Herrera *et al*. 2008; Kribs-Zaleta, 2010), but for wild canid and felid species, the only available information is that they are exposed to the *T. cruzi* transmission cycles in different environments, as expressed by positive serological tests (Brown *et al*. 2010; Herrera *et al*. 2011). In essence, carnivores have great potential to be important reservoirs due to the high diversity in their ecological niches that might range from insectivorous to carnivorous diet in different forest strata and habitats (Nowak, 2005), favouring contact with different components of the *T. cruzi* transmission net. Besides, top predators can be bioaccumulators of parasites (Cleaveland *et al*. 2006), and this may be also the case for *T. cruzi*, since the oral transmission is a highly efficient route for this parasite. Along with their huge biomass and broad home range, these characteristics give them a great potential to amplify and spread the parasite populations.

The Serra da Canastra National Park (SCNP) is a natural landscape conservation unit in Minas Gerais state, one of the oldest known endemic areas for Chagas disease in Brazil. Herein, the aim of this study was to evaluate *T. cruzi* transmission in both peridomestic and sylvatic environments in the SCNP region. The role played by the different components of the *T. cruzi* reservoir net: wild carnivores, small mammals and sympatric domestic dogs and the maintenance of distinct *T. cruzi* lineages in the area are discussed.

MATERIALS AND METHODS

Study area

The study was conducted within the Serra da Canastra National Park – SCNP (UTM 23K 345499/7764402) and adjacent areas, in Minas Gerais state, southeastern Brazil (Fig. 1). It is an important remnant of the Cerrado biome and shelters huge populations of some vulnerable mammalian species, such as the maned wolf (*Chrysocyon brachyurus*) and the giant anteater (*Myrmecophaga tridactyla*). Many streams and rivers, including the São Francisco river, originate in the highlands of the SCNP. The vegetation is basically made up of highland grasslands, with some spots of stone fields, scrub savanna and riparian vegetation occurring sparsely alongside the river courses. The altitude varies from 700 to 800 meters above sea level in valleys and above 1000 meters on the plateau. The climate is tropical, the dry season occurs from March to October and the wet season from November to February. Annual rain precipitation ranges from 1200 to 1800 mm and average temperature is around 22–23 °C (IBAMA. Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis, 2005).

The park was created in 1972 with a total area of 2000 ha of which only 715 ha are managed by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio), whereas the remaining areas are still privately owned. The park is surrounded by small rural properties (< 100 ha) whose economy is based on cattle ranching for artisanal cheese production and coffee plantations. Total rural population is 5500 inhabitants (Bizerril *et al*. 2011).

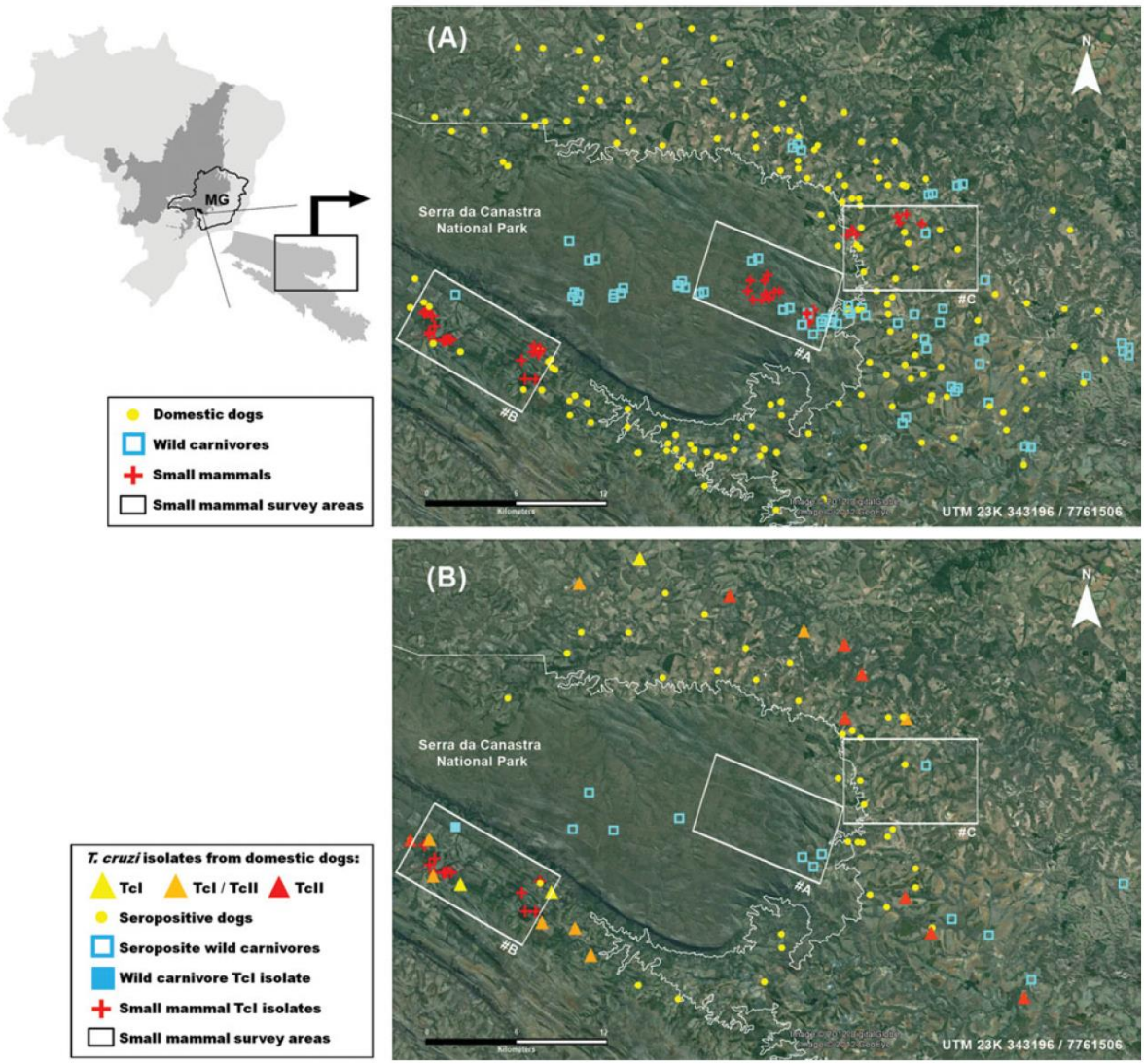

Fig. 1. Map of the spatial distribution of *Trypanosoma cruzi* infection in wild mammals from Serra da Canastra National Park (SCNP) and its surroundings. (A) All mammals sampled; (B) *T. cruzi* infected mammals. Triangles represent domestic dog isolates (genotypes according to the figure legend); blue squares represent infected wild carnivores and red crosses represent infected small mammals. The white squares indicate small mammal survey areas: #A – within SCNP, #B – Vão dos Cândidos region and #C – Cerradão/São Roque region. In the upper left figure the study site in Brazil, the grey shade corresponds to the limits of the Cerrado biome and the black contour shows Minas Gerais state (MG) limits.

Dog surveys

A house-to-house census of dogs was undertaken in farms located 5 to 20 km from the park border during annual rabies vaccination campaigns from 2007 to 2010. The annual average domestic dog population for the 4 years follow-up was 557 ± 59 individuals. After owner consent, blood samples were collected by puncture of the cephalic vein through a Vacutainer® system. Age class was based on the owner information and confirmed with dental condition status. We considered as juveniles dogs younger than 6 months and adults as the dogs older than that.

Our sample included 214 dogs, composed by 177 adults and 39 juveniles, ranging from 3 months to 14 years. The sex ratio was 3:1 (161 males/53 females). In the calculation of the prevalence of *T. cruzi* infection, resampled infected dogs were counted once.

The majority of adult dogs (71%) were used in cattle raising and slept outside the house. Owners also reported that their dogs hunt and that they go out by themselves for several consecutive days. The juvenile dogs were reported to be restricted only to peridomestic areas.

Capture of wild mammals

Wild carnivores. These were captured from March 2007 to August 2011 using box traps made with

galvanized wire mesh baited with sardine and boiled chicken. Traps were disposed both inside the park and on adjacent farmlands. We immobilized the animals with an intramuscular injection of a combination of zolazepan and tiletamine (Zoletil®) at dosages of 3 mg/kg for maned wolves, 8·3 mg/kg for ocelots (*Leopardus pardalis*) and 10 mg/kg for hoary foxes (*Lycalopex vetulus*) and crab-eating foxes (*Cerdocyon thous*). We also used a subcutaneous injection of 0·04 mg/kg of atropine sulphate, whenever necessary. Anaesthetized animals were weighed, measured and had their teeth condition assessed in order to estimate age and were marked with ear-tags or radiocollars for individual identification. We took blood samples by puncture of the cephalic vein stored in Vacutainer® tubes for haemoculture and serological tests. Animals were released at the site of capture after recovery from anaesthesia. Total capture effort was 3819 traps/night.

Small wild mammals. These were captured using live traps (Sherman® – H. B. Sherman Traps, Tallahassee, FL, USA and Tomahawk® Tomahawk Live Traps, Tomahawk, WI, USA) baited with a mixture of banana, peanut butter, oat, bacon/sardines. Traps were set for 5 consecutive nights along linear transects, placed on the ground at 10 m intervals and alternating between trap type, in 3 field expeditions (May 2010, February 2011 and August 2011). Traps were placed into distinct habitat types inside SCNP (gallery forest, stone fields, savanna, and grasslands) as well as in 2 vicinal regions under anthropogenic influence, 'São Roque/Cerradão' and 'Vão dos Cândidos'. The last region is within the official limits of the SCNP, but not managed as a conservation unit, since there are still several privately owned farmlands. Total capture effort was 3126 traps-nights, equally distributed among the 3 expeditions. We calculated the relative abundance of small mammals as the number of individuals of each species divided by the total number of individuals multiplied by 100. Identification of specimens was based on external and cranial morphological characters and on karyological analyses as described by Bonvicino *et al*. (2005). Voucher specimens were deposited in the Mammal Collection of the National Museum – UFRJ (Rio de Janeiro, Brazil). Blood samples were collected by cardiac puncture after anaesthesia with an intramuscular injection of ketamine (10–30 mg/kg) associated with acepromazine (5–10 mg/kg) for rodents (proportion 9:1) or xylazine (2 mg/kg) for marsupials (1:1).

All animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian government environmental agency (Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO license number 18635–3) and was endorsed by the ethics committee of the Oswaldo Cruz Institute/FIOCRUZ (CEUA P-292–06), in accordance to Brazilian regulations. Appropriate biosecurity techniques and individual protection equipment were used during all procedures involving animals and biological sample collecting and handling.

Trypanosoma cruzi *infection*

The *T. cruzi* infection survey was performed by parasitological and/or serological methods. If insufficient blood was collected, priority was given to haemoculture. Parasitological tests were based on examination of fresh blood smears (microscopic analysis) and haemoculture (HC), the latter performed as follows: 0·3 ml of blood from each animal was cultured in 2 tubes containing Novy-McNeal-Nicole (NNN) medium with liver infusion tryptose (LIT) overlay. Tubes were examined every 15 days up to 5 months. When positive, parasites were amplified, cryopreserved and deposited in the Collection of Trypanosomatids from wild mammals, domestic animals and vectors – COLTRYP (Fundação Oswaldo Cruz, Rio de Janeiro-RJ, Brazil).

For the detection of anti-*T. cruzi* IgG antibodies in sera we used the Indirect Fluorescent Antibody Test (IFAT) as previously described by Camargo (1966) and the Enzyme-Linked Immunoabsorbent Assay (ELISA, Biomanguinhos, Rio de Janeiro-RJ, Brazil). We also searched for IgM antibodies through the IFAT to identify recent *T. cruzi* infection among HC-positive dogs and other dogs from the same farms. The antigen used in serological assays for all species was obtained from a *T. cruzi* isolate derived from dogs of this study area harvested from axenic culture. For small mammals, rodent sera were tested with a commercial anti-rat IgG conjugate (FITC, Sigma-Aldrich®, St Louis, MO, USA), whereas marsupial sera were tested with an intermediary anti-opossum serum raised in rabbits followed by a commercial anti-rabbit conjugate. Wild canids and felids were tested using domestic dog and cat conjugates, respectively. The cut-off value titre adopted for IFAT was 1:40 for dogs and marsupials and 1:10 for rodents (Herrera *et al*. 2005). The cut-off value for ELISA was optical absorbance $\geqslant$ 0·200 mean $\pm$ 3 S.D. For wild carnivores, the test was performed with a non-specific conjugate and since there are no available data on IFAT cut-off values for some carnivore species of this study, we performed a PCR in all ELISA-positive serum samples besides a subsample of ELISA negative samples ($n = 5$). Therefore, the cut-off value adopted was 1:20, as it was the lowest serum dilution in which parasites could be detected by PCR. Each reaction included 2 positive and 2 negative control sera.

In order to detect mixed-infection and/or cross-reaction with *Leishmania* spp. we performed an

IFAT test using *L. infantum* and *L. braziliensis* parasites harvested from axenic culture as antigens (cut-off: 1:40). For wild and domestic canids, we also used the Rapid Test for Diagnosis of Canine Visceral Leishmaniasis (TR DPP®, BioManguinhos, Rio de Janeiro, Brazil).

Samples were considered positive when parasites were isolated by HC or when the sera showed reactivity in at least 2 of the serological tests. Samples that had IFAT-IgG concomitant positive values for *T. cruzi* and *Leishmania* sp. with non-corresponding ELISA, DPP or that displayed borderline results were attributed to cross-reaction and were considered indeterminate.

Trypanosoma cruzi *molecular characterization*

Genomic DNA was extracted from cultures and wild carnivore serum samples using standard phenol-chloroform protocols (Vallejo *et al*. 1999). Characterization was carried out in 3 steps: (1) multiplex PCR amplification of the mini-exon gene following conditions described by Fernandes *et al*. (2001) for the identification of 3 DTU *T. cruzi* groups: Tc1 (TcI – 200 basepairs), Tc2 (TcII/TcV/TcVI – 250 basepairs) and Zymodeme 3 (TcIII/TcIV- 150 basepairs), besides *T. rangeli* (100 basepairs) or mixed infections; (2) PCR amplification of nuclear 1f8 gene followed by restriction fragment length polymorphism (RFLP) analysis of fragments digested by Alw21I enzime (Rozas *et al*. 2007) to discriminate TcII from hybrids (TcV and TcVI) DTU's in isolates previously typed as Tc2 or mixed Tc1/Tc2 in mini-exon assays and (3) PCR-RFLP of histone H3/AluI (Westenberger *et al*. 2005) to rule out hybrids (TcV and TcVI) in mixed Tc1/Tc2 infections, as it could possibly overlap digested fragments in 1f8/Alw21I assay. Both PCR-RFLP 1f8/Alw21I and histone H3/AluI were performed with minor modifications in the conditions described by Rozas *et al*. (2007). Each reaction included a negative control and positive control samples from those *T. cruzi* strains representing the DTUs to be typed. PCR products were visualized in 2% agarose gel after ethidium bromide staining and visualized under ultraviolet light.

Spatial and statistical analyses

In order to verify the spatial distribution of trypanosomatid infection, locations of each individual captured were accessed through a hand-held GPS receiver using the WGS 84 Datum geodetic coordinate system. Locations were analysed in a Geographic Information System platform using GPS trackmakerPRO® software (Geostudio Tecnologia, Brazil) juxtaposed on a base map modified from Google earth® software (v. 6.2, Google Inc., USA)

To examine the distribution pattern of *T. cruzi*-infected dogs, the mean geographical distance was compared among infected dog locations ($n=76$) to the mean distance distribution across 10000 randomly assigned samples of the same size using R 2.13 software. Spatial autocorrelation of seropositive dogs was tested with Moran's I. Maps with discriminated locality of infected hosts and parasite genotypes were also prepared. Statistical tests were conducted with $\alpha=0{\cdot}05$.

The degree of concordance between IFAT-IgG and ELISA assays was assessed by the kappa statistic using SYSTAT 11 for Windows. To test for the influence of sex and age class on *T. cruzi* infection rates in dogs the 3-dimensional Chi-square contingency table was used. We also compared the *T. cruzi* infection among domestic dogs, carnivores, rodents and marsupials and applied a Chi square test to verify whether infection rate is independent of taxonomic group.

RESULTS

Trypanosoma cruzi *infection in dogs*

Dogs are included in a well-established *T. cruzi* transmission cycle in all the geographical regions surrounding the Serra da Canastra National Park, as demonstrated by the high parasitological and serological *T. cruzi* infection prevalence (Table 1, Fig 1).

Trypanosoma cruzi was isolated from the blood of 19 dogs (7·9%, $n=214$), collected on the same expedition (September/2010). Genotyping revealed the presence only of 2 main *T. cruzi* lineages: TcI ($n=3$) and TcII ($n=8$), besides mixed TcI/TcII infections ($n=8$) (Figs 1 and 2). After 5 months, we re-examined 10 out of the 19 dogs that previously displayed positive HC and none of them tested positive. Among the remaining HC-positive dogs, 6 died and 3 disappeared.

Prevalence was calculated considering the total number of examined dogs during the study. A total of 63 dogs (29·4%) were seropositive for *T. cruzi* (Table 1), including 8 (12·7%) individuals that were co-infected with *Leishmania* sp. Once infected, *T. cruzi*-positive testing dogs maintained serological titres, as observed during re-sampling. Seventeen dog samples were considered indeterminate by the serological assays. We found no significant difference in the *T. cruzi* infection rate between the dogs' sex or age class (Chi square = 2·69; D.F. = 7; $P=0{\cdot}9$). The agreement between IFAT-IgG and ELISA was 78% with a kappa value of 0·522 (moderate agreement). ELISA presented higher sensitivity to detect the dog's acute phase since it was positive in 15 out of 19 HC-positive dogs, whereas IFAT detected infection in only 4 of them.

Six juvenile dogs (3–6 months old) displayed positive HC, demonstrating that *T. cruzi*

Table 1. *Trypanosoma cruzi* infection assessment through serology (IgG – IFAT/ELISA) and haemoculture (HC) in mammals from the Serra da Canastra National Park and surrounding areas, Brazil

Species (common name)	*N*	Capture location[1]	Small mammals relative abundance (%)[2]	Serology (Positive/*N*)	HC (Positive/*N*)	Genotype
Akodon montensis	27	a,b	20·0	0/17	1/27	TcI
Akodon spp.[3]	13	a,b	9·6	0/5	1/13	TcI
Calomys spp.[4]	14	b	10·4	0/3	3/14	TcI
Cerradomys subflavus	15	a,b	11·1	0/13	4/15	TcI
Necromys lasiurus	28	a,b	20·7	0/21	0/28	–
Nectomys squamipes	3	a,b	2·2	0/3	0/3	–
Oligoryzomys spp.[5]	4	a,b	3·0	0/2	0/4	–
Oxymycterus delator	9	a,b	6·7	1/8	0/9	–
Total rodents	113		83·7	1/72 (1·4%)	9/113 (7·9%)	–
Caluromys philander	1	b	0·7	0/0	1/1	TcI
Didelphis albiventris	4	a,b	3·0	0/4	0/4	–
Gracilinanus agilis	4	a	3·0	0/2	0/4	–
Lutreolina crassicaudata	1	a	0·7	0/1	0/1	–
Marmosops incanus	5	b	3·7	4/4	3/5	TcI
Monodelphis spp.[6]	7	a,b	5·2	2/5	0/7	–
Total marsupials	22		16·3	6/16 (37·5%)	4/22 (18·1%)	–
Chrysocyon brachyurus (Maned wolf)	39	a,b	–	8/39	0/30	–
Cerdocyon thous (Crab-eating fox)	8	a,b	–	4/8	0/3	–
Lycalopex vetulus (Hoary fox)	10	a,b	–	0/10	0/6	–
Leopardus pardalis (Ocelot)	1	b	–	1/1	1/1	TcI
Conepatus semistriatus (Skunk)	2	a,b	–	0/0	0/2	–
Total wild carnivores	60		–	13/58 (22·4%)	1/42 (2·4%)	–
Canis lupus familiaris (Dog)	214		–	63/214 (29·4%)	19/214 (7·9%)	TcI (3) TcII (8) TcI-TcII (8)

[1] Capture location site: a, SCNP; b, Farmlands; (–), not applicable.
[2] Number of individuals of each species divided by the total number of individuals * 100.
[3] *Akodon* sp. (*n* = 6; a, b), *A. lindberghi* (*n* = 5; a), *A. cursor* (*n* = 1; a).
[4] *Calomys* sp. (*n* = 3; b) *C. tener* (*n* = 11; b).
[5] *Oligoryzomys* sp. (n = 2, b), *O. nigripes* (*n* = 1; a), *O. rupestris* (*n* = 1; a).
[6] *Monodelphis americana* (*n* = 3; b), *M. domestica* (*n* = 3; b) and *M. sorex* (*n* = 1; a).

transmission also occurs within the peridomestic environment. An active transmission in that region was confirmed by, among other factors, the serological conversion observed in 4 dogs 1 year after the first examination and the 4 HC-positive dogs that displayed concomitant positive IFAT-IgM, indicative of recent infection. Further, 28·3% (*n* = 46) of the dogs amid farms with HC-positive dogs had IFAT-IgM antibodies (corroborated by ELISA), suggestive of recent infection in this scenario. The *T. cruzi* infection in dogs was spatially autocorrelated (Moran's I: observed = 0·674; expected = – 0·004; S.D. = 0·076; *P*-value = 0) and not homogeneously distributed ($P < 0{\cdot}004$), suggesting that transmission occurred throughout the SCNP surrounding areas, though with hotspot transmission foci (Fig. 1, and see Supplementary Material, online version only).

Trypanosoma cruzi *infection in wild mammals*

A total of 60 wild carnivores belonging to 5 species were examined for *T. cruzi* infection. The only felid species examined, the ocelot, tested positive both in fresh blood preparations and at the first HC reading (7 days after blood culture) along with an elevated serology titre (1:160); altogether indicative of high parasitaemia levels. The ocelot's *T. cruzi* isolate was genotyped as TcI (Table 1).

The wild canids were exposed to infection as demonstrated by serology but none were parasitologically positive by HC suggesting they may not be infective to vectors. The crab-eating fox had the highest serum prevalence rate (50% – 4/8) followed by the maned wolf (20·5% – 8/39) (Table 1). Titres ranged from 1:20 to 1:80 (IFAT). All hoary fox samples were seronegative. Three (5·8%) maned wolf

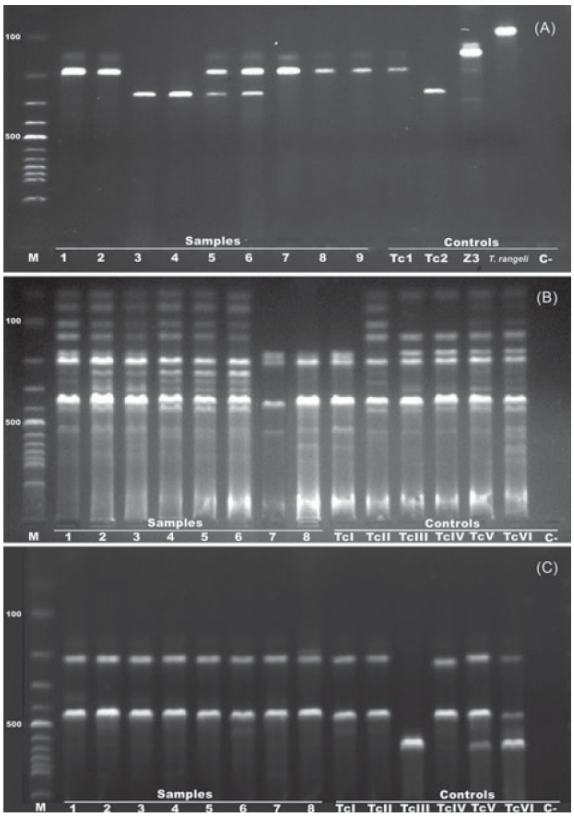

Fig. 2. *Trypanosoma cruzi* genotyping of domestic and wild mammal isolates from the Serra da Canastra National Park and surroundings, Brazil. (A) PCR products of the Mini-exon gene analysed by agarose electrophoresis gel stained with ethidium bromide. Lanes: M, Molecular weight markers (100 bp DNA ladder); 1–6, dog isolates; 7, wild carnivore isolate (*Leopardus pardalis*); 8, rodent isolate (*Akodon montensis*); 9, marsupial isolate (*Marmosops incanus*); Control samples: Tc1 (TcI – 200 bp), Tc2 (TcII/TcV/TcVI – 250 bp), Z3 (TcIII/TcIV – 150 bp), *T. rangeli* (100 bp). (B) PCR-RFLP products of 1f8 gene/Alw21I. Lanes: 1–3, dog isolates characterized as mixed Tc1/Tc2 in Mini-exon gene assay; 4–6, dog isolates characterized as Tc2; 7–8, dog isolates characterized as Tc1. Control samples: PCR-RFLP 1f8/Alw21I digestion patterns of TcI to TcVI. (C) PCR-RFLP products of histone H3/AluI. Lanes: 1–8, dog isolates characterized as mixed Tc1/Tc2 infection in Mini-exon gene assay. Control samples: PCR-RFLP H3/AluI digestion patterns of TcI to TcVI.

samples were considered indeterminate. The agreement between IFAT-IgG and ELISA was 82% with a kappa value of 0·628 (substantial agreement). The 2 specimens of the skunk (*Conepatus semistriatus*) examined only by HC tested negative.

Concerning the small mammals, relative abundances and fauna richness were comparable inside SCNP and farmlands (Table 1). The marsupials displayed high parasitaemia levels, mainly *Marmosops incanus* since 3 out of 4 seropositive individuals were positive on both fresh blood examination and HC. Positive HC was also achieved from the only captured *Caluromys philander*. Infection by IFAT was detected in the marsupial species *Marmosops incanus*, *Monodelphis americana* and *Monodelphis domestica* (prevalence of 37·5%), titres ranged from 1:40 to 1:320. Rodents from *Akodon*, *Calomys* and *Cerradomys* species also presented parasitaemias detected by HC in 9 individuals (Table 1). Of these positive rodents, we could collect sufficient blood to perform the IFAT assay from only 3 that tested negative. One *Oxymycterus delator* was the only seropositive sample (prevalence of 1·4%). The small mammal isolates were all characterized as TcI (Table 1, Fig. 1).

All *T. cruzi*-infected small mammals were collected in the 'Vão dos Cândidos' region as well as the infected ocelot (Fig. 1). The infected crab-eating foxes and 4 maned wolves were captured in farmland regions. Four serologically positive maned wolves displayed home range areas exclusively inside the SCNP, as confirmed by our parallel observations using radio telemetry techniques (May-Junior *et al.* 2009).

Comparing the 2 farmland areas, in the 'Vão dos Cândidos' region both small mammals and dogs presented high prevalence of positive HC (13/65–20% and 6/25–24%, respectively) whereas in the 'Cerradão/São Roque' region, neither small mammals ($n=7$) nor dogs ($n=21$) displayed positive HC. Overall, the proportion of *T. cruzi* infection varied significantly in different taxonomic groups. Domestic dogs tended to have more positive diagnoses than predicted by chance, whereas rodents had fewer positive diagnoses (Chi square = 21·56; D.F. = 3; $P<0·001$).

DISCUSSION

In the present study we describe a well-established and broadly distributed *T. cruzi* cycle in all geographical regions surrounding SCNP, which includes wild and domestic animals. The presence of dogs with high parasitaemia, as demonstrated by positive haemoculture (HC), contrasts with previous studies in Brazil, in which none or a minority of individuals displayed positive HC (Herrera *et al.* 2005; Roque *et al.* 2008; Xavier *et al.* 2012). The herein positive HC dogs reflect probably the initial phase of infection, typically characterized by a short period of detectable parasitaemia (Machado *et al.* 2001). A similar pattern in naturally infected dogs was observed in Monte Alegre, in the state of Pará, in Northern Brazil (Xavier *et al.* 2012). Besides, serological conversion observed during the follow up attested that they are continually being exposed to *T. cruzi* infections.

Dogs may become infected by the contaminative route (Gurtler *et al.* 2007) or by ingesting infected triatomines, a highly efficient transmission route

(Pineda *et al*. 2011). Another possible infection route might be by hunting of infected small mammals (Herrera *et al*. 2011). Actually, with regards to the 2 areas where we simultaneously sampled for small mammals and dogs, dog infection was coincident with small mammal infection. In the SCNP, *T. cruzi* transmission might occur not only in the wild, but also in the peridomestic environment, as demonstrated by the infected juvenile dogs. Herein, besides acting as sentinels hosts, as already described in other Brazilian areas (Xavier *et al*. 2012), dogs were also able to signal the presence of the 2 main *T. cruzi* genotypes in the area, TcI and TcII.

In the SCNP, the huge distance among the infected dogs along with the fact that the foci of peridomiciliary vectors are residual and submitted to regular spraying rule out the existence of a *T. cruzi* transmission cycle supported solely by dogs. Thus, the finding of TcII genotype infecting only dogs, contrasting with TcI which was also found infecting wild mammals is an apparent paradox. At a first glance the explanation could rely on TcII circulation exclusively in mammalian groups not sampled in this study, such as armadillos and bats (Yeo *et al*. 2005; Lisboa *et al*. 2008). However, this seems an oversimplified explanation; a similar picture was observed in a Chagas Disease outbreak in Santa Catarina state, where TcII was found in humans and triatomines but not in the mammalian fauna (Roque *et al*. 2008; Steindel *et al*. 2008). Moreover, reports of TcII in different wild mammal species and biomes demonstrate that this genotype is also maintained in wild cycles (Lisboa *et al*. 2006; Herrera *et al*. 2008). This raises the question on where the TcII is hidden in nature. This could reflect the transmission strategy of this genotype. In analogy with the r-k ecological selection theory, the parasitaemia curve of TcII within its mammal hosts resemble an r strategist – one precocious and short period of high parasitaemia (Andrade and Magalhaes, 1996) and ultimately would impact on the dispersion strategy of this genotype in the wild. This kind of strategy does not impede TcII transmission in the wild, but hampers parasite detection, as a consequence, it may be underestimated in nature. Further, we cannot rule out the oddities in host-parasite interactions. For instance, the golden lion tamarin (*Leontopithecus rosalia*) maintains long-lasting TcII infection with high prevalence of positive HC throughout (Lisboa *et al*. 2006), whereas the opossum *Didelphis aurita* can control and even eliminate TcII in experimental conditions (Jansen *et al*. 1991). Here, we report for the first time TcII isolation and molecular characterization in dogs from Brazil, a well-studied *T. cruzi* host. This reinforces the view that the spectrum of mammal host infected by this genotype is currently underestimated.

Undoubtedly, from our results, none is more puzzling than the high number of dogs in early stages of infection in broadly distributed and unlinked areas simultaneously. This raises the idea that still unknown variables must be involved in the dispersion of *T. cruzi* among several host species and that these variables are included in a broader phenomenon. For instance, only recently El Niño Southern Oscillation (ENSO) and similar phenomena have been taken into account to influence living organisms (Hanf *et al*. 2011). Whatever the cause, the frequent occurrence of unsolved questions in biological systems may be related to our limitation in analysing them detached from a linear, Cartesian focus. Parasitic transmission nets are clearly complex systems since they are essentially dynamic, multivariate, non-linear and unpredictable, rendering a reductionist and deterministic focus interpretation of this phenomenon worthless. Indeed focusing these phenomena in the light of the chaos theory could perhaps fill the several gaps in the current knowledge of this issue (Mazzocchi, 2008). Taking into account the presence of several dogs geographically separated and recently infected by *T. cruzi* may be a stochastic phenomenon, or the signal that the maximum transmission fitness was achieved in that moment; indeed, a feature described to be the characteristic of the edge of the chaos.

The role of each wild mammal species in the *T. cruzi* transmission networks will depend on the ability of the parasite to be transmitted to its vector, as well as the abundance and distribution of its mammal host species. In SCNP, rodents and marsupials were shown to be equally important for the maintenance of *T. cruzi*. The marsupials, regardless of their low relative abundance, displayed high prevalence of positive HC, in particular the arboreal *Marmosops incanus* and *Caluromys philander*. Rodents also displayed positive HC, mainly in highly abundant species, i.e. the terrestrial *Akodon montensis* and *Cerradomys subflavus*, pointing to the epidemiological importance of these mammals in the local transmission net. Moreover, *T. cruzi* infection was spread among terrestrial and arboreal mammals, demonstrating that parasite transmission was occurring in both strata. We observed high *T. cruzi* infection rates in the area despite the low relative abundance of the common *Didelphis* genus, generally described as one of the most important and competent mammalian reservoir of the parasite (Yeo *et al*. 2005). This emphasizes the characteristic dynamics of *T. cruzi* transmission cycles that should be examined as an unique ecological system.

Herein, we observed that 3 wild carnivore species, the ocelot, the crab-eating fox and the maned wolf were infected by *T. cruzi* in the SCNP region, but they probably play distinct roles as a result of their pattern of *T. cruzi* infection and peculiar ecological characteristics. The ocelot was the only carnivore that displayed patent parasitaemia (positive fresh blood examination and HC). To the best of our knowledge this is the first report of an ocelot naturally infected

with *T. cruzi*. In comparison to the other 2 carnivore species of this study, the ocelot is the one which better exemplifies the bioaccumulator role in a prey-predator chain since it has a more carnivorous diet, consuming mainly small mammalian prey, besides birds (Rocha-mendes *et al*. 2010). In fact, this infected ocelot was the only carnivore captured in the 'Vão dos Cândidos' region, where small mammals displayed high parasitaemia levels. Probably, *T. cruzi* infection in top predators with a more restricted carnivorous diet, such as the ocelot, is highly dependent on the prevalence of infection of the local mammal fauna that can be preyed, since infection by the contaminative route is less probable if we consider that this animal is nocturnal, very active and generally does not use dens (except during birthing). In the Pantanal, where small mammals had low infection rates, none of the 10 ocelots tested were positive in HC (Herrera *et al*. 2011).

The crab-eating fox seems to be highly exposed to *T. cruzi* infections, as demonstrated in this study and also in the Pantanal region of Brazil (Herrera *et al*. 2011). This might be related to its capacity to exploit different ecological niches. The crab-eating fox is known to be one of the most plastic carnivore species: it has an omnivorous diet – including insects and small mammals, opportunistic behaviour and is a habitat generalist (Juarez and Marinho, 2002). Also, it has great flexibility in the use of disturbed habitats (Michalski *et al*. 2006). These traits increase the probability of contact with a variety of components of the *T. cruzi* cycle pointing the crab-eating fox as a good sentinel for *T. cruzi* transmission areas.

The maned wolves were highly exposed and can also be considered a good sentinel for transmission. Accounting for its omnivorous diet and home range areas of 80 km^2 on average (Jacomo *et al*. 2009), this species can play a unique role that is to signal the transmission in large areas, in particular in wild environments which are generally difficult to access. This was the case of maned wolves from our study that signalled the *T. cruzi* transmission both inside SCNP and its surroundings. The distinct prevalence rates between them indicate that the transmission was occurring mostly outside of the conservation unit, given that only 16% (4/25) of the maned wolves that were captured and recaptured within the park area over the 5-year follow-up tested positive, whereas 28% (4/14) of the wolves from outside SCNP tested positive. Taken together with the finding that no other mammal captured inside the park was positive for *T. cruzi* infection, we can conclude that the *T. cruzi* cycle inside SCNP is less expressive than in its surroundings.

This study reports the current *T. cruzi* enzootic transmission in one of the oldest endemic areas for Chagas disease in Brazil. We surmise that the *T. cruzi* transmission is well established all around the SCNP region, and that this transmission includes 2 genotypes of the parasite: TcI and TcII. Therein, dogs, small mammals and carnivore species were shown to participate in the *T. cruzi* transmission net and parasite transmission was occurring in both arboreal and terrestrial strata, as well as in the peridomicile. An understanding of the peculiar characteristics of this net, as well as each host-parasite relationship, is the key to identify the risk of disease outbreaks. This is the first study to corroborate evidence that dogs can be used not only to report *T. cruzi* transmission areas but also the genotypes present in the area, which reinforces their role as sentinels for surveillance programmes. The observed *T. cruzi* eco-epidemiological profile should increase awareness of the necessity for continuous surveillance in order to prevent re-emergence of Chagas disease in this area.

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ANEXO 5 – Artigo: Trypanosoma cruzi among wild and domestic mammals in different areas of the Abaetetuba municipality (Pará Atate, Brazil), an endemic Chagas disease transmission area (2013). Veterinary parasitology 193:71-77

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Trypanosoma cruzi among wild and domestic mammals in different areas of the Abaetetuba municipality (Pará State, Brazil), an endemic Chagas disease transmission area

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ABSTRACT

The presence of acute Chagas disease (ACD) due to oral transmission is growing and expanding in several South American countries. Within the Amazon basin, the Abaetetuba municipality has been a site of recurrent cases spanning across distinct landscapes. Because Chagas disease is primarily a zoonotic infection, we compared the enzootic *Trypanosoma cruzi* transmission cycles in three different environmental areas of Abaetetuba to better understand this new epidemiological situation. *Philander opossum* was the most abundant mammalian species collected (38% of the collected mammals) with a *T. cruzi* prevalence of 57%, as determined by hemocultures. *Didelphis marsupialis* was abundant only in the area with the higher level of environmental disturbance (approximately 42%) and did not yield detectable parasitemia. Despite similarities observed in the composition of the small mammalian fauna and the prevalence of *T. cruzi* infection among the studied areas, the potential of these hosts to infect vectors differed significantly according to the degree of land use (with prevalences of 5%, 41%, and 64% in areas A3, A1 and A2, respectively). Domestic mammals were also found to be infected, and one canine *T. cruzi* isolate was obtained. Our data demonstrated that the transmission of *T. cruzi* in the Amazon basin is far more complex than had been previously taught and showed that the probability of humans and domestic mammals coming into contact with infected bugs can vary dramatically, even within the same municipality. The exposure of dogs to *T. cruzi* infection (indicated by positive serology) was the common feature among the studied localities, stressing the importance of selecting domestic mammals as sentinels in the identification of *T. cruzi* transmission hotspots.

1. Introduction

The classic picture of Chagas disease associated with *Triatoma infestans* in Brazil could be eliminated by routine insecticide spraying in campaigns associated with housing improvement – as was the case with the Southern Cone Initiative (Dias, 2007). The current epidemiological profile of Chagas disease, mainly based on oral transmission represents a new challenge for public authorities because previously employed control measures are not effective against this new phenomenon. The number of cases attributed to the oral infection route has recently increased in Brazil, particularly in the Amazon Basin, which was formerly considered free of infection (Aguilar et al., 2007; Pinto et al., 2009). In fact, Chagas disease is currently considered a foodborne illness (Pereira et al., 2009).

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Trypanosoma cruzi, the etiological agent of Chagas disease, is a multi-host parasite that is adaptable to hundreds of mammalian species that are found at all forest strata and canopy levels. Furthermore, the species displays marked heterogeneity, and six discrete typing units (DTUs) are currently recognized among *T. cruzi* isolates (Zingales et al., 2012). These traits result in distinct occurrences of *T. cruzi* transmission in the wild that depend on such factors as the composition of local fauna and the habitat characteristics (Jansen and Roque, 2010). Thus far, TcI, TcIII and TcIV are the unique DTUs known to circulate in the Amazon Basin (Póvoa et al., 1984; Marcili et al., 2009; Monteiro et al., 2010).

The Amazon Basin contains the greatest biodiversity in the world and comprises a mosaic of various faunas, habitats and biomes, in addition to culturally diverse human behavior (Betts et al., 2008). In the Brazilian Amazon region, the enzootic transmission of *T. cruzi* among wild mammals has been recognized for many decades (Deane, 1964; Lainson et al., 1979; Miles et al., 1981), but the first autochthonous case of Chagas disease was not described until 1969 (Shaw et al., 1969). This region was the source of 91% of the Brazilian cases of acute Chagas disease (ACD) reported from 2007 to 2010 (SVS-MS, 2011). Cases from the Amazon region are related to the ingestion of contaminated food (mainly açaí and bacaba fruit juices), hunting activities, the harvest of babaçu and piassaba palm, and transmission within homes from invading sylvatic bugs (Coura et al., 2002; Nobrega et al., 2009; Valente et al., 2009). The common factor among all of these distinct sources is human exposure to the wild transmission cycle resulting from the activities of humans in the natural environment.

Other than Belém (the capital of Pará State), the Abaetetuba municipality in the Amazon Basin is the municipality in which the majority of Brazilian ACD cases has occurred. According to the Secretary of Health from Pará State, 119 ACD cases were confirmed in Abaetetuba between 2006 and 2011. Within Abaetetuba, Chagas disease occurs frequently as outbreaks in a seasonal and recurrent fashion and is spread across distinct localities that include peripheral districts as well as preserved areas and regions that are intermittently or continuously exploited by humans. *T. cruzi* transmission in Abaetetuba depends on the maintenance and transmission of the parasite among the local wild mammalian hosts and is consistently associated with non-domiciliated bugs. Because Chagas disease is primarily a zoonotic infection, and considering the various characteristics of the habitats in which the *T. cruzi* transmission cycle occurs in Abaetetuba, the aim of the present study was to evaluate *T. cruzi* infection in domestic (dogs and pigs) and wild mammals from three areas with different ecological landscapes and different degrees of human interference and occupation. The risk of human exposure to the wild transmission cycle of *T. cruzi* is discussed for these distinct situations.

2. Materials and methods

2.1. Study areas

The Abaetetuba municipality is located in Pará State (S01°43′05″/W48°52′57″) and has an area of 1610 km^2 that includes more than 45 islands. The climate is tropical-humid with annual rainfall of 2000–2500 mm. The rainy period is from January to August, whereas October and November receives the lowest rainfall. The urban section of the municipality is surrounded by peripheral regions that display secondary vegetation and agricultural areas with several remaining patches of the original Amazonian forest. The remainder of the municipality is represented by islands composed of freshwater swamp forests (nestled between diverse riverbanks) and areas with original and secondary vegetation (Amazonian forest).

Three localities were selected according to a gradient of land-use based on a governmental land-use map (Fig. 1) and were classified as follows: (A1) the Genipaúba locality, which is a more preserved area with low human occupation density; the landscape is composed mainly of typical Amazonian forest surrounded by freshwater rivers with restricted areas of environmental disturbance; (A2) the Ajuaí locality, which is an area presenting sparse human habitation along a river bank; the landscape is composed of vegetation that is reminiscent of the Amazonian forest and is close to areas exploited for fruit harvest (mainly açaí) and subsistence plantations along small freshwater rivers; and (A3) peripheral districts with high human occupation densities in the urban section of the municipality; the landscape is composed of regions with dense human occupation surrounded by secondary vegetation in the periphery and contains areas that were exploited for açaí harvest and have been replaced by pastures and agriculture (Fig. 1). Expeditions were conducted to each locality twice: once during the wet season (April (A1 and A2) and August (A3)) and once during the dry (November (A1 and A2) and December (A3)) of 2008. Wild and domestic animals from each location were analyzed for *T. cruzi* infection.

2.2. Sample collection

Small mammals were collected with baited Tomahawk® (Tomahawk Live Traps, Tomahawk, WI, USA) and Sherman® (H. B. Sherman Traps, Tallahassee, FL, USA) traps distributed in linear transects, with capture points established 20 m apart from each other. The traps were placed on the ground and in the understory, and the total capture efforts (combining the two expeditions for each area) yielded 1656, 1856 and 2868 traps-night for areas A1, A2, and A3, respectively. When possible, bats were also collected with special mist nets near the small-mammal capture transects from 6 pm to 10 pm. The morphological characteristics and body measurements of all captured specimens were recorded for age estimations and taxonomic identification. The taxonomic status of rodents and bats was subsequently confirmed by karyological analyses (Bonvicino et al., 2002).

With the informed consent of their owners, blood was collected from domestic mammals (dogs and pigs) by puncturing their cephalic or lateral saphenous veins using heparinized vacutainer tubes. Wild mammals were anesthetized (9:1, 10% ketamine chloridate and 2% acepromazine), and their blood was collected by cardiac puncture in dry tubes. In a field laboratory set up exclusively for this purpose, blood samples were processed as follows:

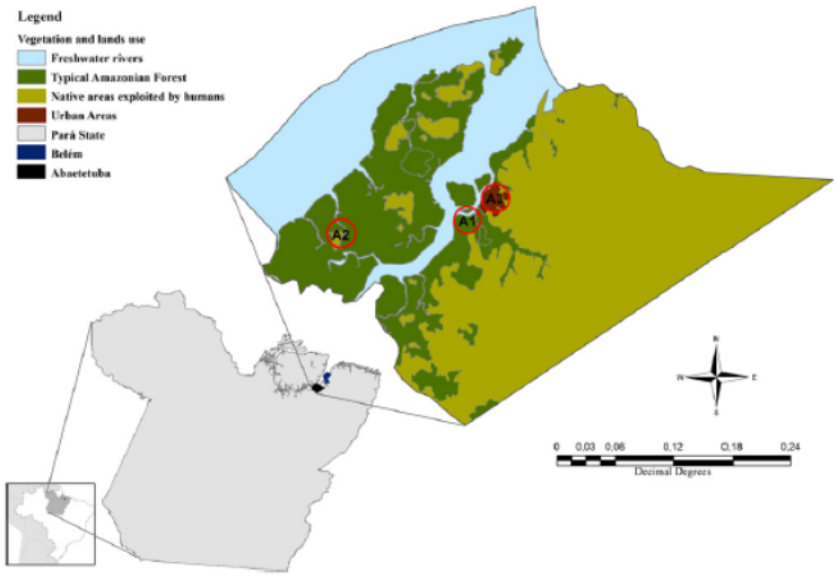

Fig. 1. Land-use map from the Abaetetuba municipality, modified from the Secretary for Strategic Projects from Pará State (SEPE/PA, http://www.sepe.pa.gov.br/zee/shapes.asp, accessed (07.6.11)) and illustrating the three studied areas in a gradient of environmental disturbance: Genipaúba locality (A1), Ajuaí locality (A2), and peripheral districts (urban area) (A3).

(i) 0.6 ml of blood was cultured in two tubes containing Novy-Mc Neal-Nicole medium (NNN) with a liver infusion tryptose medium (LIT) overlay (hemoculture), and (ii) the remaining blood was centrifuged, and the serum (wild mammals) or plasma (dogs and pigs) that was obtained was stored at −20 °C prior to analysis in serological assays. All procedures were based on protocols that were approved by the FIOCRUZ Committees of Bioethics (license 0015-07), and wild animal captures were licensed by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA/CGFAU/LIC) (license 3665-1).

2.3. Parasitological and serological diagnostic procedures

Hemocultures were analyzed every other week for three (for seronegative mammals) or five (for seropositive mammals) months. Positive hemocultures were amplified for molecular characterization and cryopreservated in the *Trypanosoma* from Sylvatic and Domestic Mammals and Vectors Collection, Oswaldo Cruz Foundation (ColTryp).

Serological diagnoses were performed using an indirect immunofluorescence antibody test (IFAT) performed using an adapted IFI-Chagas Bio-Manguinhos Kit® (Fiocruz, Rio de Janeiro, Brazil) (Camargo, 1966). *Sigmodontinae* and *Murinae* rodent sera were tested using anti-rat IgG fluorescein isothiocyanate (FITC), and domestic animals were tested using their respective anti-species IgG (Sigma®) conjugate. *Echimyidae* rodents and marsupials were tested with rabbit anti-*Thrichomys* spp. and anti-opossum specific intermediary antibodies, respectively, and reactions were visualized using an FITC anti-rabbit IgG conjugate (Sigma®). The cut-off values adopted were 1:40 for domestic mammals and marsupials and 1:10 for rodents, as previously described (Roque et al., 2008). Dogs and marsupials were also screened for *Leishmania* spp. infection using an IFI-*Leishmania* Bio-Manguinhos Kit® (Fiocruz, Rio de Janeiro, Brazil) and antigens derived from a mixture of *Leishmania infantum* and *L. braziliensis* parasites. Animals with serological titers for *Leishmania* spp. equal or higher the titers for *T. cruzi* were not considered to be infected by *T. cruzi* if the *T. cruzi* titers were ≤1:80. Animals were considered to be infected by both *T. cruzi* and *Leishmania* sp. if the titers were >1:80 in both assays. For each assay, samples from experimentally infected and non-infected animals that were born in captivity were used as positive and negative controls, respectively. Animals were considered to be infected by *T. cruzi* when the serological analysis and/or hemoculture were positive.

2.4. Molecular characterization of parasites

Seventeen positive hemocultures were amplified in LIT liquid medium at 28 °C for a maximum of three passages. Total genomic DNA was prepared from logarithmic phase cultures using standard phenol–chloroform protocols, as described elsewhere (Lisboa et al., 2006). Due to bacterial contamination, two positive hemocultures from A3 could not be amplified and characterized. A mini-exon multiplex PCR was performed using a reverse primer located in the conserved region of the mini-exon gene 5′TACCAATATAGTACAGAAACTG 3′ and the forward primers TcI 5′ACACTTTCTGTGGCGCTGATCG3′, TcII 5′TTGCTCGCACACTCGGCTGATCG3′, Z3 5′CCGCGWACAACCCCTMATAAAAATG3′, *Trypanosoma rangeli*

5′CCTATTGTGATCCCCATCTTCG3′ (Fernandes et al., 2001). The amplified PCR products were analyzed by ethidium bromide-stained agarose gel electrophoresis (2%) and visualized under UV light.

2.5. Statistical analysis

The comparison of the parasitological (positive hemocultures) and serological (positive IFAT) data from the three studied regions was performed using 2×2 contingency tables and the Chi-square statistical analysis. The test was performed using the software R (Version 2.11.1, R Development Core Team, 2010) with the level of significance set at $p < 0.05$. The capture prevalence of the small mammals (not including bats and anteaters) and the ratio of *T. cruzi* infection in the Abaetetuba municipality were calculated using the absolute number of captured specimens or positive tests divided by the total number of collected mammals or examined specimens.

3. Results

3.1. T. cruzi maintenance in the three studied areas

The differences shown by the land-use map (Fig. 1) and locally confirmed by us was associated with the observed differences in the relative abundance of *M. murina* and *D. marsupialis*. *M. murina* was the second most-captured species in the more preserved area (A1) and represented nearly 30% of the total captured species. Despite their proximity and similarity in composition of small mammalian fauna and seropositive rate of *T. cruzi*, the three examined regions displayed distinct enzootic *T. cruzi* transmission characteristics. Although the prevalence of positive IFAT was similar ($p = 0.08$), the prevalence of mammals with detectable of *T. cruzi* parasitemia (which denotes infectivity potential) that were identified by positive hemocultures was significantly greater in A2 (64%) and A1 (41%) than in the area with more extensive environmental disturbance, namely A3 (5%) ($p < 0.001$) (Table 1). Consequently, the region with an intermediate degree of disturbance (A2) also displayed a higher prevalence of positive IFAT among domestic mammals ($p < 0.001$). Notably, A2 was also the area in which we obtained the unique *T. cruzi* isolate from dogs (Table 2).

3.2. T. cruzi infection in wild and synanthropic mammals

The population of small mammalian fauna collected in the three study areas was predominantly composed of marsupial species (83.6% of the collected mammals). *Philander opossum* was the main *T. cruzi* reservoir in Abaetetuba, as it was the most abundant mammalian species captured in both wet and dry seasons (38.2% of the collected mammals) and displayed the potential to infect a vector (a prevalence of 57.1% in the positive hemocultures) (Table 1). Only in A3 was *Didelphis marsupialis* the most abundant mammalian species collected (41.6%), but all of these animals yielded negative hemocultures. Even in this area, *P. opossum* was abundant (29.1% of the collected mammals), with 71.4% serologically positive to *T. cruzi*, and one positive hemoculture. Among the other captured species, only *Marmosa murina* in A1 was relatively abundant; however, despite infection of this species with *T. cruzi* (66.7% [2/3] IFAT positive), no positive hemocultures were obtained (Table 1). Other less abundant species, such as *D. marsupialis* in A1 and A2, *M. murina* and *Micoreus demerarae* in A2, and *Artibeus planirostris* and *Tamandua tetradactyla* in A3, also had detectable parasitemia (as demonstrated by positive hemocultures). Eleven juvenile marsupials were captured, and eight of them (all from A3) were seropositive. All the rodents were negative in both the serological and parasitological assays to *T. cruzi* (Table 1).

Fifteen parasitic isolates were identified as *T. cruzi* (TcI), and two isolates were identified as *T. rangeli*. Two other isolates, from *P. opossum* and *Artibeus* spp. captured in A3, were positive via hemoculture, but parasitic amplification and molecular characterization were not achieved (Table 1).

3.3. T. cruzi infection in domestic mammals

The presence of anti-*T. cruzi* antibodies demonstrates that both dogs and pigs had been exposed to *T. cruzi* infection in all three regions. The prevalence of positive IFAT was highest in A2 ($p < 0.001$), whereas the other two areas displayed similar *T. cruzi* serological prevalence ($p = 0.35$) (Table 2). Only one dog from A2 yielded positive hemocultures. The same dog was examined 7 months later and remained positive by hemoculture. Both cultures were characterized as TcI and consistently had positive serological IFAT tests. Another 4 dogs from A2 were evaluated twice (2 positive and 2 negative), and their serological titers remained the same in both examinations. The pigs showed a higher serum prevalence (higher exposure to the *T. cruzi* transmission cycle) than the dogs in A2 ($p = 0.004$). This difference was not statistically significant in the other two areas, due to the low number of dogs ($n = 4$) and pigs ($n = 9$) that were evaluated in A1 and A3, respectively (Table 2).

4. Discussion

The presence of *Didelphis* species has been recognized as a strong indicator of environmental disturbance (Austad, 1988; Olifiers et al., 2005). and as expected, the *Didelphis* species was the most prevalent mammalian species in A3. The prevalence of *T. cruzi* detected by IFAT (indicating the exposure of the wild mammals to the *T. cruzi* transmission cycle) was similar among the studied localities. In contrast, the prevalence of positive hemocultures (which indicates the potential of these hosts to infect vectors) demonstrated that the chance of human contact with infected bugs should be quite different among the studied localities. At least three important conclusions can be drawn from the observed enzootic situations: (i) regardless of the area and its degree of land-use, *P. opossum* was the most important *T. cruzi* reservoir host, as seen in its high relative abundance, high prevalence of *T. cruzi* infection and detectable parasitemia. This marsupial species is associated with gallery forests and can be found in terrestrial and arboreal strata, (ii) the fauna composition and relative abundance alone are insufficient for identifying risk areas because, although exposure to the parasite (positive IFAT)

Table 1
Capture and prevalence of infection by *T. cruzi* in the small mammalian fauna examined in Genipaúba (A1), Ajuaí (A2) and peripheral districts (A3) localities, Abaetetuba municipality, in wet (W) and dry (D) seasons.

Small mammalian fauna		Capture prevalence (number of animals)			IFAT (positive mammals)			Hemoculture (parasite recovery)			Parasite characterization		
Order	Species	A1	A2	A3	A1	A2	A3	A1	A2	A3	A1	A2	A3
Marsupialia	*D. marsupialis*	11.1% (2^{D})	15.4% (2^{D})	41.6% ($10[5^{W}5^{D}]$)	100% (1/1)	100% (2/2)	100% (10/10)	100% (1/1)	100% (2/2)	0% (0/10)	TcI (1)	TcI (2)	–
	M. murina	27.9% ($5[1^{W}4^{D}]$)	7.7% (1^{W})	4.2% (1^{W})	66.7% ($1^{W}+1^{D}/3$)	100% (1/1)	0% (0/1)	0% (0/5)	100% (1/1)	0% (0/1)	–	TcI (1)	–
	Metachirus nudicaudatus	5.5% (1^{D})	n.c.	4.2% (1^{W})	n.d.	n.c.	0% (0/1)	0% (0/1)	n.c.	0% (0/1)	–	n.c.	–
	Micoureus demerarae	n.c.	7.7% (1^{D})	4.2% (1^{W})	n.c.	100% (1/1)	0% (0/1)	n.c.	100% (1/1)	0% (0/1)	n.c.	TcI (1)	–
	P. opossum	38.9% ($7[2^{W}5^{D}]$)	53.8% (7^{W})	29.1% ($7[3^{W}4^{D}]$)	85.7% ($2^{W}+4^{D}/7$)	57.1% (4/7)	71.4% ($3^{W}+2^{D}/7$)	85.7% ($2^{W}+4^{D}/7$)	71.4% (5/7)	14.3% ($1^{D}/7$)	TcI (6)	TcI (3) *T. rangeli* (2)	n.d.
Rodentia	*Mus musculus*	n.c.	7.7% (1^{W})	n.c.	n.c.	0% (0/1)	n.c.	n.c.	0% (0/1)	n.c.	n.c.	–	n.c.
	Nectomys rattus	n.c.	n.c.	4.2% (1^{D})	n.c.	n.c.	0% (0/1)	n.c.	n.c.	0% (0/1)	n.c.	n.c.	–
	Hylaeamys megacephalus	n.c.	7.7% (1^{D})	n.c.	n.c.	0% (0/1)	n.c.	n.c.	0% (0/1)	n.c.	n.c.	–	n.c.
	Oecomys sp.	5.5% (1^{D})	n.c.	n.c.	n.d.	n.c.	n.c.	n.d.	n.c.	n.c.	–	n.c.	n.c.
	Proechimys roberti	11.1% (2^{D})	n.c.	n.c.	0% (0/1)	n.c.	n.c.	0% (0/2)	n.c.	n.c.	–	n.c.	n.c.
	Rattus rattus	n.c.	n.c.	4.2% (1^{W})	n.c.	n.c.	0% (0/1)	n.c.	n.c.	0% (0/1)	n.c.	n.c.	–
	Rhipidomys sp.	n.c.	n.c.	8.3% (2^{D})	n.c.	n.c.	n.d.	n.c.	n.c.	0% (0/2)	n.c.	n.c.	–
Chiroptera	*Artibeus* sp.[b]	n.c.	–[a] (1^{W})	–[a] ($22[16^{W}6^{D}]$)	n.c.	n.d.	n.d.	n.c.	0% (0/1)	4.5% ($1^{D}/22$)	n.c.	–	n.d.
	Carollia perspicillata	n.c.	n.c.	–[a] (3^{W})	n.c.	n.c.	n.d.	n.c.	n.c.	0% (0/3)	n.c.	n.c.	–
	Molossus rufus	–[a] (1^{D})	n.c.	–[a] (2^{D})	n.d.	n.c.	n.d.	0% (0/1)	n.c.	0% (0/2)	–	n.c.	–
	Sturnira lilium	n.c.	n.c.	–[a] (2^{D})	n.c.	n.c.	n.d.	n.c.	n.c.	0% (0/2)	n.c.	n.c.	–
Pilosa	*T. tetradactyla*	n.c.	n.c.	–[a] (1^{W})	n.c.	n.c.	n.d.	n.c.	n.c.	100% (1/1)	n.c.	n.c.	TcI
	Total	19 ($3^{W}16^{D}$)	14 ($10^{W}4^{D}$)	54 ($32^{W}+22^{D}$)	75.0% (9/12)	61.5% (8/13)	68.2% (8/22)	41.2% (7/17)	64.3% (9/14)	5.5% (3/54)	TcI (7)	TcI (7) *T. rangeli* (2)	TcI (1) n.d. (2)

nc: not captured; n.d. = not determined; W: data from wet season (April (A1 and A2) and August (A3) of 2008); D: data from dry season (November (A1 and A2) and December (A3) of 2008).

[a] Not included in the calculation of capture prevalence.

[b] *Artibeus planirostris* (19) and *Artibeus literatus* (4).

Table 2
Prevalence of *T. cruzi* infection by IFAT and hemocultures in domestic animals examined in Abaetetuba municipality in wet (W) and dry (D) seasons.

Localities	Serum prevalence	Hemoculture prevalence
A1	17.2% (5/29)	0% (0/29)
Dogs	$0\%^{W}$ (0/4)//n.d.^{D} (0/0)	0% (0/4)
Pigs	$57.1\%^{W}$ (4/7)//$5.5\%^{D}$ (1/18)	0% (0/25)
A2	63.3% (31/49)	3.7% (2/54[a])
Dogs	$57.1\%^{W}$ (4/7)//$27.3\%^{D}$ (3/11[a])	11.1% (2/18[a])
Pigs	$92.0\%^{W}$ (23/25)//$36.4\%^{D}$ (4/11)	0% (0/36)
A3	25.3% (39/154)	0% (0/158)
Dogs	$45.1\%^{W}$ (23/51)//$17.0\%^{D}$ (16/94)	0% (0/145)
Pigs	$0\%^{W}$ (0/9)//n.d.^{D} (0/0)	0% (0/9)

[a] Considering 5 animals that were re-evaluated 7 months later; W: data from wet season (April (A1 and A2) and August (A3) of 2008); D: data from dry season (November (A1 and A2) and December (A3) of 2008).

was similar in all three areas, the potential of these hosts to infect vectors (expressed by positive hemocultures) was different, and (iii) the abundant presence of *D. marsupialis*, considered to be the main reservoir of *T. cruzi*, does not indicate epidemiological risk because the area (A3) that displayed the greatest abundance of this mammalian species was also the area that presented the lowest prevalence of positive hemocultures among examined mammals. Additionally, it was striking that in the Amazon Basin, where TcI is predominant in both human and mammals, the presence of *D. marsupialis* was not as critical for the maintenance of *T. cruzi* in the endemic Abaetetuba area, as has been described for other non-Amazonian regions (Fernandes et al., 1999).

The higher the prevalence of mammals with positive hemocultures in the wild, the greater was the chance that a given vector could acquire the parasite while feeding on an infected mammal. In places where the population reported hunting activities, *T. cruzi* transmission may occur during the cleaning of meat or the contamination of cooking utensils, rather than during ingestion of the meat, which has never been reported to be consumed raw or undercooked. Domestic mammals may become infected by (i) the classical contaminative route, (ii) the ingestion (accidental or not) of infected bugs, (iii) the ingestion of foodstuff contaminated by the feces of infected bugs, and (iv) predation while hunting for infected small wild mammals (only for dogs). The scenario observed in Abaetetuba favor the first two hypotheses. The predation of infected mammals does not seem to be an important infection route for dogs because the majority of dogs' owners did not report this type of behavior. In addition, *T. cruzi* infection was detected in young dogs (that do not yet hunt), and infected bugs are common near human dwellings. Thus, humans and domestic mammals most likely become infected after the accidental ingestion of infected triatomines.

The scenario observed in A2, in which the natural environment is progressively being modified by açaí monocultures, is the most common situation throughout rural areas of Pará State. We were unable to explain why this moderately degraded area displayed higher prevalences of positive hemocultures among wild mammals and positive IFATs in domestic mammals when compared to the other two areas for the following reasons: (i) the dilution-effect hypothesis, based on the reduction of mammalian diversity and the selection of suitable *T. cruzi* reservoirs (Keesing et al., 2006; Roque et al., 2008), does not fit here because the richness and composition of the fauna were similar among the areas, (ii) the recent occupation of the landscape could not be responsible because this area has been exploited by the local population for decades, and (iii) the capture success (approximately 1%) was similar among the three studied areas, demonstrating no bias in between-area comparisons.

This puzzling scenario stresses the importance of selecting domestic mammals as sentinels for identifying areas at risk for the emergence of Chagas disease (Roque and Jansen, 2008). In this sense, in Abaetetuba, dogs act as sentinel hosts because they present a serologically detectable infection and can indicate areas in which *T. cruzi* transmission is occurring, thus enabling the identification of hotspot regions (Xavier et al., 2012). The isolation of *T. cruzi* from the same dog at a 7-month interval and the characterization of the parasite (TcI, which is associated with human infection in this area) indicate that dogs may act as *T. cruzi* reservoirs, being able to infect vectors in A2. When the distinct situations of the three studied localities were considered, the common factor among these areas was the exposure of dogs to *T. cruzi* infection, as expressed by their positive serology. This finding supports an important measure that can be immediately employed in the identification of *T. cruzi* transmission areas: the routine use of domestic animals as sentinels to monitor the epidemiological risk of Chagas disease (Xavier et al., 2012).

The use of dogs as sentinels has already been proposed for Chagas disease (Estrada-Franco et al., 2006; Gurtler et al., 2007) and other zoonotic diseases (Scotch et al., 2009). These animals are usually easy to handle, and their movements can be monitored relatively consistently. Blood collection and subsequent serological assays (or sending the serum samples to governmental central laboratories for diagnosis) do not require great resources or high levels of infrastructure (Jansen and Roque, 2010). The recognition that seropositive dogs reflect exposure to *T. cruzi* and thus, may indicate *T. cruzi* transmission hotspots. Within Abaetetuba, the various enzootic situations each reflect a distinct opportunity for human contact with infected bugs and indicate the importance of adopting unique and micro-regional measures for disease control.

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Conflict of interest

The authors declare that they have no conflict of interest.

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