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Estudo enzoótico em uma área de doença de Chagas aguda, no município de Guarapari (Espírito Santo): plasticidade biológica e diversidade da classe Kinetoplastea

MARIA AUGUSTA DARIO

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MARIA AUGUSTA DARIO

ESTUDO ENZOÓTICO EM UMA ÁREA DE DOENÇA DE CHAGAS AGUDA, NO MUNICÍPIO DE GUARAPARI (ESPÍRITO SANTO): PLASTICIDADE BIOLÓGICA E DIVERSIDADE DA CLASSE KINETOPLASTEA

ORIENTADOR: Prof. Drª. Ana Maria Jansen

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Rio de Janeiro, 31 de outubro de 2017

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Ata da defesa de tese de doutorado em Biologia Parasitária de **Maria Augusta Dario**, sob orientação da Drª. Ana Maria Jansen Franken. Ao trigésimo primeiro dia do mês de outubro de dois mil e dezessete, realizou-se às treze horas, no Auditório Emmanuel Dias/FIOCRUZ, o exame da tese de doutorado intitulada: "**Estudo enzoótico em uma área de doença de chagas aguda, no município de Guaraparí (Espírito Santo): Plasticidade biológica e diversidade da classe kinetoplastea**" No programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutora em Ciências - área de concentração: Ecologia e Epidemiologia, na linha de pesquisa: Variabilidade Genética de Parasita, Vetores e Hospedeiros. A banca examinadora foi constituída pelos Professores: Drª. Constança Felícia de Paoli de Carvalho Britto - IOC/FIOCRUZ (Presidente), Dr. Marcelo Salabert Gonzalez - UFF/RJ, Drª. Maria Maria Geraldes Teixeira - USP/ISP e como suplentes: Dr. Bernardo Rodrigues Teixeira – IOC/FIOCRUZ e Dr. José Roberto Machado e Silva – UERJ/RJ. Após argüir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela APROVAÇÃO da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutora em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 31 de outubro de 2017.

Drª. Constança Felícia de Paoli de Carvalho Britto (Presidente da Banca):

Dr. Marcelo Salabert Gonzalez (Membro da Banca):

Drª. Maria Maria Geraldes Teixeira (Membro da Banca):

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À vida, por ser bela e generosa

Aos meus pais, Cesar e Lourdes, por acreditarem nos meus sonhos

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“Para se ter sucesso, é necessário amar de verdade o que se faz...”

Steve Jobs

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Estudo enzoótico em uma área de doença de Chagas aguda, no município de Guarapari (Espírito Santo): plasticidade biológica e diversidade da classe Kinetoplastea

RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Maria Augusta Dario

Os protozoários do gênero *Trypanosoma* são capazes de infectar uma diversidade de hospedeiros vertebrados e invertebrados. Dentro desse universo, destaca-se o clado *Trypanosoma cruzi* que tem como um dos seus componentes o *T. cruzi*, agente etiológico da doença de Chagas (DC) nas Américas. *Trypanosoma cruzi* é um parasito heterogêneo, multihospedeiro, capaz de infectar centenas de espécies de mamíferos e ser transmitido por dezenas de espécies de triatomíneos. O mesmo inclui sete genótipos ou Unidades Discretas de Tipagem (DTUs) (TcI-VI e TcBat). Casos de DC vêm ocorrendo em cenários enzoóticos distintos, no qual cada região apresenta uma rede de transmissão específica, tornando o conhecimento dessas redes fundamentais para possível orientação de moradores e agentes de saúde, evitando novos casos da doença. O nosso objetivo foi compreender a ecologia do ciclo enzoótico de tripanosomatídeos, com foco especial em *T. cruzi*, no ambiente silvestre da Mata Atlântica, no municipío de Guarapari, estado do Espírito Santo (ES), em uma área de ocorrência de um caso fatal de DC aguda, por via oral. Nós obtivemos o tecido cardíaco do paciente que veio a óbito e realizamos a caracterização molecular do DNA de *T. cruzi*. Foi determinada a composição e a abundância relativa de espécies de pequenos mamíferos não-voadores e voadores. Através de exames parasitológicos e sorológicos, avaliou-se o papel desses e também dos cães no ciclo de transmissão do *T. cruzi*. Triatomíneos foram examinados para determinação de sua taxa de infecção pelo parasito. As amostras de mamíferos e triatomíneos foram caracterizadas por métodos moleculares, para determinação de DTUs de *T. cruzi*, bem como outras espécies de tripanosomatideos. Devido a diversidade de tripanosomatídeos observada em morcegos, nós caracterizamos amostras de sangue total utilizando o sequeciamento de nova geração. Além disso, avaliamos a distribuição geográfica das DTUs de *T. cruzi* TcIII e TcIV em hospedeiros mamíferos e triatomíneos em diferentes biomas brasileiros. Foi relatado o encontro de infecção mista por quatro DTUs de *T. cruzi* e *T. dionisii* em humano. Infecções mistas em humanos devem ser levadas em consideração, uma vez que as mesmas podem ser muito mais frequentes do que se tem relatado. *Trypanosoma dionisii* é capaz de infectar tecido cardíaco humano. Nós observamos que o ciclo enzoótico de *T. cruzi* ocorre longe das residências, não há transmissão peridomiciliar e os hospedeiros vertebrados e invertebrados apresentam infecções mistas. Os morcegos são os hospedeiros de *Trypanosoma* spp. na região. Espécies de cinetoplastídeos aparentam ser muito menos específicas quanto ao hospedeiro, do que se tem relatado até o momento e a espécie *Bodo Saltans*, cinetoplastídeo de vida-livre, pode estar passando por processo de adaptação. Por

fim, as DTUs TcIII e TcIV são muito mais dispersas em hospedeiros e biomas, não apresentando nenhum tipo de associação.

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Enzootic study in an acute Chagas disease area, Guarapari municipality (Espírito Santo state): Kinetoplastea class plasticity biology and diversity

ABSTRACT

PHD THESIS IN PARASITARY BIOLOGY

Maria Augusta Dario

Trypanosoma genus protozoa are capable to infect a variety of vertebrate and invertebrate hosts. In this context, we highlight the *Trypanosoma cruzi* clade, in which includes *T. cruzi*, the aetiological agent of Chagas disease (CD) in the American continent. *Trypanosoma cruzi* is heterogeneous, multi-host, capable of infecting hundreds of mammals' species and it is transmitted by dozens of triatomines. This species includes seven genotypes or Discrete Typing Units (DTUs) (TcI-VI and TcBat). Chagas disease cases are occuring in different enzootic scenarios, in which each region presentes a specific transmission web making its knowledge essencial to residents and health service orientation, avoiding new disease's cases. The aim of this study was to understand tripanosomatids ecological enzootic cycle, focus on *T. cruzi*, in the sylvatic enviromment on the Atlantic forest of Guarapari municipality, Espírito Santo (ES) state, in an area where a fatal acute CD occured by oral route. We obtained the pacient cardiac tissue and performed DNA molecular characterization for *T. cruzi* identification. We determinated non-volant mammals and bats species composition and their relative abundance. By parasitological and serological exams, we evaluated the role played by these mammals and dogs on *T. cruzi* transmission cycle. Triatomines were examined to determinate parasite infection rate. Mammal and triatomine samples were submitted to molecular characterization for *T. cruzi* DTU and other tripanosomatids species identification. Due to tripanosomatid diversity observed in bats, we characterized bats blood sample using next generation sequencing. In addition, we evaluated *T. cruzi* TcIII and TcIV DTUs geographical distribution in mammals and triatomines in Brazilian biomes. Human mixed infections should be taken into consideration, since they can be more frequent than has been reported. *Trypanosoma cruzi* DTUs I, II, III and IV and *T. dionisii* were found infecting human. *Trypanosoma dionisii* is capable to infect human cardiac tissue. We observed that *T. cruzi* enzootic cycle is occuring far from the residences, there is no peridomiciliar transmission and invertebrate and verterbrate hosts present mixed infections. Bats are the main *Trypanosoma* spp. host in the area. Kinetoplastid species appear to be less specific than has been reported until now and *Bodo saltans* species, a free-living kinetoplastid, may be going through adaptation process. *Trypanosoma cruzi* DTUs TcIII and TcIV are more dispersed in hosts and biomes, not presenting any association type.

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LISTA DE SIGLAS E ABREVIATURAS

kDNA	Cinetoplasto
DC	Doença de Chagas
DCA	Doença de Chagas aguda
DTU	Unidade Discreta de Tipagem
DNA	Ácido desoxirribonucleico
ES	Estado do Espírito Santo
gGAPDH	Gliceraldeído-3-fosfato desidrogenase glicossomal
HSP	Proteína de choque térmico
MLEE	*Multilocus enzyme electrophoresis*
NGS	*Next generation sequencing*, sequenciamento de nova geração
RAPD	*Randomly amplified polymorphic* DNA
RNA	Ácido ribonucleico
rRNA	RNA ribossomal
SL	*Spliced-leader*
SSU	Subunidade menor

1 INTRODUÇÃO

1.1 A classe Kinetoplastea

A classe Kinetoplastea (Excavata: Euglenozoa) (Cavalier-Smith, 1981) é considerada singular, uma vez que apresenta desde espécies de protozoários considerados de vida-livre até espécies capazes de infectar hospedeiros vertebrados, invertebrados e plantas. Os protozoários dessa classe apresentam como principal característica a presença de uma organela rica em DNA extranuclear dentro de uma mitocôndria única denominada cinetoplasto (kDNA) (Vickerman, 1976). O kDNA apresenta diferentes padrões de organização, que são definidos pelos seguintes termos: eucinetoplasto – o DNA do cinetoplasto está compactado perto da base do flagelo; policinetoplasto – quando o DNA do cinetoplasto está disperso na mitocôndria, em vários corpúsculos idênticos; ou pancinetoplasto: quando o DNA do cinetoplasto está disseminado desigualmente como uma massa difusa (Vickerman,1977; Lukes *et al.*, 2002; Simpson *et al.*, 2002; Moreira *et al.*, 2004, d'Avila-Levy *et al.*, 2015).

De acordo com os estudos taxonômicos, os cinetoplastídeos foram classificados dentro da ordem Kinetoplastida (Honigberg, 1963), subdividida em duas subordens (Vickerman, 1976): i) a subordem Bodonida e ii) a subordem Trypanosomatina, (Tabela 1).

Tabela 1: Classificação da ordem Kinetoplastida baseada nas suas características morfológicas e ciclos de vida, de acordo com Vickerman, 1976.

Subordem	Características
Bodonida	Protozoários biflagelados de vida-livre ou parasitas
Trypanosomatina	Protozoários uniflagelados parasitas obrigatórios

A utilização de ferramentas moleculares mais acuradas e a inclusão de novas espécies de cinetoplastídeos levaram a uma nova divisão taxônomica desses microrganismos (Callahan *et al.*, 2002; Simpson *et al.*, 2002, 2004; Dávila e Lukes, 2003; Dykova *et al.*, 2003; Moreira *et al.*, 2004). A ordem Kinetoplastida passou a ser denominada classe Kinetoplastea e a mesma foi dividida em duas subclasses: Prokinetoplastina e Metakinetoplastina. A primeira possui uma única ordem

denomidada Prokinetoplastida, que é formada por cinetoplastídeos ectoparasitas de peixe pertencentes ao gênero *Ichthyobodo*, além de um endossimbionte do gênero *Perkinsiella*. A subclasse Metakinetoplastina foi subdividida em quatro ordens (Figure 2): a) Trypanosomatida – ordem composta pelos tripanosomatídeos parasitas obrigatórios, dentre eles os de importância médica e veterinária como *Trypanosoma cruzi*, *Trypanosoma brucei* e as diversas espécies de *Leishmania*; b) Neobodonida – composta por cinetoplastídeos dos gêneros *Cruzella*, *Dimastigella*, *Neobodo*, *Rhynchobodo* e *Rhynchomonas*; c) Parabodonida – composta por cinetoplastídeos de vida-livre ou comensais/parasitas dos gêneros *Cryptobia*, *Parabodo*, *Procryptobia* e *Trypanoplasma* e; d) Eubodonida – composta pelos cinetoplastídeos de vida-livre do gênero *Bodo* (Moreira *et al.*, 2004; Adl *et al.*, 2005, 2012; Simpson *et al.*, 2006).

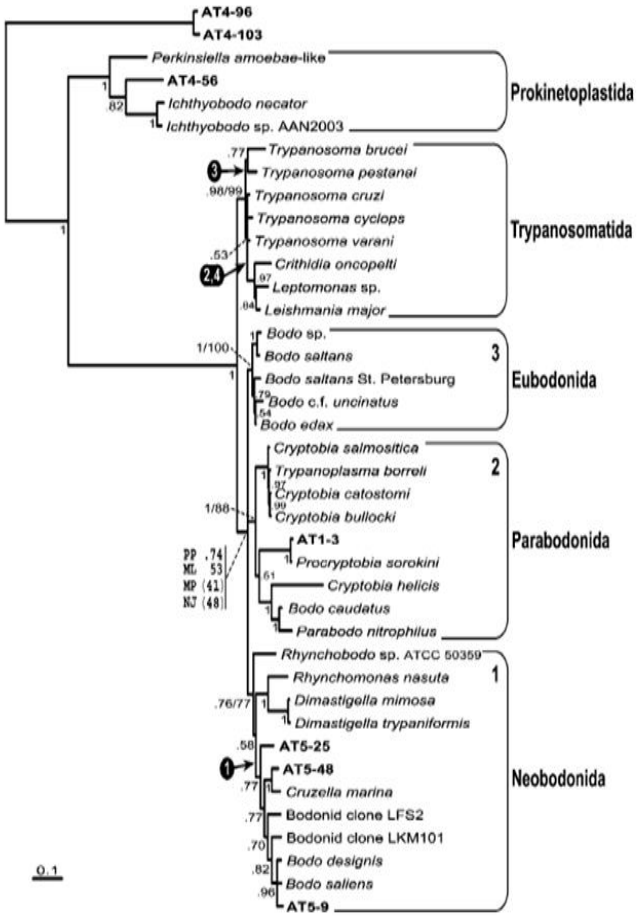

Figura 1: Árvore filogenética representativa da classe Kinetoplastea e suas cinco ordens. Fonte: Moreira *et al.*, 2004.

Bodo saltans é um cinetoplastídeo de vida-livre encontrado em ambientes marinhos e de água doce no mundo, e que se alimenta de bactérias (Mitchell *et al.*, 1988). Essa espécie apresenta características típicas dos cinetoplastídeos como: presença de flagelo duplo originário da bolsa flagelar, o cinetoplasto está localizado na base do flagelo, é capaz de realizar edição do RNA mitocondrial, apresenta glicossomo e microtúbulos (Vickerman, 1976, 1991; Blom *et al.*, 2000; Jackson *et al.*, 2008). Estudos filogenéticos demonstraram que *B. saltans* é o protozoário de vida-livre mais próximo dos tripanosomatídeos parasitas obrigatórios e em diversos estudos esse protozoário é utilizado como grupo externo. Além disso, relata-se que a origem do parasitismo em tripanosomatídeos tenha surgido a partir dos eubodonídeos (Simpson *et al.*, 2002, 2004, 2006; Stevens, 2008; Deschamp *et al.*, 2011; Lukes *et al.*, 2014). Isso faz com que o *B. saltans* seja uma espécie chave para estudos sobre a origem do parasitismo em tripanosomatídeos (Simpson *et al.*, 2004; Jackson *et al.*, 2008, 2016; Brown *et al.*, 2014; Opperdoes *et al.*, 2016).

1.2 Ordem Trypanosomatida e o gênero *Trypanosoma*

A ordem Trypanosomatida é composta por tripanosomatídeos de 19 genêros e que apresentam diferentes tipos de hospedeiros e ciclos evolutivos (Kaufer *et al.*, 2017). Os parasitos dos gêneros *Angomonas*, *Blastocrithidia*, *Blechomonas*, *Crithidia*, *Herpetomonas*, *Kentomonas*, *Leptomonas*, *Lotmaria*, *Novymonas*, *Paratrypanosoma*, *Sergeia*, *Streigomonas*, *Wallaceomonas* e *Zelonia* são considerados monoxênicos, uma vez que possuem apenas um hospedeiro definitivo, os invertebrados, sendo na sua maioria pertencente as ordens Diptera e Hemiptera (Wallace, 1966; Vickerman, 1976; Wallace *et al.*, 1983; Olsen, 1986; Podlipaev, 2001; Svobodova *et al.*, 2007; Teixeira *et al.*, 2011; Flegontov *et al.*, 2013; Maslov *et al.*, 2013; Votýpka *et al.*, 2013, 2014; Kostygov *et al.*, 2014, 2016; Lukes *et al.*, 2014; Schwarz *et al.*, 2015; Espinosa *et al.*, 2016).

Os parasitos dos gêneros *Endotrypanum*, *Phytomonas*, *Leishmania, Porcisia* e *Trypanosoma*, por apresentarem dois hospedeiros, são considerados parasitos heteroroxênicos. Aqueles do gênero *Phytomonas* são transmitidos por insetos fitófagos as plantas. Os parasitos do gênero *Porcisia* apresentam hospedeiros invertebrados desconhecidos e seus hospedeiros vertebrados descritos até o momento são o porco-espinho e a preguiça. Os gêneros *Endotrypanum*, *Leishmania* e *Trypanosoma* são transmitidos por insetos hematófagos aos hospedeiros

vertebrados (Wallace, 1966; Vickerman, 1976; Arias *et al.*, 1985; Camargo, 1998; Espinosa *et al.*, 2016).

O gênero *Trypanosoma* compreende organismos capazes de infectar todas as espécies de vertebrados e são em sua maioria transmitidos por diferentes espécies de vetores invertebrados (Hoare, 1972; Simpson *et al.*, 2006). Os tripanosomatídeos que compõem esse gênero são divididos em dois grupos, de acordo com seu desenvolvimento no inseto vetor e sua forma de transmissão, sendo denominados Salivaria e Stercoraria (Hoare, 1972). A secção Salivaria é composta por parasitos dos subgêneros *Duttonela*, *Trypanozoon*, *Tejeraia*, *Pycnomonas* e *Nannomonas*, que são capazes de se desenvolver no tubo digestivo médio e glândulas salivares ou probócide dos seus insetos vetores (moscas na África e triatomíneos nas Américas). Sua transmissão ocorre pela inoculação de formas tripomastigotas metacíclicas junto com a saliva durante o repasto sanguíneo. Dentro dessa secção temos a espécie *Trypanosoma brucei*, responsável pela doença do sono em humanos e nagana em animais, *Trypanosoma evansi* e *Trypanosoma vivax*, responsáveis por doenças em animais na África e na América, e *Trypanosoma rangeli*, capaz de ser patogênico nos triatomíneos nas Américas. A secção Stercoraria é formada por parasitos dos subgêneros *Schizotrypanum*, *Megatrypanum* e *Herpetomonas*. Os parasitos dessa secção se desenvolvem no intestino posterior do seu hospedeiro invertebrado, no qual as formas infectantes, tripomastigotas metacíclicas, são eliminadas nas fezes durante a alimentação sanguínea nos vertebrados. Dentro dessa secção está a espécie de importância médica *Trypanosoma cruzi*, agente etiológico da doença de Chagas (CD) (Tobie, 1970; D'Alessandro, 1976; Vickerman, 1994; Eichler e Schaub, 1998; Stuart *et al.*, 2008).

A origem do gênero *Trypanosoma* é um assunto controverso, sendo estudado e discutido por vários grupos. Os primeiros estudos filogenéticos baseados em marcadores ribossomais foram pioneiros nesse assunto e demonstraram que os parasitos desse gênero apresentavam origem parafilética (Fernandes *et al.*, 1993; Maslov *et al.*, 1994, 1996; Vickerman, 1994). A partir desses estudos e com a inclusão de novas espécies, foi possível determinar relações evolutivas entre espécies distantes e com isso, observou-se que o gênero *Trypanosoma* apresentava uma origem monofilética, ou seja, todos os tripanosomatídeos desse gênero, independente da sua espécie hospedeira, se originaram de um único ancestral (Lukes *et al.*, 1997; Haag *et al.*, 1998; Stevens *et al.*, 1999, 2001). A utilização de

outros marcadores moleculares, no caso o gliceraldeído-3-fosfato desidrogenase glicossomal (gGAPDH) e as proteínas de choque-térmico (*heat shok proteins* – hsp 70 e 90), também demonstrara que a origem do gênero *Trypanosoma* seria monofilética, sendo essa a teoria mais aceita. (Hamilton *et al.*, 2004, 2007; Simpson e Roger, 2004; Simpson *et al.*, 2006).

Até o momento, dez clados do gênero *Trypanosoma* foram reconhecidos a partir de análises filogenéticas (Telleria e Tibayrenc, 2017): a) clado aquático – composto por tripanosomatídeos isolados de peixes, ornitorrinco e anfíbios; b) clado lagartos e cobras – formado por tripanosomatídeos isolados de cobras e largatos, como *Trypanosoma cascavelli* e *Trypanosoma serpentis*; c) clado crocodiliano – composto por tripanosomatídeos isolados de crocodilianos na África e América do Sul. O seu principal representante é o *Trypanosoma grayi*; d) clado *Trypanosoma lewisi* – representante do subgênero *Hepertosoma*, é composto por tripanosomatídeos de roedores e coelhos; e) clado *Trypanosoma theileri* – representante do subgênero *Megatrypanum*, composto por tripanosomatídeos isolados de bois, antílopes, cervídeos, ovelhas, marsupiais australianos, sanguessuga e macaco. O clado é subdivido em clado *Trypanosoma cyclops* e clado *T. theileri*; f) clado aviário: composto por tripanosomatídeos isolados de aves, com ampla distribuição mundial e subdividido em clado *Trypanosoma avium* e clado *Trypanosoma corvi*; g) clado *Trypanosoma pestanai*: composto por tripanosomatídeos de texugo, carrapatos no Japão, marsupiais australianos e cães no Brasil. As principais espécies desse clado são *T. pestanai*, *Trypanosoma caninum*, *Trypanosoma gilletti* e *Trypanosoma vegrandis*; h) clado *Trypanosoma irwini*: composto por tripanosomatídeos de falcão americano, de coalas e primatas sul americanos, tendo as espécies *T. irwini*, *Trypanosoma bennetti* e *Trypanosoma minasense*; i) clado *Trypanosoma brucei* – composto por tripanosomatídeos de diversas espécies de mamíferos africanos e pertencentes a secção Salivaria e; j) clado *T. cruzi* – composto por representantes do subgênero *Schizotrypanum* e outras espécies de tripanosomatídeos de mamíferos (Ayala, 1970; Hoare, 1972; Minter-Goedbloed *et al.*, 1993; Noyes *et al.*, 1999; Stevens *et al.*, 1998, 1999, 2001; Jakes *et al.* 2001; Votýpka *et al.*, 2002, 2004a, 2004b, 2015; Hamilton *et al.*, 2005a, 2005b, 2008, 2009; Rodrigues *et al.*, 2005; Sato *et al.*, 2005, 2008; Stevens e Brisse, 2005; Bray *et al.*, 2007; Thekisoe *et al.*, 2007; McInnes *et al.*, 2009, 2011; Viola *et al.*, 2009a, 2009b; Gibson *et al.*, 2010; Van den Bossche *et al.*, 2010; Lizundina *et al.*, 2011; Paparini *et al.*, 2011; Lima *et al.*, 2012, 2013, 2015b; Martinkovic *et al.*, 2012;

Botero *et al.*, 2013, 2016; Barros *et al.*, 2015; Austen *et al.*, 2016; Barbosa *et al.* 2016).

1.3 Clado *Trypanosoma cruzi*

O clado *T. cruzi* (Figura 2) é um clado heterogêneo, composto por uma diversidade de espécies de *Trypanosoma*, com ampla diversidade de hospedeiros mamíferos e distribuição mundial. O clado inclui tripanosomatídeos do subgênero *Schizotrypanum*, composto pelas espécies *T. cruzi*, *T. dionisii* e *T. erneyi*. O clado *T. cruzi* inclui também um grupo denominado *T. rangeli*/*T. conorhini*, representado pelas espécies *T. rangeli*, *T. conorhini*, *T. vespertilionis* e três representantes isolados de um macaco, uma civeta e um morcego africano; um grupo formado por espécies de tripanosomatídeos isolados de morcegos neotropicais, conhecidos como *neobats*; um grupo formado por espécies de tripanosomatídeos isolados de marsupiais australianos; além de *T. livingstonei*, descrito em morcegos africanos e pela espécie *T. teixeirae* descrita em morcego australiano (Hoare, 1972; Noyes *et al.*, 1999; Stevens *et al.*, 1999a, 1999b; Hamilton *et al.*, 2007, 2009; Paparini *et al.*, 2011; Lima *et al.*, 2012, 2013, 2015b; Botero *et al.*, 2013, 2016; Barbosa *et al.*, 2016).

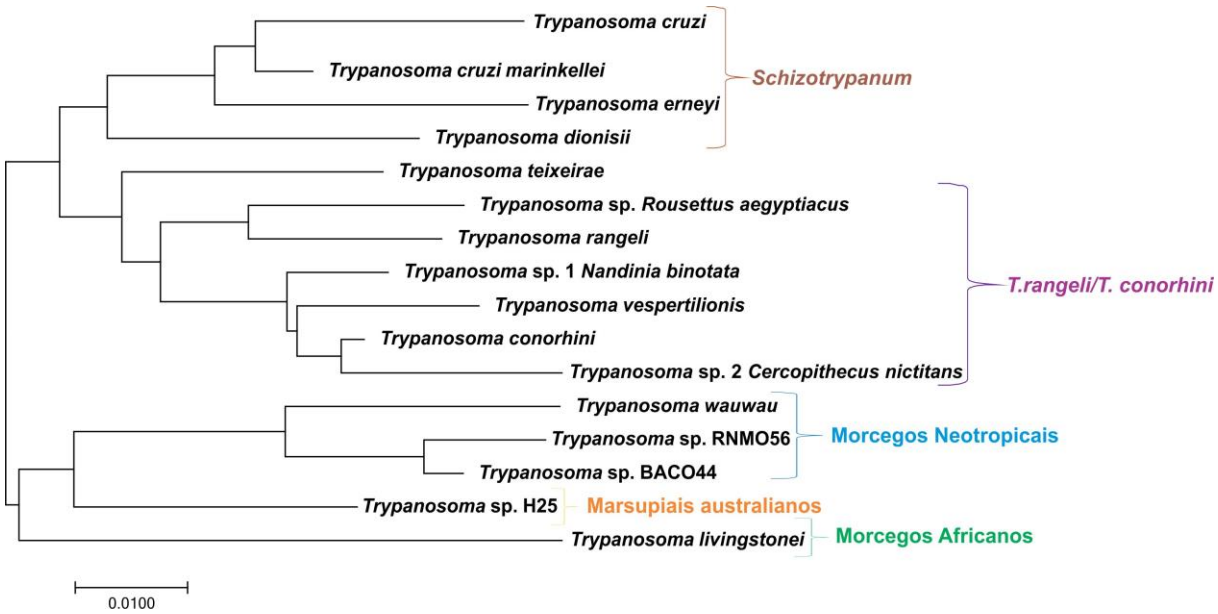

Figura 2: Árvore filogenética representativa do clado *T. cruzi*. Os cincos grupos estão representados, a partir das seguintes cores: vinho – subgênero *Schizotrypanum*; roxo – grupo *T. rangeli/T. conorhini*; azul – morcegos Neotropicais; laranja – marsupiais australianos; verde – morcegos africanos. Fonte: Dario MA, 2017.

O subgênero *Schizotrypanum* compreende, com exceção do *T. cruzi*, de espécies que são supostamente associadas exclusivamente a morcegos. Essas espécies são morfologicamente semelhantes entre si e os tripanosomatídeos desse subgênero foram denominados *T. cruzi*-like (Hoare, 1972; Marinkelle, 1976; Molyneux, 1991). Uma característica comum das espécies desse subgênero é a capacidade de desenvolver as formas amastigotas e tripomastigotas em hospedeiros mamíferos (Molyneux, 1991; Lima *et al.*, 2012).

Trypanosoma cruzi, agente etiológico da DC, é um parasito multi-hospedeiro, capaz de infectar centenas de espécies de hospedeiros mamíferos, de oito diferentes ordens, transmitido por várias espécies de triatomíneos hematófagos (família Reduviidae, subfamília Triatominae) (Jansen *et al.*, 2015). Sua ocorrência é descrita em todo o continente americano, desde o sul dos Estados Unidos até a Argentina e o Chile (Llewellyn *et al.*, 2009; Zingales *et al.*, 2012). Taxonomicamente, a espécie *T. cruzi sensu lato* está dividida em duas subespécies: *T. cruzi cruzi* (*sensu strictu*) e *T. cruzi marinkellei* (Baker *et al.*, 1978).

O ciclo de vida do *T. cruzi* é complexo. O parasito apresenta quatro estágios de desenvolvimento nos seus hospedeiros: amastigota e tripomastigota sanguíneas nos hospedeiros mamíferos; e epimastigota e tripomastigota metacíclica no inseto vetor. As formas amastigotas e tripomastigotas (metacíclicas e sanguíneas) são as

reponsáveis por causar infecção nos seus hospedeiros (Mortara *et al.*, 2008; Herrera *et al.*, 2011; Fernandes *et al.*, 2012). No vetor, o ciclo de vida do parasito se inicia quando ocorre a alimentação com sangue contendo as formas tripomastigotas sanguíneas oriundas do hospedeiro mamífero infectado. Essas formas ingeridas sofrem diferenciação no intestino anterior do triatomíneo, transformando-se em epimastigotas, que por sua vez, migram para o intestino médio, onde irão se multiplicar por fissão binária. Com uma intensa replicação, as formas epimastigotas aderem ao epitélio do intestino posterior, onde sofrem diferenciação em formas tripomastigotas metacíclicas. As formas tripomastigotas metacíclicas são eliminadas juntamente com as fezes, durante um novo repasto sanguíneo. As formas metacíclicas contidas nas fezes são capazes de penetrar em qualquer solução de continuidade da pele ou em mucosas. Uma vez no interior das células hospedeiras dos mamíferos, as formas metacíclicas formam um vacúolo parasitário, onde se diferenciam nas formas amastigotas e proliferam no citoplasma celular. Com o aumento da população de amastigotas no interior das células, estas se rompem e ocorre a diferenciação de amastigotas em tripomastigotas sanguíneas, os quais podem permanecer na corrente sanguínea, invadir outras células ou serem ingeridos por outro triatomíneo durante seu repasto sanguíneo (Figura 3) (Brener, 1971; Tyler e Engman, 2003; Rassi *et al.*, 2010).

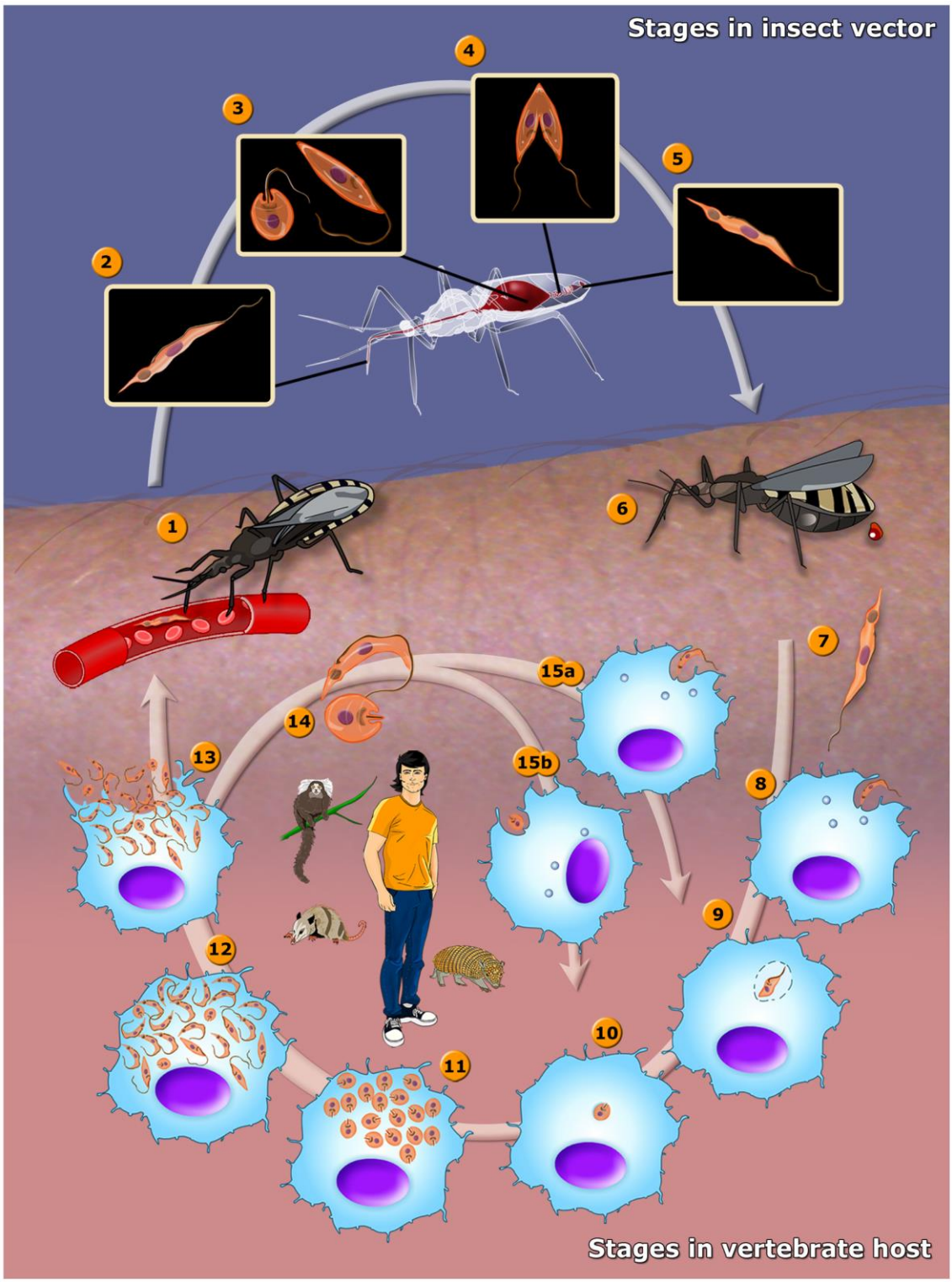

Figura 3: Ciclo de vida do *Trypanosoma cruzi* e seus estágios de desenvolvimento no hospedeiro mamífero e no inseto vetor, o triatomíneo. Fonte: Teixeira *et al.*, 2012.

Trypanosoma cruzi é um parasito heterogêneo. Estudos baseados nas características morfológicas e biológicas do parasito em camundongos infectados, as populações de *T. cruzi* foram classificadas em três tipos ou biodemas: I, II e III (Andrade, 1974). Miles *et al.* (1977, 1978, 1980) utilizando análise eletroforética dos

perfis de isoenzimas, também mostram que *T. cruzi* se agrupava em três grupos distintos, sendo esses denominados zimodemas (Z). Zimodema 1 e Zimodema 3 foram correlacionados ao ciclo silvestre de transmissão do *T. cruzi*, enquanto Z2 ao ciclo domiciliar. Essa diversidade também foi observada quando outros loci isoenzimáticos foram utilizados (Tibayrenc *et al.*, 1986) e quando outras metodologias foram aplicadas, como a análise de fragmentos de restrição de kDNA (Morel *et al.*, 1980), *blotting* cromossômico (Henriksson *et al.*, 1993, 1995), DNA *fingerprint* (Macedo *et al.*, 1992) e marcadores moleculares (Souto *et al.*, 1996; Brisse, 1997; Brisse *et al.*, 1998; Fernandes *et al.*, 1998; dos Santos e Buck, 1999). Ficou estabelecido que *T. cruzi* apresentava duas linhagens distintas: *T. cruzi* I – associada ao ciclo silvestre de transmissão e *T. cruzi* II – associada ao ciclo domiciliar de transmissão (Anonymous, 1999). Os isolados denominados como Z3 ou que apresentavam características híbridas, não foram classificadas dentro desses dois grupos principais.

Com o avanço nos estudos sobre as populações de *T. cruzi*, Brisse *et al.* (2000) utilizando *multilocus enzyme electrophoresis* (MLEE) e *Randomly amplified polymorphic* DNA (RAPD) observaram que *T. cruzi* se subdividia em seis genótipos: TcI correspondente à linhagem *T. cruzi* I; e a linhagem *T. cruzi* II foi subdividida em cinco subgrupos (TcIIa-TcIIe), no qual TcIIb corresponde a linhagem *T. cruzi* II e os subgrupos TcIIa, IIc-e, correspondem às cepas híbridas e aquelas pertencentes ao grupo Z3. Essa subdivisão também foi observada utilizando os genes mini-exon e ribossomais (Brisse *et al.*, 2001). Seis genótipos de *T. cruzi* foram reconhecidos, denominados Unidades Discretas de Tipagem (DTUs) (Zingales *et al.*, 2009). Unidade Discreta de Tipagem tem como conceito o conjunto de populações que são geneticamente semelhantes e podem ser identificadas por marcadores genéticos, imunológicos e moleculares em comum (Tibayrenc, 1998). As DTUs foram nomeadas TcI (TcI), TcII (TcIIb), TcIII (IIa), TcIV (IIc), TcV (IId) e TcVI (IIe). No mesmo ano do novo consenso de nomenclatura dos genótipos de *T. cruzi*, foi descrito um novo genótipo, denominado TcBat (Marcili *et al.*, 2009a; Zingales *et al.*, 2012). Estudos utilizando marcadores nucleares e mitocondriais questionam a existência das seis DTUs de *T. cruzi* e discutem se de fato somente três genótipos (TcI, TcII e TcIII-VI) possam ser considerados (Barnabé *et al.*, 2016).

As DTUs TcI e TcII representam os genótipos de *T. cruzi* mais antigos, também conhecidos como genótipos parentais. Não se sabe ao certo quando essas DTUs se separaram. Estudos apontam que essa separaração tenha ocorrido entre

3-10 milhões de anos (Briones *et al.*, 1999; Machado e Ayala 2001; de Freitas *et al.*, 2006; Lewis *et al.*, 2011). A DTU TcI é a mais abundante e pode ser encontrada desde os Estados Unidos até o Chile (Miles *et al.*, 2009; Cura *et al.*, 2010; Tomasini *et al.*, 2010; Brenière *et al.*, 2016). Foi proposto que a DTU TcI esteja relacionada ao ciclo de transmissão silvestre arbóreo e a marsupiais do genêro *Didelphis* (Yeo *et al.*, 2005; Zingales *et al.*, 2012). Porém, essa DTU já foi isolada em diferentes espécies de mamíferos e triatomíneos, e já foi encontrada em todos os estratos florestais (Jansen *et al.*, 2015). Essa DTU é a responsável por casos de DC nos países do norte da América do Sul, assim como na região Amazônica no Brasil (Añez *et al.*, 2004; Montilla *et al.*, 2002; Coura *et al.*, 2002; Teixeira *et al.*, 2006, Costales *et al.*, 2015).

TcI apresenta uma diversidade intra-específica (Llewellyn *et al.*, 2009a). Estudos moleculares empregando diferentes marcadores demonstraram a elevada heterogeneidade dessa DTU. Em amostras obtidas na Colômbia, TcI foi dividida em quatro grupos ou haplótipos, Ia a Id, que correspoderam a haplótipos de infecção humana, de triatomíneos do genêro *Rhodnius* no ambiente silvetre, ao ciclo de transmissão de *T. cruzi* no ambiente peridomiciliar e a espécie de triatomíneo *Triatoma dimidiata*, e aos ciclos de transmissão silvestres (Herrera *et al.*, 2007). A existência do haplótipo TcIc é questionada, pois o mesmo foi observado somente uma vez (Falla *et al.*, 2009; Herrera *et al.*, 2009). No Chile, um novo haplótipo (Ie) foi descrito, associado ao ciclo doméstico de TcI no Chile e ao ciclo silvestre na Bolívia (Cura *et al.*, 2010). Estudos filogenéticos utilizando marcadores nucleares e mitocondriais demonstraram a existência de uma população de TcI: TcI_{dom} – que ocorre na América Central até a América do Sul e está associada ao ciclo doméstico de transmissão (Ramírez *et al.*, 2012c; Zumaya-Estrada *et al.*, 2012; Segovia *et al.*, 2013). Atualmente, dois genótipos de TcI são reconhecidos: TcI_{dom} associado ao ciclo de transmissão doméstico e TcI silvestre associado ao ciclo de transmissão silvestre (Ramírez *et al.*, 2012a, 2012b, 2012c; Zumaya-Estrada *et al.*, 2012; Villa *et al.*, 2013; León *et al.*, 2015).

Segundo o estudo realizado por Brenière *et al.* (2016), a DTU TcII é a terceira DTU de maior ocorrência no continente americano. No Brasil esse perfil não é observado, já que esta DTU representa a segunda mais isolada no país (Jansen *et al.*, 2015). Foi proposto que TcII esteja associada ao ciclo doméstico de transmissão de *T. cruzi*, com ocorrência nas regiões centrais e sul da América do Sul, sendo a mesma responsável pelos casos graves de DC (Zingales *et al.*, 2012). Entretanto,

TcII já foi descrita na região Amazônica brasileira, na Colômbia, no México e nos Estados Unidos (Dorn *et al.*, 2007; Zafra *et al.*, 2008; Ramírez *et al.*, 2014; Ramos-Ligonio *et al.*, 2012; Ibanez-Cervantes *et al.*, 2013; Lima *et al.*, 2014; Herrera *et al.*, 2015). Apesar da suposta associação ao ciclo doméstico, TcII tem sido encontrada no ambiente silvestre em mamíferos e vetores. O seu encontro já foi descrito em primatas, roedores, marsupiais, morcegos, carnívoros e em triatomíneos dos gêneros *Triatoma* e *Rhodnius* (Jansen *et al.*, 1991; Herrera *et al.*, 2008; Roque *et al.*, 2008, Dario, 2013; Lima *et al.*, 2014; Herrera *et al.*, 2015; Jansen *et al.*, 2015; Lima *et al.*, 2015a; Lisboa *et al.*, 2015; Argibay *et al.*, 2016).

A origem das DTUs TcIII e TcIV ainda é uma questão a ser resolvida, uma vez que é considerado que esses genótipos tenham surgido a partir de eventos de hibridização entre as DTUs TcI e TcII (Westenberger *et al.*, 2005, 2006; Lewis *et al.*, 2011). de Freitas *et al.* (2006) consideram a DTU TcIII como uma linhagem ancestral. A DTU TcIII apresenta ampla distribuição na natureza, sendo encontrada no México (Bosseno *et al.*, 2002, 2009; Ramos-ligonio *et al.*, 2012; Ibanez-Cervantes *et al.*, 2013) e desde a Venezuela até a Argentina (Llewellyn *et al.*, 2009b). Foi proposto que essa DTU esteja associada ao ciclo de transmissão terrestre e a espécie *Dasypus novemcinctus* (Marcili *et al.*, 2009b; Llewellyn *et al.*, 2009b). No Brasil, essa DTU já foi descrita nos biomas Mata Atlântica, Caatinga, Amazônia e Pantanal e em diferentes espécies de mamíferos, incluindo mamíferos arbóreos (Lisboa *et al.*, 2009; Rocha *et al.*, 2013; Jansen *et al.*, 2015). A mesma já foi isolada de cães domésticos (Cardinal *et al.*, 2008) e de triatomíneos dos gêneros *Panstrogylus*, *Rhodnius* e *Triatoma* (Martins *et al.*, 2008; Câmara *et al.*, 2010; Dario, 2013; Brenière *et al.*, 2016).

A DTU TcIV foi descrita nos Estados Unidos da América (Barnabé *et al.*, 2000; Roellig *et al.*, 2013; Herrera *et al.*, 2015; Shender *et al.*, 2016), na Guatemala (Higo *et al.*, 2004; Iwagami *et al.*, 2007) e na América do Sul, onde a sua ocorrência foi observada até na região Amazônica (Zingales *et al.*, 2012). Esta DTU já foi encontrada na Mata Atlântica do sudeste brasileiro, circulando em triatomíneos da espécie *Triatoma vitticeps* (Dario, 2013). De acordo com Lewis *et al.* (2009) e Marcili *et al.* (2009), TcIV está subdivida em dois genótipos: um relacionado a isolados da América do Norte e outro a isolados da América do Sul. Foi proposto que TcIV esteja associado ao ciclo de transmissão arbóreo, a primatas e triatomíneos do gênero *Rhodnius* (Yeo *et al.*, 2005; Marcili *et al.*, 2009c; Zingales *et al.*, 2012). Casos de DC

por essa DTU foram descritos na Amazônia (Monteiro *et al.*, 2012), Bolívia (Santalla Vargas *et al.*, 2012) e Colômbia (Ramírez *et al.*, 2013).

As DTUs TcV e TcVI foram propostas como sendo associonadas a casos de DC na região centro-sul da América do Sul e descritas principalmente na Argentina (Cardinal *et al.*, 2008; Lucero *et al.*, 2016), Bolívia (Barnabé *et al.*, 2011), Chile (Coronado *et al.*, 2009; Arenas *et al.*, 2012; Toledo *et al.*, 2013) e Paraguai (Del Puerto *et al.*, 2010; Fernández *et al.*, 2014). As mesmas já foram encontradas no México, Colômbia, Brasil, Equador e Peru (Barnabé *et al.*, 2000; Garzon *et al.*, 2002; Araújo *et al.*, 2011; Ramos-Ligonio *et al.*, 2012; Ramírez *et al.*, 2013; Sanchez *et al.*, 2013; Guhl *et al.*, 2014; Lima *et al.*, 2014; Jansen *et al.*, 2015), sendo isoladas no ambiente silvestre, a partir de marsupiais e roedores (Brenière *et al.*, 1998, 2016; Rozas *et al.*, 2007; Araújo *et al.*, 2011). Essas DTUs são consideradas híbridas, devido a observação de heterozigose em seus isolados (Sturm *et al.*, 2003; Sturm e Campbell, 2010). Eventos de hibridização, em que há troca genética entre TcII e TcIII, são propostos para explicar esta heterogeneidade (Westenberger *et al.*, 2005; de Freitas *et al.*, 2006).

A DTU TcBat é a mais recente e foi descrita pela primeira vez em morcegos dos gêneros *Myotis* e *Noctilio* nos estados de São Paulo e Mato Grosso do Sul (Marcili *et al.*, 2009a). Sabe-se que sua distribuição é mais ampla, havendo relatos do seu encontro em outros estados brasileiros (Lima *et al.*, 2015a), como também no Panamá (Pinto *et al.*, 2012) e na Colômbia (Trejo-Varón *et al.*, 2013; Ramírez *et al.*, 2014a). Antes restrita a morcegos, essa DTU já foi descrita em humanos, em múmia humana (Guhl *et al.*, 2014; Ramírez *et al.*, 2014b) e sua ocorrência também foi descrita em triatomíneos da espécie *T. sordida* (Cominetti *et al.*, 2014). Análises filogenéticas demonstraram TcBat como uma DTU independente, sendo a mesma geneticamente mais próxima a DTU TcI (Pinto *et al.*, 2012; Franco *et al.*, 2015; Lima *et al.*, 2015a).

Trypanosoma cruzi marinkellei é uma subespécie de *T. cruzi* que está associada a morcegos (Baker *et al.*, 1978). Apresenta ampla distribuição na América Central e América do Sul, sendo relatada em países como Bolívia, Brasil (regiões norte, nordeste e central), Colômbia, Panamá e Venezuela (Marinkelle, 1976, 1982; Barnabé *et al.*, 2003; Cavanzza Jr *et al.*, 2010; García *et al.*, 2012; Ramírez *et al.*, 2014). Sua transmissão na natureza está relacionada a triatomíneos do gênero *Cavernicola*, gênero associado a colônia de morcegos presente em cavernas e palmeiras (Marinkelle, 1976, 1982; Molyneux 1991).

Trypanosoma dionisii, espécie restrista a morcegos até o momento, sua transmissão está associada aos insetos da família Cimicidae (Gardner e Molyneux, 1988b). Essa espécie é capaz de invadir células e se diferenciar na forma tripomastigota (Glauert *et al.*, 1982; Baker, 1985; Oliveira *et al.*, 2009), formar cistos no tecido muscular e cardíaco de seus hospedeiros mamíferos (Gardner e Molyneux, 1988a).

A última espécie reconhecida do subgênero *Schizotrypanum* foi o *Trypanosoma erneyi*, descrita em morcegos da família Molossidae, dos gêneros *Mops* e *Tadarida*, em Moçambique (Lima *et al.*, 2012). Não se conhece os possíveis vetores dessa espécie na natureza. Triatomíneos não foram capazes de se infectar pela mesma. Como os demais *Schizotrypanum*, *T*. *erneyi* foi capaz de se multiplicar na forma amastigota dentro da célula hospedeira, se transformar em tripomastigota e romper a célula infectada, porém essa espécie não foi capaz de infectar camundongos (Lima *et al.*, 2012).

Trypanosoma rangeli, assim como o *T. cruzi*, é um flagelado capaz de infectar diferentes espécies de mamíferos e também apresenta como vetor os triatomíneos, com distribuição na América Central e do Sul (Guhl *et al.*, 1987; D'Alessandro e Saravia, 1992). Esse parasito já foi classificado dentro do subgênero *Herpetosoma* (Hoare, 1972; D'Alessandro e Saraiva, 1992), embora Añez (1982) classifique esse protozoário dentro do grupo Salivaria, levando a classificação de *T. rangeli* para o subgênero *Tejeraia*. *Trypanosoma rangeli* também apresenta uma ampla variedade genética demonstrada por marcadores nucleares e mitocondriais (Macedo *et al.*, 1993; Steindel *et al.*, 1994; Henriksson *et al.*, 1996; Grisard *et al.* 1999, Vallejo *et al.* 2002, 2003; Maia da Silva *et al.*, 2004a, 2004b). Variabilidade nos minicírculos de kDNA, no caso, a presença ou ausência do minicírculo KP1, fez com que *T. rangeli* fosse separado em dois grupos, denominados KP1(+) e KP1(-) (Vallejo *et al.*, 2002 2003, 2007, 2009; Urrea *et al.*, 2005; Cabrine-Santos *et al.*, 2009). O uso de marcadores nucleares *spliced-leader* (SL) e SSU rRNA, mostraram que *T. rangeli* pode ser subdividido em cinco linhagens, denominadas A, B, C, D e E (Maia da Silva *et al.*, 2004a, 2004b, 2007, 2009).

A distribuição de *T. rangeli* na natureza, muitas vezes se sobrepõe com *T. cruzi*, levando a ocorrência de infecções simples ou mistas em hospedeiros mamíferos e triatomíneos, em uma dada região geográfica (Guhl *et al.*, 1987; Grisard *et al.*, 1999; Machado *et al.*, 2001; Ramirez *et al.*, 2002). Até o momento, somente em triatomíneos do gênero *Rhodnius* spp., as formas metacíclicas de *T*.

rangeli foram encontradas nas glândulas salivares e com isso, a transmissão do parasito está associada a esse gênero de triatomíneos (Guhl e Vallejo, 2003). Apesar de *T. rangeli* causar infecção no homem, o parasito não é patogênico, porém a sua infecção pode resultar na produção de anticorpos que podem causar reação-cruzada com os antígenos de *T. cruzi*, levando a um diagnóstico falso-positivo da DC, principalmente em locais onde os seus ciclos se sobrepõem (Hudson *et al.*, 1988; O'Daly *et al.*, 1994; Moraes *et al.*, 2008). No Brasil, cinco casos humanos foram registrados: três na região Amazônica (Coura *et al.*, 1996) e dois casos fora desta região (de Sousa *et al.*, 2008). Além da infecção em humanos, *T. rangeli* foi relatado no estado de Santa Catarina, em roedor da espécie *Phyllomys dasythrix* e em triatomíneo da espécie *Panstrongylus megistus* (Steindel *et al.*, 1991, 1994), na região central e sudeste do Brasil em marsupial da espécie *Didelphis marsupialis* e *Rhodnius neglectus* (Ramirez *et al.*, 2002; Gurgel-Gonçalves *et al.*, 2004, 2012), em *Rhodnius nasutus* no nordeste (Dias *et al.*, 2007) e em quatis no Mato Grosso (comunicação pessoal).

Trypanosoma conorhini é um flagelado que foi descrito em ratos domésticos e sua ampla dispersão está associada a esse hospedeiro (Hoare, 1972; Stevens *et al.*, 1999b). Essa espécie foi descrita infectando triatomíneos, sendo associado a espécie *Triatoma rubrofasciata* (Dias e Seabra, 1943; Hoare, 1972). *Trypanosoma vespertilionis* é uma espécie que foi descrita infectando morcegos no continente europeu (Garder e Molineux, 1988a). Estudo filogenético realizado em isolados de tripanosomatídeos de mamíferos africanos (macaco e civeta) demonstrou que esses tripanosomatídeos se agrupavam dentro do clado *T. cruzi*. Os isolados de *Cercopithecus nictitans* (HochNdi1) e *Nandinia binotata* (NanDoum1) estão filogeneticamente mais relacionados ao *T. vespertilionis* e ao *T. conorhini*, respectivamente. Um isolado de tripanosomatídeo de morcego africano da espécie *Rousettus aegyptiacus* também faz parte desse grupo (Hamilton *et al.*, 2009).

Trypanosoma teixeirae foi o tripanosomatídeo descrito mais recentemente dentro do clado *T. cruzi*. Essa espécie foi isolada de morcegos australianos da espécie *Pteropus scapulatus*. Estudos filogenéticos demonstraram que *T. teixeirae* agrupou-se dentro do clado *T. cruzi*, próximo ao grupo *T. rangeli*/*T. conorhini* (Barbosa *et al.*, 2016).

Foi inserido dentro do clado *T. cruzi* um grupo de tripanosomatídeos isolados de morcegos neotropicais (Lima *et al.*, 2015b). Esse grupo é composto pelo *Trypanosoma wauwau* e por outros tripanosomatídeos do gênero *Trypanosoma* cuja

a espécie não foi determinada até o momento. A espécie *T. wauwau* foi descrita pela primeira vez no Brasil, em morcegos da família Mormoopidae, do gênero *Pteronotus* spp. no estado de Rondônia. Há relatos dessa espécie em outros estados (Pará, Tocantins, Mato Grosso, Rio Grande do Norte e Sergipe) e também em outros países da América Latina, como Guyana, Suriname, Panamá e Guatemala (Lima *et al.*, 2015b; da Costa *et al.*, 2016). Essa espécie não foi capaz de se desenvolver em triatomíneos, de infectar camundongo e nem se desenvolver em células de mamíferos *in vitro* (Lima *et al.*, 2015b). Os tripanosomatídeos de morcegos neotropicais, também conhecidos como *neobats*, foram encontrados em morcegos da família Phyllostomidae, dos gêneros *Trachops* e *Artibeus* no Brasil, no estado do Rio Grande do Norte, na Colômbia e no Panamá (Lima *et al.*, 2015b).

Estudos filogenéticos concluíram que os isolados de marsupiais australianos também estão inseridos dentro do clado *T. cruzi* (Stevens *et al.*, 1999). A primeira espécie inserida foi o *Trypanosoma* sp. H25 (Noyes *et al.*, 1999), atualmente nomeada como *Trypanosoma noyesi* (Botero *et al.*, 2016), descrita inicialmente na espécie de canguru *Macropus giganteus*. Populações de *Trypanosoma* sp. H25 foram isoladas de outras espécies de marsupiais e as mesmas também foram incluídas dentro do clado *T. cruzi*: *Trypanosoma* sp. D15, 17 e 64 isoladas da espécie *Trichosurus vulpecula* (*Cusu comum*) e G8 isolado das espécies de marsupiais *Lagostrophus fasciatus*, *Bettongia lesueur* e *Bettongia penicillate* (Noyes *et al.*, 1999; Paparini *et al.*, 2011; Botero *et al.*, 2013). Apesar de Botero *et al.* (2016) relatarem o desconhecimento dos possíveis vetores de tripanosomatídeos australianos, estes já foram isolados de moscas da família Tabanidae, mosquitos e flebotomíneos (Thompson, 2014).

Trypanosoma livingstonei é a espécie mais basal entre os tripanosomatídeos que compõe o clado *T. cruzi*. Pouco se conhece dessa espécie na natureza. Foi descrita pela primeira vez em morcegos africanos da família Rhinolophidae, da espécie *Rhinolophus landeri*, mas a espécie também já foi relatada em morcegos da família Hipposideridade, na espécie *Hipposideros caffer*, todas em Moçambique (Lima *et al.*, 2013). Infecção experimental em triatomíneos por *T. livingstonei* demonstrou que o mesmo não foi capaz de se desenvolver nesse vetor. É provável que outros vetores estejam envolvidos na sua transmissão (Lima *et al.*, 2013). Esse parasito não foi capaz de infectar células de mamíferos *in vitro* e nem camundongos.

Muito se tem discutido e estudado sobre a origem do clado *T. cruzi*. Os primeiros estudos relacionados a esse assunto demonstravam que esse clado teria

se originado a partir da teoria dos supercontinentes sul. Esta teoria sugere que as espécies de tripanosomatídeos desse clado apareceram a partir de uma espécie de *Trypanosoma* presente em marsupiais, quando a América do Sul, a Austrália e a Antártica formavam um único continente. A teoria ganhou força, uma vez que espécies de tripanosomatídeos de marsupiais australianos foram incluídas no clado *T. cruzi*, agrupando-se em sua região basal (Stevens *et al.*, 1998a, 1999c, 2001). A descrição de espécies de tripanosomatídeos de mamíferos africanos e de novas espécies de tripanosomatídeos em morcegos africanos e americanos, dentro do clado *T. cruzi* (Hamilton *et al.*, 2009; Lima *et al.*, 2012, 2013, 2015b), resultaram na proposta que as espécies do clado *T. cruzi*, tiveram origem a partir de um tripanosomatídeo de morcego e que este foi se adaptando a outras espécies de mamíferos em vários eventos distintos. Essa teoria é conhecida como *bat-seeding hypothesis* (Hamilton *et al.*, 2012).

1.4 Epidemiologia da doença de Chagas

Descoberta há mais de 100 anos por Carlos Chagas, a DC é ainda um problema de saúde pública na América Latina, onde aproximadamente seis milhões de pessoas apresentam a doença e mais de 70 milhões sofrem o risco de adquiri-la (WHO, 2015). A tripanossomíase por *T. cruzi* é considerada primeiramente uma enzootia de animais silvestres, no qual o homem foi incluído no ciclo de transmissão com a sua chegada ao continente americano, há aproximadamente 15 mil anos (Guhl *et al.*, 2000; Afderheide *et al.*, 2004). O número de casos da doença vem aumentando fora da América Latina, como no Japão, Austrália Estados Unidos, Canadá e em países da Europa, se tornando um problema mundial (Schmunis, 2007; Schmunis *et al.*, 2010; Basile *et al.*, 2011; Requena-Mendez *et al.*, 2015; Antinori *et al.*, 2017).

Apesar de autores relatarem a existência de três ciclos de transmissão de *T. cruzi* (silvestre, peridoméstico e doméstico) (Coura, 2008; Coura e Dias, 2009), de fato, a plasticidade do parasito resulta em ciclos de transmissão complexos que envolvem múltiplas variáveis em diferentes perfis epizoóticos. Deste modo, podem ser observados ciclos de transmissão independentes ou sobrepostos, determinados pela ecologia dos hospedeiros, em um mesmo estrato florestal (Herrera *et al.*, 2011; Perez et al., 2012; Rocha *et al.*, 2013). Devido a essa complexidade, cada ciclo de transmissão do parasito deve ser analisado como um evento único.

A DC pode ser consequente a mecanismos de transmissão que vão além da via vetorial contaminativa clássica. Os outros mecanismos de transmissão são: a) congênita; b) transfusional e transplante de órgãos; c) via oral e; e) por acidente de trabalho (Freitas *et al.*, 1952; Nery-Guimarães *et al.*, 1968; Azogue e Darras, 1991; Wendel e Brener, 1992; Riarte *et al.*, 1999; Coura *et al.*, 2002; Bern *et al.*, 2009; Dias e Amato Neto, 2011; Huprikar *et al.*, 2013; Luquetti *et al.*, 2015). As vias de transmissão congênita, transfusional e por transplante de órgãos têm ganhado importância nas duas últimas décadas, devido ao aumento do movimento de migração de indivíduos infectados, habitantes em áreas endêmicas, para regiões consideradas não-endêmicas e esse fato levou ao aparecimento de casos de DC distribuídos globalmente (Gascon *et al.*, 2010; Basile *et al.*, 2011).

O triatomíneo da espécie *Triatoma infestans* foi o principal transmissor intradomiciliar de *T. cruzi* para o homem nas Américas. Nos últimos anos, através de programas de controle da transmissão vetorial, associados à melhoria das condições de moradia das populações e o controle dos bancos de sangue em diversos países da América Central e do Sul, levaram a uma diminuição da incidência da doença em diversos países da América Latina (WHO, 2002; Yamagata e Nakagawa, 2006; Abad-Franch *et al.*, 2013; Salvatella *et al.*, 2014). Porém, em algumas regiões, os casos de DC voltaram a aumentar, como na região do Gran-Chaco, onde *T. infestan* apresenta resistência ao inseticida piretróide (Gurevitz *et al.*, 2013).

No Brasil, casos e surtos de DC emergiram e têm aumentado em áreas antes não consideradas endêmicas. Essas regiões não apresentavam a circulação da espécie *T. infestans* no domicílio, porém outras espécies de triatomíneos que circulam nessas áreas, são capazes de invadir os domicílios e transmitir o parasito (Borges-Pereira *et al.*, 2002; Sangenis *et al.*, 2015). Provavelmente isso seja uma consequência da maior exploração pelo homem do ambiente silvestre, pelo desmatamento, agricultura, caça e ingestão de animais silvestres e até mesmo por ecoturismo. Em todas estas situações, o homem passa a ter um maior contato com o ciclo de transmissão do parasito. A este fato, associa-se a diminuição da fauna de mamíferos, ou seja, a perda de fonte alimentar dos triatomíneos (Roque *et al.*, 2008).

A Amazônia, uma região antes considerada não-endêmica, nos últimos anos tem apresentado elevado casos/surtos de DC, por via vetorial contaminativa e por via oral, esta última principalmente pela ingestão de açaí e bacaba contaminados com fezes de triatomíneos infectados (Coura *et al.*, 2002; Dias, 2006; Beltrão *et al.*,

2009; Pinto *et al.*, 2008; Nóbrega *et al.*, 2009; Valente *et al.*, 2009; Shikanai-Yasuda e Carvalho 2012; Xavier *et al.*, 2014). Além dos casos registrados na região Amazônica, casos extra-Amazônia foram relatados nos últimos anos, como na Bahia, Ceará e Santa Catarina, todos associados ao consumo de alimentos contaminados (Roque *et al.*, 2008; Steindel *et al.*, 2008; Shikanai-Yasuda e Carvalho, 2012).

Nesse novo cenário epidemiológico, são necessárias medidas de vigilância mais complexas, pois cada ciclo de transmissão de *T. cruzi* ocorre em um cenário distinto e as medidas de controle anteriormente utilizadas já não se aplicam. Nesse novo cenário, é necessário conhecer as variáveis que determinam o ciclo de transmissão. Isso significa que o conhecimento da ecologia e do papel dos mamíferos na área é fundamental. Tal conhecimento se dá por meio por meio da determinação da diversidade de espécies mamíferos e seu papel (reservatório) no ciclo de transmissão do parasito. No processo de identificação, a ocorrência e distribuição dos principais genótipos de *T. cruzi* nesses animais é obrigatória (Xavier, 2014).

1.5 Reservatórios silvestres

Definir uma espécie como reservatório é um desafio para qualquer tipo de parasito e a espécie *T. cruzi* não foge desse cenário. Desde de sua descoberta por Carlos Chagas, diversos grupos de pesquisa tentam definir quais espécies de mamíferos atuam como fonte de infecção para os vetores e outros hospedeiros em uma determinaria área. Diversas espécies de mamíferos silvestres já foram encontradas infectadas por *T. cruzi*. Nesse caso, os mamíferos silvestres podem apresentar diferentes papeis na importância como fonte de infecção na natureza e que uma mesma espécie de mamífero pode desempenhar funções na manutenção e transmissão do parasito no tempo e no espaço (Jansen e Roque, 2010; Jansen *et al*., 2015).

Uma só espécie de mamífero infectada pode ser responsável pela manutenção de uma determinada espécie de parasita em área, mas na maioria dos o parasito é mantido por diversas espécies de mamíferos, que passa a constituir um sistema reservatório (Ashford, 1996). Como os mamíferos podem apresentar diferentes papéis na manutenção do parasito, o sistema reservatório apresenta variações e deve ser considerado único dentro de uma escala espaço-temporal e

seu estudo é muito importante para o entendimento do ciclo de transmissão e epizoontia de um parasito (Jansen e Roque, 2010). Os padrões de infecção por *T. cruzi* variam entre as espécies de mamífero e é determinada por suas particularidades, tais como infecções concomitantes com outros parasitos, espécie, sexo, idade, comportamento, vias de transmissão e pelas condições do meio ambiente onde ocorre a interação parasito-hospedeiro (Gürtler e Cardinal, 2015).

A infectividade de uma espécie de mamífero ao vetor, no caso os triatomíneos, é realizada pela presença de formas infectantes do parasito no sangue desses animais e esses devem estar presentes na hora do repasso sanguíneo. A determinação da infectividade de uma espécie é observada através de exames parasitológicos realizados, como exame a fresco, hemocultura e xenodiagnóstico, que detectam a presença do parasito no sangue periférico e, portanto, seu potencial infectivo (Chiari e Brener, 1966; Portela-Lindoso e Shikanai-Yasuada, 2003; Xavier *et al.*, 2012; Jansen *et al.*, 2015). Estando o mamífero infectado, o mesmo se mantém infectado pelo resto da vida. A espécie de animal que apresenta sorologia positiva demonstra a exposição do mamífero ao parasito, mas esse resultado não reflete sua infectividade ao vetor (Herrera *et al.*, 2005; Rademaker *et al.*, 2009; Xavier *et al.*, 2012).

O encontro de uma espécie animal infectada na natureza não define ainda o seu papel na rede de transmissão do parasito. O mesmo dependerá das peculiaridades da interação parasito-hospedeiro e do perfil da infecção. É o conjunto desses fatores que resultará na competência de uma espécie na dispersão do parasito. A utilização de pequenos mamíferos em estudos como indicadores de parasitos multi-hospedeiros é importante, pois esses animais são abundantes na natureza e quando infectados, dependendo da sua taxa de infecção, mostram o impacto sofrido na área devido a alterações ambientais (Roque *et al.*, 2008; Jansen *et al.*, 2015).

1.6 Ordem Didelphimorphia

A ordem Didelphimorphia no continente Americano tem como representante uma única família, a Didelphidae, sendo essa uma das mais antigas e importante reservatório de *T. cruzi* (WHO, 1991; Yeo *et al.*, 2005). A família conta com mais de 70 espécies de marsupiais, podendo ser encontrada do Canadá até a Argentina (Nowak, 1999; Gardner, 2005. Voss *et al.*, 2005). De acordo com Jansen *et al.*

(2015), os didelphídeos são, de fato, importantes reservatórios do parasito, com os gêneros *Didelphis* e *Philander* seus principais representantes.

O gênero *Didelphis* apresenta uma ampla distribuição na natureza devido a sua capacidade de se adaptar a diferentes nichos ecológicos, principalmente a ambientes antroponizados (Olifiers *et al.*, 2005). Por isso, é considerado um biomarcador de ambientes degradados. Esses animais são nômades, a maioria dos machos são solitários, se refugiam em buracos e folhagem de árvores, e são excelentes escaladores. São capazes de utilizar todos os estratos florestais e com isso, estar em contato com os diferentes ciclos de transmissão de *T. cruzi* (Jansen *et al.*, 2015). Sua alimentação omnívora favorece a transmissão por via oral, pela predação de triatomíneos infectados ou de outros pequenos mamíferos. Dependendo da região, a infecção por *T. cruzi* nesses marsupiais pode variar de 11 a 90% (Fernandes *et al.*, 1999; Jansen *et al.*, 2015).

O gênero *Philander* também pode apresentar um papel importante na transmissão de *T. cruzi*, uma vez que o número de hemoculturas positivas pode chegar a 80% em algumas áreas (Jansen *et al.*, 2015). Esse gênero está associado a galerias florestais e não apresenta comportamento sinantrópico. *Monodelphis domestica* foi identificado como principal reservatório de *T. cruzi* em um surto de DC no nordeste brasileiro (Roque *et al.* 2008). *Caluromys lanatus*, *C. parvidens, Lutreolina crassicaudata*, *Marmosa* sp., *Marmosops* sp., *Metachirus nudicaudatus, Micoreus demerarae, M. paraguayanus, M. brevicaudata, Thylamys* sp. são outras espécies de marsupiais que já foram encontradas naturalmente infectadas pelo parasito (Barretto e Ribeiro, 1979; Herrera *et al.,* 2005; Marcili *et al.,* 2009a; Jansen *et al.,* 2015).

1.7 Ordem Rodentia

Considerada a ordem mais diversa dentro dos mamíferos, a ordem Rodentia pode ser encontrada nos diferentes ambientes. Animais dessa ordem colonizam vários tipos de habitats, desde florestas tropicais a desertos, áreas de planatos e planícies, ambiente silvestres e urbanos. Na natureza, são capazes de circular pelos diversos estratos florestais, sendo encontrado roedores terrestres, arbóreos e semiaquáticos (Wilson e Reeder, 2005).

No ambiente silvetre, os roedores compartilham alguns dos seus microhabitats com triatomíneos dos gêneros *Triatoma* e *Panstrongylus* (Carcavallo *et*

al., 1998). Como são os principais alvos de predação, os roedores são importantes no estudo do ciclo de transmissão de *T. cruzi*, uma vez que a mesma pode acontecer devido a via oral. Além disso, algumas espécies de roedores circulam no ambiente peridoméstico e assim participar do ciclo de transmissão nesse ambiente (Roque *et al.*, 2008).

A taxa infecção de roedores silvestres por *T. cruzi* é baixa e isso pode ser resultante do uso restrito do ambiente, que leva a um menor contato com o ciclo de transmissão ou devido a forma como os estudos utilizando esses animais são empregados (Jansen *et al*., 2015). No Brasil, a espécie de roedor mais encontrada infectada pelo parasito é do gênero *Thrychomys* spp. No entato, registros de outras espécies na América Latina já foram encontradas naturalmente infectadas por *T. cruzi*: *Agouti paca*, *Akodon montensis, A. toba*, *Baiomys musculus*, *Calomys expulsus*, *C. callosus*, *Cavia* sp, *Cerradomys subflavus, Clyomys laticeps*, *Dasyprocta* sp., *Echymis chrysurus*, *E. dasytrix*, *Galea spixii*, *Graomys chacoensis, Holochilus brasiliensis*, *Hylaeamys* sp. *Kerodon rupestris, Necromys lasiurus*, *Nectomys squamipes*, *Neotoma floridana, N. mexicana*, *N. micropus*, *Octodon degus*, *Octodontomys* sp., *Oecomys mamorae*, *Oligoryzomys chacoensis*, *O. nigripes, O. stramineus*, *Oryzomys capito*, *O. scotti*, *Peromyscus gossypinus, P. levipes, Proechimys* spp., *Rhiphidomys macrurus*, *Sigmodon hispidus*, e *Tylomys mirae* (Herrera *et al.,* 2005; Yeo *et al.,*2005; Herrera *et al.,* 2007a; Vaz *et al.,* 2007; Roque *et al.,* 2008; Ramsey *et al.,* 2012; Charles *et al.,* 2013; Orozco *et al.,* 2014; Herrera *et al.,* 2015; Jansen *et al.,* 2015).

1.8 Ordem Chiroptera

Considerada a segunda maior ordem entre os mamíferos, a ordem Chiroptera é a única que apresenta mamíferos voadores e do mesmo modo que as aves realizam migrações sazionais. São animais noturnos, se abrigam em buraco de árvores, topo de palmeira e no teto de habitações. A dieta ancestral desse grupo de mamíferos é constituída por insetos. Apresentam hábitos alimentares ecléticos, que incluem o consumo de frutas, pequenos vertebrados, insetos, néctar e sangue (Nowak, 1994; Simmons, 2005; Peracchi *et al.*, 2006; Fenton e Simmons, 2015). Uma vez que são animais de hábitos ecléticos e se adaptam a diversos ambientes, estão muito expostos a infecção por *T. cruzi.* Muitas espécies de morcegos podem compartilhar habitats com triatomíneos e isso facilitaria a sua transmissão,

principalmente pela via oral (Gardner, 1977; Carcavallo *et al.,* 1998; Thomas *et al.*, 2007).

Além de *T. cruzi*, os morcegos são encontrados infectados por outras espécies do gênero *Trypanosoma*, inclusive do clado *T. cruzi e* de espécies ainda não identificadas (Maia da Silva *et al.*, 2009; Marcili *et al.*, 2009a, 2013; Cavazzana Jr *et al.*, 2010; Lima *et al.*, 2012, 2013, 2015a, 2015b; Pinto *et al*., 2012, 2015; Acosta *et al.*, 2014; Ramirez *et al*., 2014; Barbosa *et al*., 2016; da Costa *et al*., 2016). Um grande número de espécies do subgênero *Schizotrypanum* já foram descritos em representates da família Phyllostomidae, principalmente das espécies generalistas dos gêneros *Carollia*, *Artibeus* e *Phyllostomus* (Marcili *et al.*, 2009a, 2013; Cavazzana Jr *et al.*, 2010; Lima *et al.*, 2015a; Acosta *et al*., 2014; da Costa *et al*., 2016).

1.9 *Canis familiaris*

O cão doméstico foi o primeiro modelo experimental de infecção por *T. cruzi* usado por Carlos Chagas. Os cães podem apresentar importância no ciclo de transmissão do parasito, porém essa característica pode variar de acordo com cada região. Em regiões que apresentam triatomíneos e mamíferos silvestres com altas taxas de infecção por *T. cruzi*, os cães costumam estar mais expostos a infecção. Por consequência, são considerados a última barreira entre o ciclo de transmissão silvestre e o ambiente doméstico antes do parasito chegar ao homem. O acompanhamento desses animais através de inquéritos sorológicos pode ser um indicativo de como o ciclo de transmissão do parasito está ocorrendo em uma determinada área (Roque *et al.*, 2008; Roque e Jansen, 2008; Pineda *et al.*, 2011).

No Brasil, já foi demonstrado que os cães estão expostos a infecção por *T. cruzi*, observado nos resultados positivos em exames sorológicos. Porém o isolamento do parasito por técnicas como hemocultivo e xenodiagnóstico é raro (Roque e Jansen, 2008; Noireau *et al.*, 2009; Xavier *et al.*, 2012). Já na Argentina, especialmente na região do Gran Chaco, o cenário é totalmente o oposto, os cães nessa região são um dos principais reservatórios de *T. cruzi* (Gürtler *et al.*, 2007; Gürtler e Cardinal, 2015). Isso mostra como o papel do hospedeiro pode variar em cada região.

1.10 O ciclo enzoótico de *Trypanosoma cruzi* e a doença de Chagas no estado do Espírito Santo

A Mata Atlântica do sudeste brasileiro apresenta ciclos de transmissão enzoóticos robustos de *T. cruzi* (Lisboa *et al.*, 2000, 2006; Monteiro *et al.*, 2006). Embora este seja o bioma com alta biodiversidade no país (Bovendorp *et al.*, 2017), pouco se conhece do papel desempenhado por hospedeiros e vetores nesse ecossistema. Isso se deve a esta região nunca ter sido considerada endêmica para DC e provavelmente ao fato de que o perfil epidemiológico clássico da transmissão de *T. cruzi* e as medida de controle serem bem conhecidos. Porém, devido ao atual cenário de transmissão do *T. cruzi*, nesse caso, a via oral de transmissão e sua complexa epidemiologia, as variáveis que regulam o seu ciclo enzoótico estejam longe de serem conhecidas. Visto isso, é necessário o entendimento de todos os componentes participantes desse ciclo, desde os hospedeiros/reservatórios capazes de manter e dispersar a infecção, vetores e mecanismos de transmissão, até o ambiente onde o caso ocorreu. Esse conhecimento deve abranger a diversidade de mamíferos da área, sua infecção e a infeção de vetores circulantes na área, e quais as DTUs de *T. cruzi* envolvidas no ciclo. Só com o conhecimento desses fatores é que será possível implementar medidas de controle eficazes contra a transmissão da doença.

No estado do Espírito Santo (ES), sete espécies de triatomíneos são relatadas: *Cavernicolla pilosa*, *Panstrongylus diasi*, *P. geniculatus*, *P. megistus*, *Rhodnius domesticus*, *Triatoma tibiamaculata* e *T. vitticeps* (Galvão *et al.*, 2003; Gurgel-Gonçalves *et al.*, 2012). A invasão domiciliar por triatomíneos, na zona rural do ES, acontece com frequência, principalmente nas localidades que apresentam relevo irregular, nas regiões montanhosas do estado (Santos *et al.*, 2006; Leite *et al.*, 2011), sem que se conheçam as variáveis reguladoras deste fenômeno. A espécie de triatomíneo *T. vitticeps* é a mais capturada. A presença de colônias de *T. vitticeps* já foi observada no peridomícilio em alguns municípios do estado (Santos *et al.*, 1969; Silveira *et al.*, 1983; Sessa e Carias, 1986). No ano de 2005, em dois municípios, espécimes adultos e ninfas estavam infectados por *T. cruzi* (Santos *et al.*, 2005). Os espécimes adultos de *T. vitticeps* apresentaram altas taxas de infecção por *T. cruzi* (Silveira *et al.*, 1983; Sessa e Carias, 1986; Dias *et al.*, 1989; Santos *et al.*, 2006; Dario, 2013). Uma diversidade de DTUs de *T. cruzi* foi observada em espécies de triatomíneos coletados no domicílio e peridomicílio: *T.*

vitticeps apresentou infecção por quatro DTUs - TcI, TcII, TcIII e TcIV, e *P. geniculatus* por duas DTUs – TcII e TcIII (Dario, 2013).

2 JUSTIFICATIVA

O estado do ES não é considerado endêmico para DC, uma vez que poucos casos autóctones foram registrados e segundo inquéritos sorológicos, a soroprevalência da população em diferentes idades encontrou-se baixa (Barros *et al.*, 1975; Camargo *et al.*, 1984; Sessa *et al.*, 2002). Porém, a transmissão por via oral tem adquirido importância epidemiológica, como demonstrado pelo elevado número de casos e surtos de doença de Chagas aguda (DCA) no Brasil (Coura *et al.*, 2002; Dias, 2006; Valente *et al.*, 2009; Shikanai-Yasuda e Carvalho, 2012, Coura e Junqueira, 2015). A falta de informação da população sobre a via oral de transmissão, associado ao conceito de que *T. vitticeps* seria um vetor secundário (de menor importância epidemiológica) (Santos *et al.*, 2004) da DC, resultou em 2012, no óbito de uma criança de dois anos de idade decorrente de DCA adquirida por via oral, no município de Guarapari. A criança manuseou um triatomíneo infectado, levando à boca a mão suja com as excretas contaminadas do mesmo (dado não publicado da Secretaria de Estado da Saúde Sesa/ES).

Pode-se observar o seguinte cenário eco-epidemiológico no estado do ES: a presença de um ciclo silvestre de transmissão do parasito, os triatomíneos silvestres altamente infectados invadem as residências, porém não se tem o conhecimento de como essa enzootia é mantida na natureza e quais espécies de mamíferos envolvidas na manutenção desse ciclo. A esta situação enzoótica, soma-se a falta de informação da população sobre a transmissão do parasito e com isso, o surgimento de infecção em humanos. Por isso, é preciso preencher essas lacunas do conhecimento da enzootia de *T. cruzi* nessa região. A proposição de medidas de controle só é possível com o conhecimento de todos os aspectos que envolvem o ciclo enzótico deste parasito. Devido a essa complexidade da relação parasito-hospedeiro envolvendo os tripanosomatídeos, inclusive *T. cruzi*, o seu estudo é de interesse na área científica, pois essa relação é multidisciplinar e serve de interface para o conhecimento e modelo para outros estudos que envolvem essa relação.

3 OBJETIVOS

3.1 Objetivo Geral

Compreender a ecologia do ciclo enzoótico de tripanosomatídeos, com foco especial em *Trypanosoma cruzi*, no ambiente silvestre na Mata Atlântica do estado do Espírito Santo, incluindo uma área de ocorrência de um caso fatal de doença de Chagas aguda, por via oral.

3.2 Objetivos Específicos

- Obter e caracterizar por métodos moleculares o DNA de *T. cruzi* de tecido cardíaco em bloco de parafina, de um caso fatal de doença de Chagas aguda, na zona rural do município de Guarapari;
- Determinar o papel exercido por espécies de mamíferos silvestres não-voadores e domésticos no ciclo enzoótico de *Trypanosoma* sp. na área descrita acima e em suas adjacências;
- Ampliar o estudo do ciclo enzoótico de *Trypanosoma* sp., a partir da inclusão da captura de mamíferos silvestres voadores, na área onde ocorreu o caso fatal de doença de Chagas aguda e em duas áreas com diferentes níveis de invasão de triatomíneos nas residências;
- Realizar a coleta, a identificação das espécies de triatomíneos que circulam nas áreas descritas acima e avaliar sua taxa de infecção por *Trypanosoma* sp.;
- Caracterizar os isolados de tripanosomatídeos obtidos de mamíferos silvestres e vetores, a nível de espécie e, no caso de *T. cruzi*, de DTUs (I-VI e TcBat);
- Avaliar, pelo método de sequenciamento de nova geração, a diversidade de espécies de cinetoplastídeos que circulam na Mata Atlântica do estado do ES;
- Recaracterizar isolados de *T. cruzi* previamente definidos como zimodema 3 para determinar a distribuição geográfica das DTUs de *T. cruzi* TcIII e TcIV, seus hospedeiros mamíferos e triatomíneos em diferentes biomas brasileiros.

4 RESULTADOS

Os resultados obtidos serão apresentados no formato de artigos:

Artigo 1. Ecological scenario and *Trypanosoma cruzi* DTU characterization of a fatal acute Chagas disease case transmitted orally (Espírito Santo state, Brazil). Parasites & Vectors 9 (2016) 477: doi: 10.1186/s13071-016-1754-4.

Artigo 2. High *Trypanosoma* spp. diversity is maintained by bats and triatomines, Espírito Santo state, Brazil. Submetido a revista PLoS One.

Artigo 3. Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats. PLoS Neglected Tropical Diseases (2017) e0005790. doi: 10.1371/journal.pntd.0005790

Artigo 4. Identification of novel mammalian hosts and Brazilian biome geographic distribution of *Trypanosoma cruzi* TcIII and TcIV. Acta Tropica 172 (2017) 173-179. doi: 10.1016/j.actatropica.2017.05.003.

Artigo 1. Ecological scenario and *Trypanosoma cruzi* DTU characterization of a fatal acute Chagas disease case transmitted orally (Espírito Santo state, Brazil).

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No ano de 2012, houve a notificação de um caso de DCA por via oral, na zona rural do município de Guarapari, ES. Três meses após a notificação, foi feita uma primeira viagem ao local para coleta de pequenos mamíferos silvestres não-voadores e domésticos para avaliar o perfil da infecção por *T. cruzi* por métodos parasitológicos e sorológicos. Triatomíneos foram examinados para avaliação da infecção por *T. cruzi*, uma vez que moradores relatam sua frequente invasão as residências. O estudo foi conduzido na área de ocorrência do caso fatal de DCA e em quatro áreas adjacentes, onde há relatos por moradores de triatomíneos invadindo as residências. A hipótese testada seria de que o ciclo enzoótico de *T. cruzi* estaria ocorrendo próximo das residências.

Além disso, nós obtivemos o tecido cardíaco embebido em parafina do paciente que veio a óbito e decidimos realizar a extração de DNA para identificar a DTU de *T. cruzi* que causou a doença. A hipótese testada foi, se tratando de uma infecção em humano, fora da região norte do Brasil, a DTU de *T. cruzi* responsável pelo caso foi a DTU TcII.

Dario *et al. Parasites & Vectors* (2016) 9:477
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Parasites & Vectors

RESEARCH Open Access

Ecological scenario and *Trypanosoma cruzi* DTU characterization of a fatal acute Chagas disease case transmitted orally (Espírito Santo state, Brazil)

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Abstract

Background: *Trypanosoma cruzi* infection via oral route results in outbreaks or cases of acute Chagas disease (ACD) in different Brazilian regions and poses a novel epidemiological scenario. In the Espírito Santo state (southeastern Brazil), a fatal case of a patient with ACD led us to investigate the enzootic scenario to avoid the development of new cases. At the studied locality, *Triatoma vitticeps* exhibited high *T. cruzi* infection rates and frequently invaded residences.

Methods: Sylvatic and domestic mammals in the Rio da Prata locality, where the ACD case occurred, and in four surrounding areas (Baia Nova, Buenos Aires, Santa Rita and Todos os Santos) were examined and underwent parasitological and serological tests. Triatomines were collected for a fecal material exam, culturing and mini-exon gene molecular characterization, followed by RFLP-PCR of H3/AluI. Paraffin-embedded cardiac tissue of a patient was washed with xylene to remove paraffin and DNA was extracted using the phenol-chloroform method. For genotype characterization, PCR was performed to amplify the 1f8, GPI and 18S rRNA genes. In the case of V7V8 SSU rRNA, the PCR products were molecularly cloned. PCR products were sequenced and compared to sequences in GenBank. Phylogenetic analysis using maximum likelihood method with 1000 bootstrap replicates was performed.

Results: None of the animals showed positive hemocultures. Three rodents and two dogs showed signs of infection, as inferred from borderline serological titers. *T. vitticeps* was the only triatomine species identified and showed *T. cruzi* infection by DTUs TcI and TcIV. The analysis of cardiac tissue DNA showed mixed infection by *T. cruzi* (DTUs I, II, III and IV) and *Trypanosoma dionisii*.

Conclusions: Each case or outbreak of ACD should be analyzed as a particular epidemiological occurrence. The results indicated that mixed infections in humans may play a role in pathogenicity and may be more common than is currently recognized. Direct molecular characterization from biological samples is essential because this procedure avoids parasite selection. *T. dionisii* may under certain and unknown circumstances infect humans. The distribution of *T. cruzi* DTUS TcIII and TcIV in Brazilian biomes is broader than has been assumed to date.

Keywords: Mixed infections, *Trypanosoma cruzi* DTU, *Trypanosoma dionisii*, Triatomine, Oral infection, Acute chagas

(Continued on next page)

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(Continued from previous page)
disease

Abbreviation: ACD, Acute chagas disease; BLAST, Basic local alignment search tool; CD, Chagas disease; COLTRYP, Coleção de trypanosoma de mamíferos silvestres, domésticos e vetores; DTU, Discrete typing unit; ELISA, Enzyme-Linked Immunosorbent Assay; ES, Espírito Santo state; GPI, Glucose-phosphate isomerase; IFAT, Indirect Immunofluorescent Antibody Test; LIT, Liver Infusion Tryptose; ML, Maximum likelihood; NNN, Novy Mc Neal Nicole; PCR, Polymerase chain reaction; RFLP, Restriction fragment length polymorphism; Sesa/ES, Espírito Santo state Health Department; ZCC, Zoonosis Control Center

Background

The genus *Trypanosoma* (Trypanosomatidae, Kinetoplastida), which includes the subgenus *Schizotrypanum*, is composed of numerous species that are distributed worldwide. Humans or other mammals can serve as suitable hosts. With the exception of *Trypanosoma cruzi*, other species of this subgenus are restricted to bats. Due to their morphological similarity, these other species have been classically described as *T. cruzi*-like [1, 2]. The biological cycles of *Schizotrypanum* trypanosomes are similar, differing only in the identity of their mammalian hosts and their hemipteran vectors. *Trypanosoma cruzi marinkellei* is transmitted by triatomine insects of the genus *Cavernicola*, and *Trypanosoma dionisii* is transmitted by Cimicidae. Species of *Schizotrypanum* are the only trypanosomes described thus far that infect mammalian cells and multiply inside them as amastigotes [2–4]. There is still much to study regarding *T. dionisii* and *T. c. marinkellei*. Furthermore, despite being the subject of intensive study for more than 100 years, there are still several unanswered questions pertaining to the biology of *T. cruzi*.

Trypanosomiasis by *T. cruzi* is primarily a sylvatic enzooty. This flagellate species is widely distributed in nature, occurring from the southern United States (USA) through southern Argentina and Chile [5]. *Trypanosoma cruzi* circulates among 150 mammal species and is capable of colonizing almost any tissue of its mammalian hosts. It can also be transmitted by dozens of triatomine species [6]. The parasite transmission cycle is complex in nature because, in addition to its tremendous host species diversity, *T. cruzi* is highly genetically diverse [7]. Currently, six Discrete Typing Units (DTUs), TcI to TcVI, in addition to TcBat, are recognized [8–10]. Correlations among DTUs/geographical distribution/host species and pathogenicity are still controversial. Classically, TcII, TcV and TcVI were related to severe human diseases and TcI, TcIII and TcIV were related to the sylvatic cycle [10], but in the Amazon region, Colombia and Venezuela, reports have described human disease by TcI, TcIII and TcIV [11–16]. Although diverse studies have proposed these and other correlations, this topic still requires further clarification. *Trypanosoma cruzi* populations can be selected when they are grown under laboratory conditions or even when natural infections lead to erroneous conclusions regarding DTU variety and putative associations [17, 18]. Similarly, due to the undersampling of hosts and habitats, the ecology of the DTUs of *T. cruzi* is far from well understood.

In Brazil, the efficient control of Chagas disease (CD) due to intra-domiciliary transmission of *T. cruzi* by *Triatoma infestans* has been largely achieved. However, human infection by *T. cruzi* is re-emerging as a food-borne disease in previously non-endemic areas, such as the Amazon region, where it is associated with the ingestion of Açai juice [19–21]. The oral route transmission has been demonstrated to be a highly efficient mechanism of infection [22, 23]. Acute Chagas disease (ACD) cases and outbreaks involving triatomines, which were not previously considered as the main vector species for the contaminative vectorial route, demonstrate that any triatomine can act as a vector when it is related to oral transmission [24]. Moreover, in sylvatic environments, this mechanism is likely the primary means of infection between animals [25].

In the Espírito Santo State (ES), the invasion of domiciles by infected triatomines (mainly *Triatoma vitticeps* but also *Panstrongylus geniculatus*) is frequently reported in rural areas, primarily in mountainous regions that have irregular terrain [26]. *Triatoma vitticeps* is the more prevalent species in ES and can be found in Rio de Janeiro, Minas Gerais and Bahia states [27, 28]. *Triatoma vitticeps* occasionally forms colonies associated with opossum nests in peridomiciles and has high infection rates by flagellates, such as *T. cruzi* [29, 30]. In a study conducted between 2010 and 2012 (Dario, unpublished data), 55 *T. cruzi* isolates derived from *T. vitticeps* and *P. geniculatus* collected in ES subjected to molecular characterization, demonstrated the transmission of four *T. cruzi* DTUs (TcI, TcII, TcIII and TcIV).

Despite the high *T. cruzi* infection rates, *T. vitticeps* has always been considered a secondary vector of CD due to the long time interval between feeding and defecation, reducing the success for the classical triatomine-human route transmission [31]. Since 2007,

according to the Espírito Santo state Health Department (Sesa/ES), only three cases of CD were reported in ES. The last case, in 2012, led to the death of a 2-year-old patient, and epidemiological investigation showed the cause to be ACD acquired by the oral route due to the manipulation of a recently dead (and infected) *T. vitticeps*.

This study had the following objectives: (i) to determine the *T. cruzi* DTU that was related to the fatal case that occurred in ES and (ii) to study the ecology of the transmission cycle of *T. cruzi* in the area where the ACD occurred and in nearby locations where triatomines continuously invade residences. The aim of these objectives is to contribute to the development of local control measures to prevent additional cases of CD.

Methods

Cardiac tissue sample

A cardiac fragment was collected during the *post mortem* patient's exam, embedded in paraffin and sent to Sesa/ES. The cardiac fragment was kindly donated by Dr Janaina A. Shineider Casotti from Sesa/ES.

Study area

The Guarapari municipality is located in the Central coast of the ES state. It is 594,487 km^2 in size and has a population of 105,286; of these, 4758 live in rural areas. The rural area in which the case occurred (and where we examined the domestic and wild animals) is located mostly in mountainous areas, which contain remnants of Atlantic rainforest. The residents of the rural area report banana and coffee agriculture to be the main source of their income.

The study was conducted at five localities: Rio da Prata, where the patient's case occurred; Baia Nova; Buenos Aires; Todos os Santos; and Santa Rita (Fig. 1), which, according to Zoonosis Control Center (ZCC), have registered a higher number of infected triatomines invading residences in recent years.

Small wild mammal capture

Fieldwork was conducted in June 2012, just a few months after the occurrence of the fatal case. Small wild mammals were captured using the following protocol: linear transects were designed in which capture points were established (each point 10 m apart) using alternating Shermann® (H. B. Sherman Traps, Tallahassee, FL, USA) and Tomahawk® (Tomahawk Live Traps, Tomahawk, WI, USA) live traps baited with a mixture of banana, peanut butter, bacon and sardine. The traps were placed near houses and in wild habitats. Seven transects with 10 Sherman and

Fig. 1 Representative map of the locality of the serological results of small sylvatic and domestic mammals. The positive mammals are framed in yellow. Only one rodent was positive at the Buenos Aires location and two were positive at the Baia Nova location. Two dogs were positive at the Baia Nova (n = 1) and Santa Rita (n = 1) locations

10 Tomahawk traps each were placed in the field for five nights, with a total capture effort of 840 traps-night.

All captured animals were manipulated according to the safety manual for the use of wild mammals in research [32] and were anesthetized (9:1 ketamine chlorhydrate 10 % and acepromazine 2 %) for blood sample collection (by cardiac puncture) for parasitological and serological analyses. Only those mammals that required taxonomical confirmation obtained by karyotyping were sacrificed [33].

Dog survey

An active search for dogs was performed at the following locations: Rio da Prata, Baia Nova and Santa Rita. At all of these locations, with the owner's consent, a blood sample was collected by puncturing the cephalic vein under aseptic conditions with Vacutainer® tubes with anticoagulant for serological and parasitological tests. A canine questionnaire was given to the owner that requested the following information: name of the dog, age, sex and the dog's primary function (protection, hunting or company). We considered dogs to be juveniles when they were less than one year of age and adults when they were more than one year of age. Each dog was considered a single event, even if they lived at the same house.

Trypanosoma cruzi survey

To survey for *T. cruzi*, parasitological and serological tests were performed for both small wild mammals and dogs. The following parasitological tests were conducted: (i) fresh blood examination to visualize *T. cruzi* flagellates and (ii) hemoculture, which involved the inoculation of 0.6 ml of blood into two tubes containing Novy Mc Neal Nicole (NNN) medium with Liver Infusion Tryptose (LIT) overlay. The tubes were examined every two weeks for a total of three (for seronegative animals) or five months (for seropositive animals). When positive, the parasites were amplified in LIT, cryopreserved and deposited in the Coleção de Trypanosoma de Mamíferos Silvestres, Domésticos e Vetores, Fiocruz - COLTRYP (Oswaldo Cruz Foundation, Rio de Janeiro - RJ/Brazil). Positive hemoculture results also showed that the animal exhibited notable parasitemia levels.

A serological survey for the detection of anti-*T. cruzi* IgG antibodies was performed using an Indirect Immunofluorescent Antibody Test (IFAT), as described by [34]. The antigens used in the reaction were an equal mixture of parasites derived from the strains I00/BR/00 F (TcI) and MHOM/BR/1957/Y (TcII). The sera of Murinae rodents were tested with anti-rat IgG, while the sera of dogs were tested with anti-dog IgG. All sera were conjugated to fluorescein isothiocyanate (Sigma, St Louis, MO, USA). Echimyidae rodents and marsupials sera were tested using intermediary anti-IgG antibodies for *Thrichomys* spp. and anti-IgG for Didelphidae, respectively, both of which were raised in rabbits. The reaction was revealed using anti-rabbit IgG antibodies conjugated with fluorescein (Sigma, St Louis, MO, USA). The cutoff values for serological results were 1:40 for dogs and marsupials and 1:10 for rodents [35].

To avoid possible cross-reactions with other trypanosomatids, small mammals and dogs were screened to detect anti-*Leishmania* IgG antibodies through IFAT, as described above, using antigens derived from a mixture of *Leishmania infantum* and *L. braziliensis*. Animals were considered positive for *Leishmania* spp. when the serological titers for this parasite were higher than for *T. cruzi* by at least two dilutions and were considered to present both infections when titers were > 1:40 in each assay. Animals were considered to present *T. cruzi* infection when the serological titer was higher that the cutoff value analysis and/or when hemoculture were positive.

In-house Enzyme-Linked Immunosorbent Assays (ELISA) were performed to confirm infections in dogs by *T. cruzi* and *Leishmania* sp. The mean negative control optical density, which added 20 % to this value via a dog serum panel, established the cutoff values in each plate. For each serological reaction, two *T. cruzi* and *Leishmania* sp. positive and negative control sera were added.

Trypanosoma cruzi survey in triatomines

Triatomines were collected inside houses by residents and delivered to the ZCC. Triatomine identification was performed according to the method of Lent & Wygodzinsky [36]. The intestinal contents were removed using scissors and forceps, and examined on a microscope slide with a cover slip under an optical microscope to search for flagellar forms similar to *T. cruzi*. When the exam was positive, the sample was cultured in NNN with LIT overlay and was examined every two weeks for up to five months [37, 38]. In addition, the culture was amplified, cryopreserved and deposited in COLTRYP, as described previously.

Trypanosoma cruzi molecular characterization

DNA extraction

T. cruzi DNA derived from epimastigotes in the logarithmic phase of the cultures and DNA from the cardiac tissue embedded in the paraffin was extracted using the standard phenol-chloroform method [39]. Prior to this step, the cultures were washed with phosphate-buffered saline (PBS) solution and incubated with proteinase K (100 μg/ml) and 0.5 % sodium dodecyl sulfate (SDS) at 56 °C for two hours. For paraffin removal, the cardiac tissue was washed using a previously described method [40, 41]. DNA from the cardiac tissue was quantified

using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, San Jose, CA, USA), and the final concentration was adjusted to 50, 100, 150, 200 and 250 ng/μl. To avoid contamination, only unused aerosol-resistant pipette tips were used, and PCR was conducted in a separate room free of any *T. cruzi* or *T. dionisii* DNA (we do not have *T. dionisii* isolates in our laboratory). Irradiation with ultraviolet (UV) light was also performed on all materials, such as pipets, filter tips, PCR tubes and the cabinet area where the PCR was carried out.

Culture characterization

The parasite characterization of epimastigotes from positive cultures was performed as follows. First, multiplex-PCR was performed to amplify the non-transcribed spacer of the mini-exon gene [42] for the identification of TcI (DTU I), TcII (DTU II/V/VI), zymodeme 3 (DTU III/IV) and *T. rangeli* fragments of 200 bp, 250 bp, 150 bp and 100 bp [43], respectively, as well as mixed infections. Positive samples, except for TcI, were amplified by PCR for the histone 3 (H3) gene [44] followed by restriction fragment length polymorphism (RFLP) analysis. The fragments were digested by the AluI enzyme for discrimination of Z3 (DTUs III or IV).

Electrophoresis of PCR products was carried out in a 2 % agarose gel, which was stained with ethidium bromide solution and visualized under UV light. All reactions included distilled water as a negative control. *Trypanosoma cruzi* strains, representing all DTUs (TcI-SylvioX/10cl1; TcII-Esmeraldocl3; TcIII-M5631cl5; TcIV-92122102R and TcV/VI-SC43cl1), and *T. rangeli* (Choco) samples were used as positive controls.

Cardiac tissue characterization

For DTU identification, DNA extracted from cardiac tissue was used to amplify three nuclear markers: 1f8 (950 bp), glucose-phosphate isomerase (GPI) (652 bp) and the third portion of variable regions 7 and 8 (V7V8) of 18S rRNA gene (650 bp) (45), according to previous studies [46–48]. PCR products were purified using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). In addition, the V7V8 region of SSU rRNA (750–800bp) was amplified as described [49].

V7V8 SSU rRNA PCR products were purified with a Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) and cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA) per the manufacturer's protocol. Sixteen colonies were randomly collected and minipreps were performed with Invisorb Spin Plasmid Mini Two kits (STRATEC Biomedical AG, Germany).

All of the samples were sequenced for both strands of DNA with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA, USA) on an ABI 3730 DNA sequencer available on the PDTIS/FIOCRUZ sequencing platform (Fig. 2). Two clones generated poor sequences and were excluded from the analysis.

Sequence and phylogenetic analysis

The sequences were edited, aligned and corrected using the BioEdit software. The sequences were compared with nucleotide sequences deposited in GenBank using the BLAST (Basic Local Alignment Search Tool) algorithm. Phylogenetic tree construction was performed using Mega 5 software [50]. We used the maximum likelihood (ML) method, employing the best DNA model. The best substitution model was identified as having the lowest Bayesian Information Criterion score (BIC): Hasegawa-Kishino-Yano for the 1f8 gene, Tamura 3+G (a gamma-distributed rate of variation among sites)

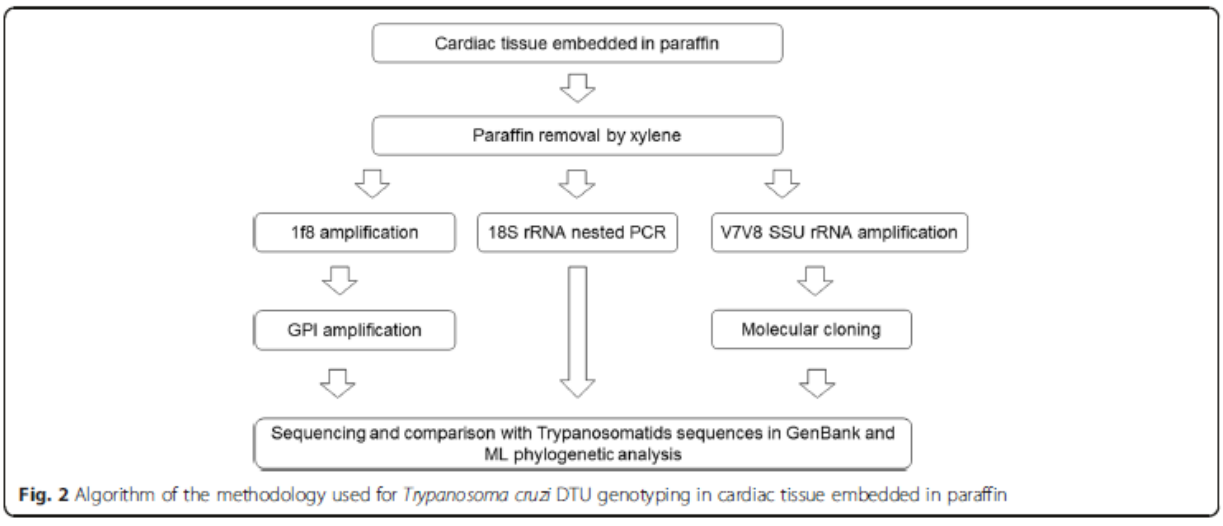

Fig. 2 Algorithm of the methodology used for *Trypanosoma cruzi* DTU genotyping in cardiac tissue embedded in paraffin

parameter for the GPI gene, Kimura 2-parameter for the 18S rRNA gene, and the Kimura 2 + G parameter for V7V8 SSU rRNA, with bootstrapping at 1000 replicates. We used *T. cruzi* (TcI to TcVI), *T. c. marinkellei*, *T. rangeli* and *T. dionisii* sequences from GenBank as references. All sequences analyzed were deposited in the GenBank database under the accession numbers KR905432–KR905446 for the 18S rRNA marker, KT737478 for GPI and KT983981 for 1f8. The GenBank accession numbers can be viewed in Additional file 1.

Results

Identification of *Trypanosoma cruzi* DTUs and *T. dionisii* in cardiac tissue

In this study, we decided to use three nuclear markers to genotype the DNA obtained from the cardiac tissue: 1f8, GPI and 18S rRNA genes. Nuclear markers cluster separately with *T. cruzi* DTUs (TcI to TcIV) [44, 51–53]. For the 18S rRNA gene, which exists as thousands of copies in the genome, we used two different regions for characterization: a variable region (V7V8) and a third portion of this variable region [45, 48]. 18S rRNA gene allows identification of different species within the subgenus *Schizotrypanum* and is considered a reliable marker to distinguish between *T. cruzi* DTUs [45, 54–57]. The 1f8 gene allows discrimination between the DTUs TcI and TcIV [44, 46]. The GPI gene, which is also considered a suitable target to distinguish between DTUs, revealed that TcI, TcII, TcIII and TcIV are robust monophyletic groups [5, 13, 52, 58, 59].

We demonstrated via a PCR method the occurrence of four sympatric *T. cruzi* DTUs (TcI, TcII, TcIII and TcIV) in the cardiac tissue of a patient who died in the acute phase of Chagas disease. This is the first time that we observed such a diversity of DTUs in a human case. Furthermore, we also detected *T. dionisii*, a *Trypanosoma* species that has only been described in bats until now, by phylogenetic and additional alignment analyses (Additional file 2).

The DTU TcIV was detected in cardiac tissue by employing 1f8 and GPI as molecular targets. The sequence obtained by amplification of the 1f8 gene was subjected to BLAST algorithm and shown to be similar to both TcIII (CM17 and M5631) and TcIV (CANIIII and M4167) strains (96–97 %). According to the ML phylogenetic analysis, the DNA sequence clustered together with the TcIV reference strain CANIII (Fig. 3a). To confirm this result, we sought to determine the

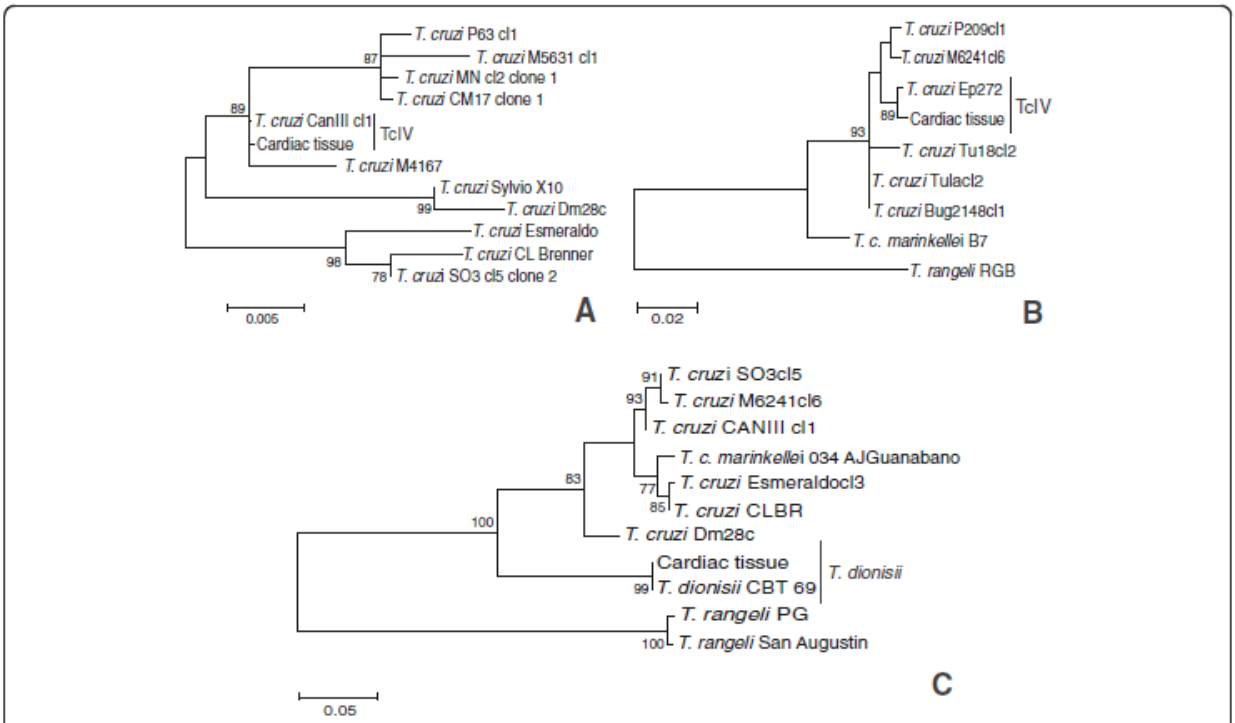

Fig. 3 Molecular phylogenetic analysis by maximum likelihood methods. **a** for 1f8 gene with HKY parameter and bootstrap value of 1000 replicates; **b** for GPI gene with T92 + G parameter and bootstrap value of 1000 replicates; and **c** for 18S rRNA K2 parameter and bootstrap value of 1000 replicates. The 1f8 and GPI DNA sequences clustered with DTU TcIV reference strains CANIII (A) and Ep272 (B), and 18S rDNA sequence clustered together with *T. dionisii* isolates. *T. rangeli* isolates RGB, PG and San Augustin were used as the outgroup, except for 1f8 gene, because no sequence is available on GenBank

occurrence of this DTU based on GPI. With this target, the nucleotide sequence showed 99 % identity to TcIV (Ep272, Saimiri3cl1 and CANIII) strains. In addition, the phylogenetic analysis clustered the sequence with the TcIV reference strain Ep272 (Fig. 3b). DNA from the cardiac tissue was subjected to PCR and molecular cloning of the V7V8 SSU rRNA region. The BLAST algorithm showed that one clone presented a similarity of 100 % to TcI (Dm28c strain), one clone presented a similarity of 99 % to TcII/VI (isolate TCC873, Tulahuencl2, TCC2558), three clones presented similarities of 99 % and 100 % to TcIII (MT3663 strain, isolates TryCC1356 and 1078) and six clones presented similarities of 99–100 % to TcIV (Jose Julio, MT4167 and CanIII strains). The phylogenetic analysis clustered the clones with TcI ($n = 01$), TcII/TcVI ($n = 01$), TcIII ($n = 03$) and TcIV ($n = 06$) (Fig. 4).

Furthermore, three clones were identified as *T. dionisii* via phylogenetic analysis using the ML method. The identification of *T. dionisii* also occurred via sequence analysis of the 18S rRNA gene. The sequence obtained from 18S rRNA nested-PCR was subjected to BLAST algorithm. This sequence showed 100 % identity with *T. dionisii* isolates CBT 63, 64 and 69. In the phylogenetic analysis, the sequence clustered with *T. dionisii* isolates, confirming the presence of *T. dionisii* in this human cardiac tissue sample (Figs. 3c and 4).

Small mammal capture and *Trypanosoma cruzi* infection

The study area clearly had a reduced mammalian fauna density and diversity. Despite an extensive capture effort involving 840 traps for five nights, only one species of Rodentia (*Trinomys paratus* (n = 5)) and four species of Didelphimorphia [*Didelphis aurita* (n = 1), *Philander frenatus* (n = 1), *Metachirus nudicaudatus* (n = 2) and *Marmosops incanus* (n = 2)] were captured. Additionally, we examined four synanthropic rodents (*Rattus rattus*) that were collected from the peridomicile area. The

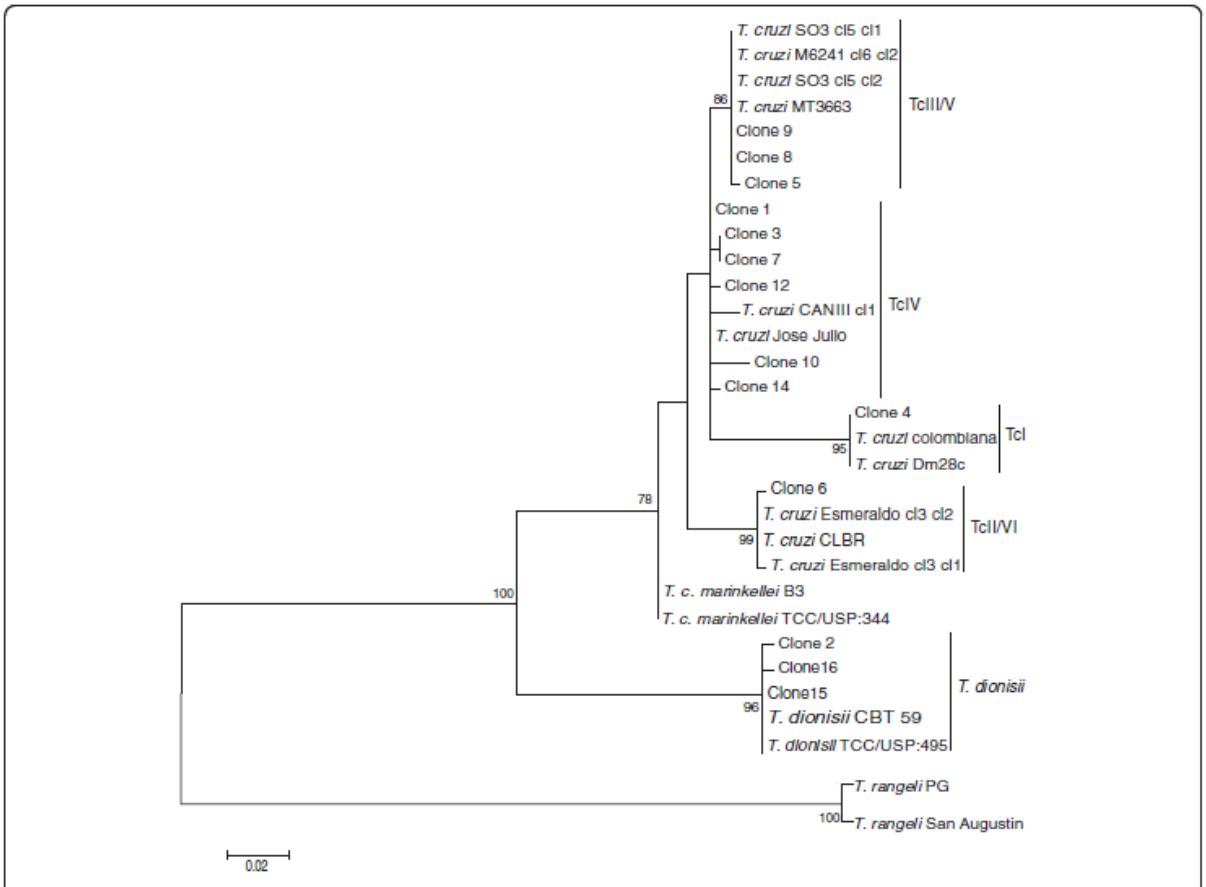

Fig. 4 Molecular phylogenetic analysis by maximum likelihood of the V7V8 SSU rRNA gene. K2 + G parameter and bootstrap value of 1000 replicates. The clones from cardiac tissue clustered with Jose Julio and CANIII strains (TcIV); SO3cl5 clone1 and clone2, M6241cl6 clone2 and MT3663 strains (TcIII/TcV); Dm28c and Colombiana strains (TcI); Esmeraldocl3 clone1 and clone2, and CLBR (TcII/TcVI); and *T. dionisii* isolates TCC/USP:495 and CBT 59. *T. rangeli* isolates PG and San Agustin were used as the outgroup

relative abundance of mammals captured was higher for Didelphimorphia, which represented 54.5 % of the mammals captured, whereas the percentage of Rodentia represented 45.5 %.

None of the sylvatic animals had parasites based on the examination of fresh blood or hemoculture. In serological tests, only three specimens of *T. paratus*, two from Baia Nova and one from Buenos Aires locations, were found to be infected with *T. cruzi* (Fig. 1). The three positive rodents presented only borderline serological titers (1:20) (Table 1).

Dogs and *Trypanosoma cruzi* infection

Dogs are considered sentinel hosts [60], signaling that *T. cruzi* cycle is occurring in a peridomicile area. Fifteen dogs were examined from the following locations: Rio da Prata -house of the infected patient (n = 10), Baia Nova (n = 2) and Santa Rita (n = 3). Of this total, only two, one from Baia Nova and the other from Santa Rita, displayed only borderline IFAT tests for *T. cruzi* in IFAT (both 1:40) and ELISA (Table 1). All of the dogs (n = 10) from the house where the patient lived were negative for *T. cruzi* based on serological and parasitological tests. This finding means that the dogs from Rio de Prata have not been exposed to *T. cruzi* infection.

Triatomine infection and molecular characterization

Five triatomine specimens were delivered to the ZCC during our expedition. All of the specimens were identified as *T. vitticeps*. These specimens were from São Miguel ($n = 1$), Rio da Prata ($n = 2$) and Baia Nova ($n = 2$). Four *T. vitticeps* (75 %) had *T. cruzi* based on the intestinal content exam. Only one sample from Rio da Prata (LBT 3214) was negative.

Four positive samples were subjected to culture and three samples presented epimastigote forms. Molecular characterization using the non-transcribed spacer of the mini-exon gene was performed, which classified the samples as TcI (DTU TcI) - LBT 3211 and Z3 (DTU TcIII/TcIV) - LBT 3198 and LBT 3210 (Fig. 5a). To discriminate between TcIII and TcIV, which is not possible with the mini-exon gene, the LBT 3198 and LBT 3210 samples were further characterized at the DTU level using the H3 marker, resulting in their classification as TcIV (Fig. 5b).

Discussion

Another fatal ACD case acquired via the oral route has been reported. Studies investigating this mechanism have attracted attention due to the high number of cases and outbreaks in Brazil, especially in the Amazon region, in addition to other South American countries [61–63].

Table 1 Serological survey in sylvatic and domestic mammals examined in rural areas of the Guarapari municipality

Mammal species	Family	Location	IFAT	ELISA
Marmosops incanus	Didelphidae	Baia Nova	1:10	NP
Marmosops incanus	Didelphidae	Baia Nova	Negative	NP
Didelphis aurita	Didelphidae	Baia Nova	Negative	NP
Trinomys paratus ($n = 2$)	Echimyidae	Baia Nova	1:20	NP
Trinomys paratus	Echimyidae	Baia Nova	1:10	NP
Trinomys paratus	Echimyidae	Baia Nova	Negative	NP
Metachirus nudicaudatus	Didelphidae	Buenos Aires	Negative	NP
Trinomys paratus	Echimyidae	Buenos Aires	1:20	NP
Rattus rattus	Muridae	Santa Rita	Negative	NP
Metachirus nudicaudatus	Didelphidae	Todos os Santos	Negative	NP
Philander frenata	Didelphidae	Todos os Santos	1:40	NP
Rattus rattus ($n = 2$)	Muridae	Todos os Santos	1:10	NP
Rattus rattus	Muridae	Todos os Santos	Negative	NP
Canis familiaris	Canidae	Baia Nova	1:20	Negative
Canis familiaris	Canidae	Baia Nova	1:40	Positive
Canis familiaris ($n = 8$)	Canidae	Rio da Prata	1:20	Negative
Canis familiaris	Canidae	Rio da Prata	Negative	Negative
Canis familiaris	Canidae	Santa Rita	1:20	Negative
Canis familiaris	Canidae	Santa Rita	1:40	Positive
Canis familiaris	Canidae	Santa Rita	1:40	Negative

Abbreviations: *NP* not performed, *IFAT* indirect immunofluorescence antibody test, *ELISA* Enzyme-Linked Immunosorbent Assay
Rodents with serological titles above 1:10 and marsupials and dogs with serological titles above 1:40 were considered positive

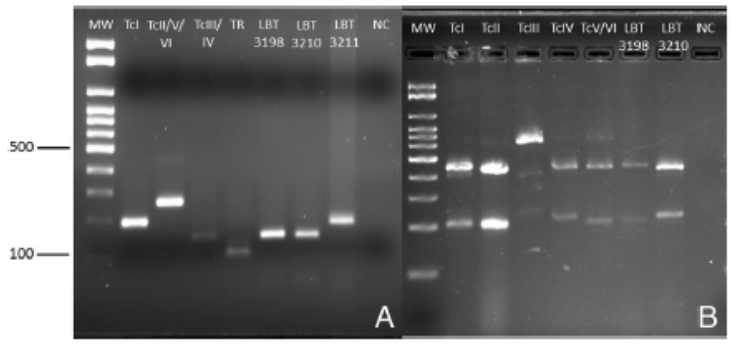

Fig. 5 *Trypanosoma cruzi* DTU molecular characterization of *T. vitticeps* isolates LBT 3198, LBT 3210 and LBT 3211. **a** Products amplified by the mini-exon gene and analyzed by electrophoresis on 2 % agarose gel stained with ethidium bromide. The sample LBT 3198 and LBT 3210 presented an amplicon size of 150 bp and was identified as zimodeme 3 (DTU TcIII-TcIV). The sample LBT 3211 was identified as TcI, as it presented an amplicon size of 200 bp. **b** PCR-RFLP with histone 3 gene/AluI restriction enzyme for *T. cruzi* DTU genotype on 3 % agarose gel stained with ethidium bromide. The samples LBT 3198 and LBT 3210 presented amplicons sizes of 210 bp and 440 bp, genotyping these samples as DTU TcIV. DTU reference strains: TcI-SylvioX/10cl1, TcII-Esmeraldocl3, TcIII-M5631cl5, TcIV-9212102R, TcV/TcVI-SC43cl1, *T. rangeli*-Choco, NC:negative control

This new/ancient epidemiological profile of the disease must be studied from a novel perspective because the control measures that are used for the elimination of *T. infestans* (domiciliary vector) are not well suited for the current threat. Moreover, all rural locations in Guarapari municipality are now at risk of experiencing CD because residents continue to observe triatomine invasions in their residences and, further, are not aware of the oral route of transmission of *T. cruzi*.

In this study, valuable information was collected regarding ACD due to the unfortunate death of a young patient. Indeed, based on the direct evaluation of infected tissue, a mixed infection by four *T. cruzi* DTUs (TcI, TcII, TcIII and TcIV) was detected in a concomitant infection with *T. dionisii*, a bat trypanosomatid. Fixed biological material, such as tissue embedded in paraffin, for diagnoses is an important source to investigate and understand epidemiology [64]. DNA recovered from this type of material is well maintained and does not result in non-specific bands [65–67]. Moreover, *T. cruzi* has already been diagnosed from mummies [68–70], whose tissue is highly degraded.

This is the first report of a mixed *T. cruzi* infection by four DTUs, identified using DNA extracted directly from human cardiac tissue. Mixed *T. cruzi* DTU infections have been described in several different mammal and triatomine species. Cura et al. [71] reported mixed infections by TcI-IV, TcI-III/IV and TcIII-TcIV in different triatomine species on the American continent. In the Amazon region, Lima et al. [72] reported that *R. pictipes* exhibited a mixed infection of TcI and TcII. Concomitant infections (TcI-TcII and TcII-TcIV) were detected in tissue samples of rodents from the USA [73]. Mixed infections by two or three DTUs in free-living wild mammals have also been described [74]. In humans, mixed infections by two or three DTUs in chronically infected patients have been described in Colombia, Argentina, Chile and in Bolivian patients' residing in Spain [75–79].

T. vitticeps specimens exhibiting mixed infections with TcI-IV, TcII-III-IV and TcI-TcII in ES have previously been observed by our group (Dario, unpublished data). In the present study, observations were conducted in Guarapari, a municipality of ES, where we observed mixed infections of *T. vitticeps* by TcI and TcIV. The finding of simultaneous infection by four *T. cruzi* DTUs in cardiac tissue is consistent with the genotype diversity observed in the state. A high diversity of *T. cruzi* DTUs is not usually observed in other regions in Brazil: in Poço das Antas (Rio de Janeiro state), where TcII is the main DTU infecting monkeys, TcI infection is rare; in Piauí state, TcI and a few cases of TcII infection have been reported; in Santa Catarina state, both TcI and TcII were reported; and in Pará state, TcI infection has been reported [21, 37, 80–82]. In addition, mixed DTU infections in *T. vitticeps* may be attributable to differentially infected blood meal sources or mixed DTU infections in mammals.

The DTUs that we detected infecting the patient have already been described in human infections in addition to presenting a large host range. TcI is the most widespread DTU in nature and is primarily responsible for human infections in the Amazon basin in Brazil, Colombia and Venezuela [11, 16, 83]. TcII, which was classically associated with the Southern cone of South America [10, 84], has already been found in the Brazilian Amazon basin, Colombia, Mexico and USA [15, 72, 73, 85, 86]. In nature, TcIII, which was classically associated with the terrestrial transmission cycle and the armadillo from *Dasypus novemcinctus*, has previously been identified infecting dogs, rodents and marsupials [5, 74, 87–89]. TcIV has also

demonstrated a much larger host range as this DTU has been isolated from primates, coati, marsupial, bats, rodent species and *Rhodnius brethesi* triatomines [73, 74, 90]. This study has shown for the first time that TcIII and TcIV are related to human infection in ES. Until now, these DTUs have only been reported in the Amazon—TcIII and TcIV [12, 13], in Bolivian patients in Spain-TcIV [79], in the Southern and Northeast parts of Brazil—TcIII [91, 92], in Minas Gerais state-TcIII [93] and in Argentina-TcIII [94]. These findings show that the distribution of TcIII and in particular TcIV is higher than has been assumed up to now and confirm that these two DTUs are involved in human infection. Moreover, this finding warns of the danger of establishing associations between a parasite species or a parasite genotype and pathogenicity, course of infection or epidemiology. Indeed, a disease is the result of the interaction of several variables, including the peculiarities of a host specimen.

For the first time, the presence of *T. dionisii* has been observed in a human sample. This species, which is closely related to *T. cruzi*, is able to invade mammalian cells as previously demonstrated experimentally [95, 96] and to form cysts in cardiac tissue [97]. We detected *T. dionisii* directly from cardiac tissue more than two weeks after the infection of the patient. This finding indicates that we demonstrated that *T. dionisii* is able to invade and differentiate in human cells. Bat trypanosomatid infections are likely self-resolving and we hypothesize that we would not have been able to detect the parasite at later stages of infection. *T. dionisii* is widely distributed in ES and has been reported in the northern part of the state (Pinheiros municipality) in the bat species *Sturnira lillium*, *Carollia perspicillata*, *Desmodus rotundus*, *Myotis nigricans* and *Lophostoma brasiliensis* [98], and particularly in Guarapari, in bats of *Carollia* species (Dario, unpublished data). The vector of *T. dionisii* is still unknown. There has only been one report on Cimicid insects that maintained an experimental infection by *T. dionisii* [99].

Monogenetic and non-human digenetic trypanosomatid species have already been described to infect humans. Trypanosomes from the subgenus *Herpetosoma* (*T. lewisi*, *T. lewisi*-like), subgenus *Dutonella* (*T. vivax*), subgenus *Trypanozoon* (*T. b. brucei* and *T. evansi*), subgenus *Nannomonas* (*T. congolense*) and the genus *Leptomonas* (*Leptomonas seymouri*) were identified as infecting humans in Africa and Asia [100–103]. *Leishmania tarentolae*, a species that typically occurs in lizards, has been identified in mummies [104]. Additionally, TcBat, a *T. cruzi* DTU reported in bats from different Latin American countries [8, 56, 105], has been described in mummies [71] and in a child from Colombia [106]. These findings show that trypanosomatids are biologically plastic and may be host generalist parasites.

Parasite maintenance via cultivation in axenic media or by passaging in experimentally infected animals results in selective pressure [17, 79, 107], making it difficult to detect the assemblage of clonal components of a given *T. cruzi* isolate. Additionally, during the course of infection, the infected host exerts selective pressures on the parasite population. As a result, during the course of the infection or due to differences in the growth rates of specific populations [108, 109], some populations may be preferentially selected over others [107]. Our study reinforces the importance of the direct characterization of biological samples. In this case, which was a case of CD acquired by the oral route; it is possible that the identification of all of the *T. cruzi* DTUs and *T. dionisii* would not have been possible if we had analyzed the hemoculture of the patient or the same cardiac tissue in the chronic phase of the disease.

In nature, the detection of several different parasite species in the same host is common. Furthermore, the impact of mixed infections on a host is still not well understood. Numerous models and experimental studies have been carried out and in general, they have concluded that mixed infections can affect the host immune response and result in increases in virulence [110, 111]. Female tamarins infected with *T. cruzi* and Acanthocephala (intestinal helminths) may experience increases in the rates of *T. cruzi* infection [112, 113]. Araujo et al. [114] observed that isolated TcI grew faster under culture conditions than TcI in mixed infections with TcII. Single infections, if they occur in nature, are rare [115, 116]. However, the complexity of this phenomenon means that there are several aspects that still require clarification. Mixed infections may occur after serial exposure to different genotypes and species of parasites at different time intervals and by distinct routes. Here, we know for sure that the patient was infected by the oral route with the four *T. cruzi* genotypes and *T. dionisii* on the same occasion. The fact that *T. cruzi* and *T. dionisii* are within the subgenus *Schyzotrypanum* and were found to occupy the same habitat leads to the hypothesis that an increase in virulence and pathogenicity may occur through competition for resources or alterations in doubling time or impairment in immune clearance, or through a combination of all of these factors.

We observed that the Atlantic rainforest remnants in the study area are suffering from degradation, as demonstrated by the low capture success (1.3 %) during the fieldwork. However, despite degradation, six different mammal species were captured, indicating that the area contains a moderate diversity of small wild mammal species. It has been reported that *T. vitticeps* presents high *T. cruzi* infection rates (more than 60 % of the triatomines are infected) [30, 117, 118]. The absence of positive hemocultures and borderline serological titers

showed that the animals examined presented low force of infection and strongly suggests that triatomines are not being infected in the peridomicile area but in distant forest fragments. In this case, the capacity for flight in *T. vitticeps* may be much higher than reported for other insects of this genus. Nothing is known concerning the flight capacity of *T. vitticeps*. *T. infestans* is capable of flying 200 m or more [119, 120] because it is capable of flying with wind assistance [121]. Another explanation for the high *T. cruzi* infection rates in *T. vitticeps* is that other non-sampled mammals, such as armadillos and bats, can be responsible for parasite maintenance [37, 87, 89, 122–124].

Conclusion

In conclusion, our results indicate that (i) mixed infections in humans may be more common than has been recognized up to now and should be taken into consideration in future studies; (ii) the distribution of *T. cruzi* TcIII and TcIV in Brazilian biomes is broader than has been assumed until now, and the putative associations between *T. cruzi* DTUs and host species, geographical distribution and pathogenicity still pose epidemiological challenges; and (iii) *T. dionisii* is able, at least, to colonize human heart muscle cells.

Additional files

Additional file 1: Table S1. *Trypanosoma cruzi* and *Trypanosoma dionisii* GenBank accession numbers for the 1f8, GPI and 18S rRNA genes. (DOCX 13 kb)

Additional file 2: Table S2. Alignment sequences from *Trypanosoma cruzi*, *Trypanosoma cruzi marinkellei*, *Trypanosoma dionisii* species isolates, and V7V8 SSU rRNA clones obtained from cardiac tissue. The dots are representing same base position for *T. cruzi*. The stars are representing same base position for *T. dionisii*. (*DOCX 17 kb*)

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Availability of data and materials

All sequences analyzed were deposited in GenBank under the accession numbers KR905432–KR905446 for the 18S rRNA gene, KT737478 for GPI and KT983981 for 1f8. The GenBank accession numbers can be viewed in Additional file 1.

Authors' contribution

MAD, ALRR and AMJ conceived and designed the experiments. PSD, ALRR and AMJ performed the fieldwork. MAD, MSR and JHSB performed and analyzed the molecular characterization. SCCX and JHSB performed and analyzed the serological characterization. MAD, ALRR and AMJ wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The capture of small wild mammals was licensed by the Sistema de Autorização e Informação em Biodiversidade - SISBIO of the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA)-permanent license number 3365-1. Blood sample collection and euthanasia were performed and supervised by the Federal Counsel of Medical Veterinary under resolution number 1.000 approved on May 11th, 2012, according to the Ethical Committee for Animal Use of the Oswaldo Cruz Foundation (license 0015-07).

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Additional file 1 *Trypanosoma cruzi* and *Trypanosoma dionisii* GenBank accession numbers for the 1f8, GPI and 18SrRNA genes.

Sample	Gene	Molecular characterization	GenBank accession number
Cardiac tissue	1f8	DTU TcIV	KT983981
Cardiac tissue	GPI	DTU TcIV	KT737478
Cardiac tissue	18S rRNA	*T. dionisii*	KR905432
Clone 1	V7V8 SSU rRNA	DTU TcIV	KR905433
Clone 2	V7V8 SSU rRNA	*T. dionisii*	KR905444
Clone 3	V7V8 SSU rRNA	DTU TcIV	KR905434
Clone 4	V7V8 SSU rRNA	DTU TcI	KR905435
Clone 5	V7V8 SSU rRNA	DTU TcIII	KR905436
Clone 6	V7V8 SSU rRNA	DTU TcII	KR905437
Clone 7	V7V8 SSU rRNA	DTU TcIV	KR905438
Clone 8	V7V8 SSU rRNA	DTU TcIII	KR905439
Clone 9	V7V8 SSU rRNA	DTU TcIII	KR905440
Clone 10	V7V8 SSU rRNA	DTU TcIV	KR905441
Clone 12	V7V8 SSU rRNA	DTU TcIV	KR905442
Clone 14	V7V8 SSU rRNA	DTU TcIV	KR905443
Clone 15	V7V8 SSU rRNA	*T. dionisii*	KR905445
Clone 16	V7V8 SSU rRNA	*T. dionisii*	KR905446

Additional file 2 Alignment sequences from *Trypanosoma cruzi*, *Trypanosoma cruzi marinkellei*, *Trypanosoma dionisii* species isolates, and V7V8 SSU rRNA clones obtained from cardiac tissue. The dots are representing same base position for *T. cruzi*. The stars are representing same base position for *T. dionisii*.

Isolate/clone	**Nucleotide position**										
	190	200	221	316	393-401	414-415	430-432	448	465	481-483	494
***T. cruzi* Dm28c**	T	A	A	C	TTATTCCA	TT	TGG	T	A	GCA	T
***T. cruzi* Y**	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
***T. cruzi* 3663**	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
***T. cruzi* CANIII cl1**	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
***T. cruzi* SO3 cl5 clone 2**	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
***T. cruzi* TCC/USP: 499**	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 1	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 3	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 4	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 5	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 6	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 7	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 8	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 9	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 10	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 12	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
Clone 14	.	.	.	.	. . . . .	. .	. . .	.	.	. . .	.
***T. c. marinkellei* TryCC 1093**	.	.	.	A	. . . . .	- -	. . .	.	.	. . .	.
***T. dionisii* TCC/USP: 495**	C	T	G	A	ATGATATC	CA	GCA	G	G	ACG	C
Clone 2	*	*	*	*	* * * * * * * *	* *	* * *	*	*	* * *	*
Clone 15	*	*	*	*	* * * * * * * *	* *	* * *	*	*	* * *	*
Clone 16	*	*	*	*	* * * * * * * *	* *	* * *	*	*	* * *	*

- - Gap position

Artigo 2. High *Trypanosoma* spp. diversity is maintained by bats and triatomines, Espírito Santo state, Brazil.

Maria Augusta Dario, Cristiane Varella Lisboa, Luciana M. Costa, Ricardo Moratelli, Monique Pereira Nascimento, Leonora Pires Costa, Yuri Leite, Martin S. Llewellyn, Samanta Cristina das Chagas Xavier, André Luiz Rodrigues Roque, Ana Maria Jansen.

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No trabalho anterior, vimos que o ciclo enzoótico de *T. cruzi* na zona rural do município de Guarapari ocorria longe das residências, uma vez que os pequenos mamíferos não-voadores e cães não apresentaram infecção pelo parasito. Nesse trabalho, nós ampliamos a busca dos possíveis hospedeiros de *Trypanosoma* sp. na área onde o caso fatal de DCA ocorreu e em duas áreas da zona rural do município de Guarapari: uma onde há relatos por moradores da invasão de triatomíneos nas residências e outra onde não há relato dessa invasão. Além disso, podemos entender um pouco mais sobre a epidemiologia da DC na região. Nós reavaliamos a infecção por tripanosomatídeos em pequenos mamíferos silvestres não-voadores, cães e triatomíneos, e decidimos estender o estudo para outra espécie de mamíferos silvetres: os morcegos.

PLOS ONE

RESEARCH ARTICLE

High *Trypanosoma* spp. diversity is maintained by bats and triatomines in Espírito Santo state, Brazil

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Abstract

The aim of this study was to reevaluate the ecology of an area in the Atlantic Forest, southeast Brazil, where Chagas disease (CD) has been found to occur. In a previous study, immediately after the occurrence of a CD case, we did not observe any sylvatic small mammals or dogs with *Trypanosoma cruzi cruzi* infections, but *Triatoma vitticeps* presented high *T. c. cruzi* infection rates. In this study, we investigated bats together with non-volant mammals, dogs, and triatomines to explore other possible *T. c. cruzi* reservoirs/hosts in the area. Seventy-three non-volant mammals and 186 bats were captured at three sites within the Guarapari municipality, Espírito Santo state. Rio da Prata and Amarelos sites exhibited greater richness in terms of non-volant mammals and bats species, respectively. The marsupial *Metachirus nudicaudatus*, the rodent *Trinomys paratus*, and the bats *Artibeus lituratus* and *Carollia perspicillata* were the most frequently captured species. As determined by positive hemocultures, only two non-volant mammals were found to be infected by *Trypanosoma* species: *Monodelphis americana*, which was infected by *T. cascavelli*, *T. dionisii* and *Trypanosoma* sp., and *Callithrix geoffroyi*, which was infected by *T. minasense*. Bats presented *T. c. cruzi* TcI and TcIII/V, *T. c. marinkellei*, *T. dionisii*, *T. rangeli* B and D, and *Trypanosoma* sp. infections. Seven dogs were infected with *T. cruzi* based only on serological exams. The triatomines *T. vitticeps* and *Panstrongylus geniculatus* were found to be infected by trypanosomes via microscopy. According to molecular characterization, *T. vitticeps* specimens were infected with *T. c. cruzi* TcI, TcII, TcIII/V, and TcIV, *T. c. marinkellei* and *T. dionisii*. We observed high trypanosome diversity in a small and fragmented region of the Atlantic Forest. This diversity was primarily maintained by bats and *T. vitticeps*. Our findings show that the host specificity of the *Trypanosoma* genus should be thoroughly reviewed. In addition, our data show that CD cases can occur without an enzootic cycle near residential areas.

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Background

The *Trypanosoma* genus comprises flagellate species that can infect diverse animal species and are transmitted by hematophagous invertebrate hosts [1–2]. These parasites are divided into two biological groups based on their development in invertebrate hosts: Salivaria and Stercoraria [3–4]. *Trypanosoma* is composed of parasite species of medical and veterinarian importance, such as *Trypanosoma cruzi cruzi*, which is responsible for Chagas disease (CD) and *Trypanosoma brucei*, which is responsible for sleeping sickness in humans and nagana in cattle in Africa [5–6].

The *T. cruzi* clade includes *T. c. cruzi*, *T. c. marinkellei*, *T. dionisii* and *T. erneyi*; a group known as *T. rangeli*/*T. conorhini*, which consists of *T. rangeli*, *T. conorhini*, *T. vespertilionis* and trypanosome species isolated from terrestrial African mammals [7–9]. It also includes trypanosomes that have been isolated from Neotropical bats [10], Australian marsupials [11–13] and *T. livingstonei*, which was isolated from African bats [14]. All trypanosomes except for *T. c. cruzi* and *T. rangeli* are known to infect specific animal groups. There are two hypotheses for the origin of the *T. cruzi* clade: the first hypothesis, i.e., the southern supercontinent hypothesis [15], proposes that *T. c. cruzi* speciated in marsupials after the separation of South America from the Australian continent. The second hypothesis is known as the bat seeding hypothesis [16] and proposes that bats were the ancestral hosts of the *T. cruzi* clade. The latter hypothesis is gaining increasing support based on the description of trypanosome species in African mammals, American bats containing members of the *T. cruzi* clade and the low diversity of species of the *T. cruzi* clade in South American terrestrial mammals [7, 9, 10, 14].

Trypanosoma cruzi cruzi has a broad distribution in the New World, extending from the southern US to Chile and Argentina. As a heterogeneous parasite, seven discrete typing units (DTUs) are recognized: TcI to TcVI and TcBat [17–18]. In Brazil, after intradomiciliary transmission of the parasite, the primary route of infection is via oral transmission, and CD is re-emerging as a food-borne disease [19–20]. In Espírito Santo (ES) state in southeastern Brazil, residents are often in contact with triatomines, as these insects are attracted by light and frequently invade residences. The primary triatomine species in the region is *Triatoma vitticeps*, which exhibits high rates of *T. c. cruzi* infection [21]. In 2012, a child died from acute Chagas disease (aCD) acquired via oral transmission [22]. The child presented with a mixed infection of *T. c. cruzi* TcI, TcII, TcIII, and TcIV and *T. dionisii* [22]. During an initial investigation, we were not able to determine the reservoirs of *T. c. cruzi* in the area, since the dogs and small wild mammals in the surrounding area tested negative. None of the animals presented patent parasitemia or positive hemocultures, contrasting with the triatomines, which presented high *T. c. cruzi* infection rates. In addition, the domestic animals (dogs) were not infected, as shown by negative serological and parasitological tests [22]. These findings led us to hypothesize that the house-invading triatomines became infected with *T. c. cruzi* by feeding on wild hosts in an area distant from the peridomiciliar area where the human case of aCD occurred. To confirm this hypothesis, we decided to diagnose *T. c. cruzi* infection in wild mammals in two other areas that were farther away from the house where the case of aCD occurred to determine which mammal taxa maintain the enzootic transmission cycle of *T. c. cruzi*. Thus, our primary objective was to build upon the previous study to identify the mammal reservoirs of *T. c. cruzi* from which the *T. vitticeps* that invaded human dwellings were becoming infected with *Trypanosoma* spp.

Materials and methods

Ethics approval and consent to participate

The sampling procedures reported herein were authorized by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) under license no. 19037–1 for bats

and license no. 10070–2 for non-volant mammals. The euthanasia and blood collection procedures met the guidelines set by the Federal Council of Veterinary Medicine, Resolution 1000 (11-05-2012), in accordance with Federal Law 11.794/2008. All procedures followed protocols approved by the Fiocruz Ethics Committee for Animal Research (L0015-07).

Study area

This study was conducted in three rural areas in the Guarapari municipality, located along the southeastern coast of Brazil, as described in [23] (Fig 1).

Sylvatic small mammal capture

Four surveys were performed in the three studied areas, with two in the dry season (May 2014 and June 2015) and two during the rainy season (October 2014 and November 2015). For the small wild mammal captures, two linear transects consisting of 15 trapping stations that were 10 m apart were established at each study site. Each trapping station had one Sherman® (H. B. Sherman Traps, Tallahassee, FL, USA) and one Tomahawk® (Tomahawk Live Traps, Tomahawk, WI, USA) trap placed on the ground and in the understory tied to vines and lianas 1–1.5 m above the ground, when possible. The traps were baited with a mixture of pineapple

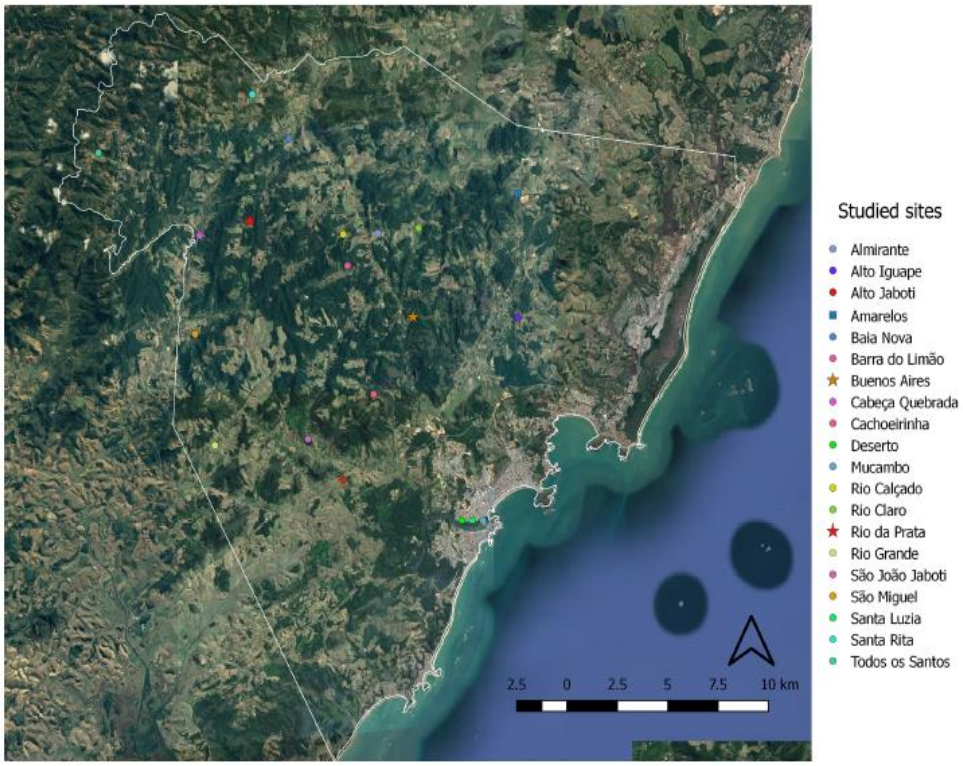

Fig 1. Guarapari municipality study locations. The stars represent locations where mammals and triatomines were captured, namely, Buenos Aires, an area where human dwelling invasion by adult triatomines (primarily by *T. vitticeps*) was reported by the residents; and Rio da Prata, the location where the aCD case occurred. The square represents Amarelos location, an area with no reports of human dwelling invasion by triatomines. The dots represent locations from which triatomines were received. Three of the triatomine collection locations (Deserto, Mucambo and Santa Luzia) were georeferenced by the municipality centroid, because we could not obtain the exact georeference of these collection site. (Source: Google Earth).

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and peanut butter and remained open for five consecutive nights during each sampling event, resulting in a trapping effort of 1,200 trap nights.

Bats were captured near the same transects using mist nets. Ten mist nets were placed in the surrounding forest and near food sources (fruit trees), shelters and flight routes, and they remained open for four hours after sunset. Bat captures were performed for two consecutive nights at each location.

For all the animals, morphological characteristics and body measurements were recorded for taxonomic identification. Rodent taxonomy was performed according to Patton and coworkers [24]. The bat identifications were confirmed following Gardner's methodology [25]. Blood was collected from all the animals under anesthesia using 9:1 ketamine chlorhydrate (10%) and acepromazine (1%). All the small mammals, including the bats, that were used in these analyses received a collection number along with the initials of the collectors (YL and RM), and the animals were prepared for fluid preservation. These materials were subsequently deposited in the mammal collection at the Federal University of Espírito Santo (small non-volant mammals) and the Nacional Museum at the Federal University of Rio de Janeiro (bats).

Dog survey

A search for dogs was conducted in houses near the locations where the wild mammals were captured. With the informed consent of their owners, blood samples were collected using Vacutainer® tubes containing EDTA by puncturing each dog's femoral vein. A questionnaire was used to record the age, sex, size, and primary function (hunting, companionship, or protection) of each dog. All dogs from the same house were considered to be a single event in this study.

Parasitological survey

Parasitological and serological methods were used to identify *Trypanosoma* species in wild mammals and dogs. The parasitological methods included the examination of (i) fresh blood and (ii) hemocultures; for the latter, 0.3 to 0.6 ml of blood was inoculated into two tubes containing NNN/LIT medium for small mammals and dogs, one tube containing NNN/LIT for the isolation of *T. cruzi* and one tube containing NNN/Schneider's medium for the isolation of trypanosomatids from bats.

The hemocultures were examined fortnightly for five months. Positive cultures, which demonstrated parasite growth, were amplified, cryopreserved, and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, COLTRYP/Fiocruz. The sediments of the positive hemocultures that did not successfully amplify the parasites were centrifuged, and the pellets were stored at -20˚C for the molecular characterization of the *Trypanosoma* species.

Serological analyses were performed only with the sera of non-volant wild mammals and dogs because no commercial anti-bat conjugate is available. For IgG antibody detection in the sera of wild mammals and dog, an indirect immunofluorescence antibody test (IFAT) assay was performed as described in [26]. Reference strains I00/BR/00F (TcI) and MHOM/BR/1957/Y (TcII) from axenic cultures were mixed in equal (1:1) proportions and used as antigens. The sera from Murinae rodents and plasma from dogs were tested with rat anti-IgG and dog anti-IgG, respectively, which were coupled with fluorescein isothiocyanate (Sigma, St. Louis, MO, USA). The sera of Echimyidae rodents and marsupials were tested as described in [20] using in-house anti-*Thrichomys* IgG and anti-*Didelphis* spp. IgG, respectively. The cut-off values for the IFAT were 1:40 for marsupials and dogs and 1:10 for rodents [27]. To confirm the dogs' serological results, an enzyme-linked immunosorbent assay (ELISA) was

performed. The cut-off value for the ELISA was the mean optical absorbance of the negative controls plus 20%. For the IFAT and ELISA, two negative and two positive control sera were added to each reaction. For the IFAT assays, specific positive and negative controls were added for each mammal order.

To exclude cross-reactions and to confirm mixed infections by *T. cruzi* and *Leishmania* sp., an IFAT using a mixture of axenic cultures containing *L. infantum* and *L. braziliensis* was performed. Mammals that presented higher serological titers for *Leishmania* sp. than for *T. cruzi* were considered to be infected by *Leishmania* sp. only when the *T. cruzi* titers were ≤1:80, and the presence of mixed infections was confirmed when both serological titers were >1:80 [28]. To test for cross-infection with *Leishmania* sp. in dogs, a rapid test for the diagnosis of canine visceral leishmaniasis (CVL) (TR DPP®, Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil) was performed.

Triatomine collection

After residents in rural locations in the Guarapari municipality reported the invasion of their residences by triatomines, health agents contacted them to discuss collection procedures and the delivery of the insects to the Zoonosis Control Center (ZCC). Between 2014 and 2015, the triatomines that were collected from distinct locations were delivered to and examined by our group (Fig 1).

The morphological identification of triatomines was performed according to [29], and the presence of flagellated *Trypanosoma* sp. forms in fecal material was observed by removing the intestinal content with scissors and forceps using optical microscopy. The intestinal content was diluted in phosphate-buffered saline (PBS) and stored at -20˚C for *Trypanosoma* spp. characterization.

Molecular characterization of cultures and intestinal content

The total genomic DNA from the mammalian blood cultures and triatomine intestinal contents was extracted using a phenol-chloroform method [30]. To identify infection of sylvatic mammals by *Trypanosoma* species, the DNA samples were subjected to a nested PCR for the small subunit (SSU) rRNA [11, 31] and gGAPDH [32] genes. For the identification of *Trypanosoma* sp. in triatomines, nested PCR was performed for only the SSU rRNA gene. All reactions included distilled water as a negative control. *Trypanosoma cruzi* strain SylvioX/10cl1 was used as a positive control.

The PCR products (~650 bp for the SSU rRNA gene and ~800 bp for the gGAPDH gene) were visualized using a 2% agarose gel stained with ethidium bromide and purified using an Illustra GFX PCR DNA and gel band purification kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). Both strands of DNA were then sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 DNA sequencer available at the PDTIS/Fiocruz sequencing platform.

The sequences were assembled and edited using SeqMan (DNASTAR Lasergene, Gatc, Konstanz, Germany) to obtain the SSU rRNA and gGAPDH consensus sequences, which were then aligned and corrected using BioEdit [33]. The sequences were compared to nucleotide sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm for initial screening. For the SSU rRNA and gGAPDH genes, phylogenies were inferred in Mega7 [34] using maximum likelihood (ML) tree inference under Kimura's two-parameter model of nucleotide substitution with gamma-distributed variation among sites (K2P + G) for triatomines and non-volant mammals and a gamma-distributed rate with invariant sites (K2P + G + I) for bats. For the gGAPDH sequences, Tamura's three-parameter model of

substitution with invariant sites (T92P + I) was inferred for the *Monodelphis americana* isolate, and Tamura's three-parameter model of nucleotide substitution with gamma-distributed variation among sites (T92 + G) was inferred for the bat isolates.

SSU rRNA amplification and deep sequencing

The c624 isolate was subjected to another nested PCR of the SSU rRNA using the primers described above [11, 31]. For deep sequencing, the PCR products were single-end barcoded, purified using agarose gel electrophoresis (PureLink Quick Gel Extraction Kit, Invitrogen), quantified using a fluorometric assay (Qubit 2.0, Thermo Fisher Scientific) and pooled to equimolar concentrations for multiplexed, paired-end (2 × 300 bp) sequencing on an Illumina MiSeq platform (Reagent Kit v2) [23].

Deep sequencing data analysis

Amplicon sequences were analyzed as described in [23]: after the sequence quality was verified in FastQC [35], the amplicons were filtered using windowed trimming in Sickle [36], retaining only full-length reads with ≥99.9% base call accuracy, which were then mapped against a *Trypanosoma* spp. reference collection from SILVA v119 [37] using Bowtie 2 [38]. Operational taxonomic unit (OTU) construction proceeded using the UPARSE algorithm in USEARCH [39] and BLAST-based taxonomic assignment in the QIIME environment [40], with run parameters established during prior *in silico* testing on trypanosomatid 18S rRNA sequences from NCBI. The samples were clustered into OTUs *de novo* at 98% sequence similarity and assigned to extant species with a confidence threshold of 80%.

After OTU establishment, the sequence read pairs for each OTU were merged and aligned in ClustalW (with the manual refinement of misplaced reads). Phylogenies were inferred in Mega7 [34] using ML tree construction under Kimura's two-parameter model of nucleotide substitution with gamma-distributed variation among sites (K2P + G) and bootstrap values for 1000 replicates. The SSU rRNA and gGAPDH reference strains used for the Sanger and deep sequencing phylogenic analyses are listed with their accession numbers in S1 Table.

Results

In this study, we observed substantial diversity among *Trypanosoma* species as well as among genotypes of *T. rangeli* and *T. c. cruzi* in a fragmented Atlantic Forest coastal area. Infection with distinct *Trypanosoma* species occurred primarily in bats, since only two non-volant wild mammal specimens from two species were infected, which constitutes an epizootic profile that is quite different from the profile that has usually been observed: the transmission cycle is occurring far from the residential areas.

Non-volant sylvatic mammal occurrence and distribution in the Atlantic rainforest

During the four surveys, 73 small, non-volant sylvatic mammals were captured and classified into 12 species. The species richness was slightly higher in marsupials (seven species) than in rodents (five species) (Table 1). The species richness was similar among the three locations, but the species composition and relative abundances were distinct. We found nine species in Rio da Prata, eight species in Buenos Aires and six species in Amarelos (Table 1); we also accidentally captured two primates (*Callithrix geoffroyi*). The non-volant mammals' relative abundances are presented in Table 1, which shows that the two most abundant species were *Metachirus nudicaudatus* (Didelphimorphia) and *Trinomys paratus* (Rodentia).

Table 1. Species richness (r) and relative abundance (%) of non-volant sylvatic mammals in Amarelos, Buenos Aires and Rio da Prata, Guarapari municipality, ES state, Brazil.

Species	Location			Total (r/%)
	Amarelos (r/%)	Buenos Aires (r/%)	Rio da Prata (r/%)	
*Didelphis aurita**	4 (30.77)	1 (2.86)	1 (4.0)	6 (8.22)
Gracilianus microtarsus	-	1 (2.86)	-	1 (1.37)
Marmosa paraguayana	5 (38.46)	-	1 (4.0)	6 (8.22)
Marmosa murina	-	-	1 (4.0)	1 (1.37)
Marmosops incanus	1 (7.69)	3 (8.57)	1 (4.0)	5 (6.85)
*Metachirus nudicaudatus**	-	13(37.15)	2 (8.0)	15 (20.55)
Monodelphis americana	-	2 (5.71)	-	2 (2.74)
Akodon cursor	1 (7.69)	4 (11.43)	7 (28.0)	12 (16.44)
Necromys lasiurus	-	-	1 (4.0)	1 (1.37)
Nectomys squamipes	1 (7.69)	2 (5.71)	5 (20)	8 (10.95)
Rhipidomys mastacalis	1 (7.69)	-	-	1 (1.37)
*Trinomys paratus**	-	9 (25.71)	6 (24)	15 (20.55)
Total	13 (17.81)	35 (47.94)	25 (34.25)	73 (100)

r/%: species richness/relative abundance

*The star represents mammals that presented positive results on the serological exam.

https://doi.org/10.1371/journal.pone.0188412.t001

Occurrence and distribution of bats

One hundred eighty-six bat specimens from 17 distinct species were examined during the four sampling events. Only seven bat species were common to the three study sites (Table 2). Moreover, the species richness differed among the three locations; Amarelos presented the highest

Table 2. Species richness and relative abundance of bats (%) in Amarelos, Buenos Aires and Rio da Prata, Guarapari municipality, ES state, Brazil.

Species	Location			Total (r/%)
	Amarelos (r/%)	Buenos Aires (r/%)	Rio da Prata (r/%)	
Anoura caudifer	3 (3.75)	-	6 (8.95)	9 (4.84)
Anoura geoffroyi	-	-	9 (13.43)	9 (4.84)
Artibeus fimbriatus	1 (1.25)	2 (5.13)	1 (1.49)	4 (2.15)
Artibeus lituratus	15 (18.75)	9 (23.08)	5 (7.46)	29 (15.59)
Carollia perspicillata	30 (37.5)	14 (35.90)	25 (37.31)	69 (37.10)
Desmodus rotundus	9 (11.25)	-	1 (1.49)	10 (5.38)
Glossophaga soricina	4 (5.00)	2 (5.13)	-	6 (3.22)
Micronycteris sp.	2 (2.50)	-	-	2 (1.07)
Myotis nigricans	2 (2.50)	1 (2.56)	1 (1.49)	4 (2.15)
Phyllostomus discolor	2 (2.50)	1 (2.56)	2 (2.98)	5 (2.69)
Phyllostomus hastatus	3 (3.75)	2 (5.13)	-	5 (2.69)
Platyrrhinus lineatus	-	-	4 (5.97)	4 (2.15)
Platyrrhinus recifinus	1(1.25)	1 (2.56)	3 (4.48)	5 (2.69)
Rhinophylla pumilio	3 (3.75)	-	7 (10.45)	10 (5.38)
Sturnira lilium	3 (3.75)	7 (17.95)	3 (4.48)	13 (6.98)
Tonatia bidens	1 (1.25)	-	-	1 (0.54)
Trachops cirrhosus	1 (1.25)	-	-	1 (0.54)
Total	80 (43.01)	39 (20.97)	67 (36.02)	186 (100)

r/%: species richness/relative abundance

https://doi.org/10.1371/journal.pone.0188412.t002

bat species richness, and Buenos Aires presented the lowest (Table 2). *Artibeus lituratus* and *Carollia perspicillata* were the most abundant bat species. The Amarelos location presented the highest number of captured bats, and *A. lituratus, C. perspicillata,* and *Desmodus rotundus* were the most abundant. At the Buenos Aires location, the primary species captured were *A. lituratus, C. perspicillata*, and *Sturnira lilium*. At the Rio da Prata location, *Anoura geoffroyi, C. perspicillata*, and *Rhinophylla pumilio* were the most abundant species (Table 2).

Trypanosoma spp. infection in sylvatic non-volant mammals and bats

The prevalence of *Trypanosoma* spp. infection was higher in bats (22.66%) than in sylvatic non-volant wild mammals (2.67%). Only two non-volant mammal specimens of two species were found to be infected with *Trypanosoma* species, as demonstrated by positive hemocultures, and they were *M. americana* (2851-c624) in Buenos Aires and *C. geoffroyi* (EAR04) in Amarelos. The SSU rRNA marker was amplified in both samples, and, to our surprise, the ML tree showed the presence of a *Trypanosoma* sp. from a reptile clade that clustered with *T. cascavelli* (Fig 2A, Fig 2B, Table 3) in the *M. americana* isolate, and the gGAPDH marker showed that this marsupial specimen was also infected with *T. dionisii* (Fig 2C). The EAR04 sample

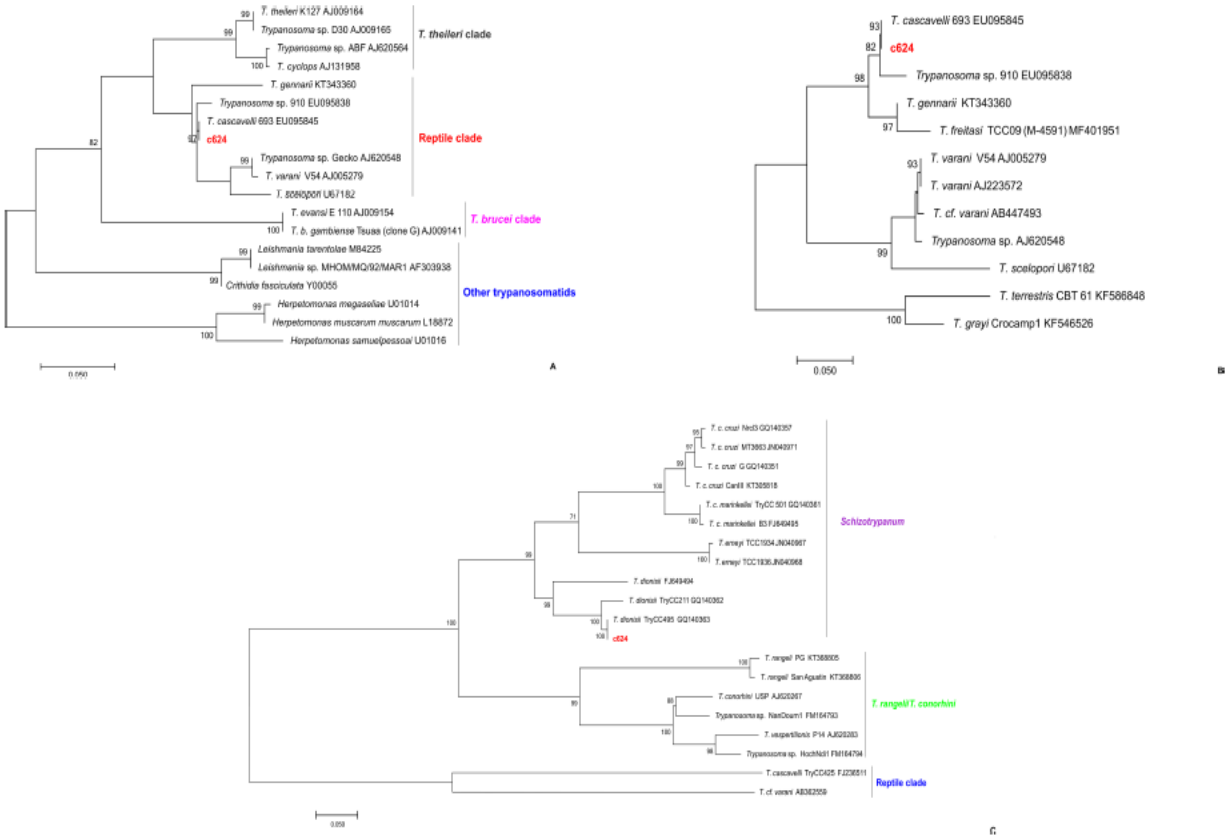

Fig 2. Phylogenetic placements of SSU rRNA and gGAPDH sequences from hemocultures of *Monodelphis americana*. The tree was inferred by maximum likelihood using the Kimura 2-parameter model with a gamma-distributed rate of variation among sites (K2P + G) for SSU rRNA and the Tamura 3-parameter model of substitution with invariant sites (T92P + I) for gGAPDH. The numbers at the nodes indicate support from 1000 bootstrap pseudoreplicates. (A) SSU rRNA showed the c624 isolate clustered in the *Trypanosoma* reptile clade; (B) based on SSU rRNA, the c624 isolate was identified as *T. cascavelli*; (C) based on gGAPDH, the c624 isolate was identified as *T. dionisii*.

https://doi.org/10.1371/journal.pone.0188412.g002

Table 3. *Trypanosoma* spp. identification using SSU rRNA and gGAPDH in sylvatic mammals captured in the Guarapari municipality, ES state, Brazil.

Species	ID	Location	SSU rRNA	gGAPDH
Anoura spp.	c596	Rio da Prata	*T. dionisii*	*T. dionisii*
	c621, c621s			
Artibeus spp.	c700	Buenos Aires	*T. cruzi* TcI	NA
Artibeu spp.	RM 837	Amarelos	*T. cruzi* TcIII/V	NA
C. geoffroyi	EAR04	Amarelos	*T. minasense*	NA
C. perspicillata	c593	Rio da Prata	*T. rangeli* D	*T. rangeli* D
	c594	Amarelos	*T. dionisii*	*T. dionisii*
	c595	Rio da Prata	*T. dionisii*	*T. dionisii*
	c597	Buenos Aires	*T. dionisii*	*T. dionisii*
	c598	Rio da Prata	*T. dionisii*	*T. dionisii*
	c622, c622s	Amarelos	*T. dionisii*	*T. dionisii*
	c623	Buenos Aires	*T. dionisii*	*T. dionisii*
	c625	Rio da Prata	*T. dionisii*	*T. dionisii*
	c626	Amarelos	*T. dionisii*	*T. dionisii*
	c681	Rio da Prata	*T. dionisii*	NA
	c688	Amarelos	*T. dionisii*	NA
	c692	Amarelos	*T. dionisii*	*T. c. cruzi* TcI
	RM851	Rio da Prata	*T. cruzi* TcIII/V	NA
	RM2028	Amarelos	*T. rangeli* B	*T. rangeli* B
	RM 2054	Buenos Aires	*Trypanosoma* sp.	*Trypanosoma* sp.
D. rotundus	c694	Amarelos	*T. cruzi* TcI	*T. cruzi* TcI
	RM823		*T. cruzi* TcIII/V	*Trypanosoma* sp.
	RM2027		*Trypanosoma* sp.	NA
G. soricina	c620	Amarelos	*T. dionisii*	*T. dionisii*
M. americana	c624	Buenos Aires	*T. cascavelli*	*T. dionisii*
M. nigricans	RM838	Amarelos	*T. c. cruzi* TcIII/V	*Trypanosoma* sp.
P. discolor	RM 742	Buenos Aires	*T. c. marinkellei*	*T. c. marinkellei*
	RM 842	Rio da Prata	*T. c. marinkellei*	NA

NA: Not amplified due low DNA quantities.

https://doi.org/10.1371/journal.pone.0188412.t003

clustered with *T. minasense* (Fig 3, Table 3). Serologically, two marsupial specimens, one *Didelphis aurita* (1:320) and one *M. nudicaudatus* (1:80) from Buenos Aires, and two *T. paratus* specimens, one from Buenos Aires (1:40) and one from Rio da Prata (1:20), were positive for *T. cruzi* infection (Table 1).

Forty-four bats from eight genera/species presented positive fresh blood smears or hemocultures. Amarelos had the highest number of bats infected with *Trypanosoma* species, with an infection rate of 45.45%. The infection rate among bats from Rio da Prata was 38.63%, and the infection rate among bats from Buenos Aires was 15.92%. *Carollia perspicillata* was the primary bat species presenting the highest number of infected bat specimens (Fig 4).

The trypanosome isolates from 26 bats were characterized using SSU rRNA, and 19 isolates were characterized using gGAPDH sequencing to identify the *Trypanosoma* species circulating in the three study locations. Three *Trypanosoma* species were identified, *T. cruzi*, *T. dionisii*, and *T. rangeli*, in addition to a not-yet-described *Trypanosoma* sp. from Neotropical bats (Fig 4A, Fig 4B). *Trypanosoma dionisii* was the most predominant species (56%) among bats (Fig 5) and was identified in bats from the three locations together with *T. c. cruzi*. *Trypanosoma cruzi*

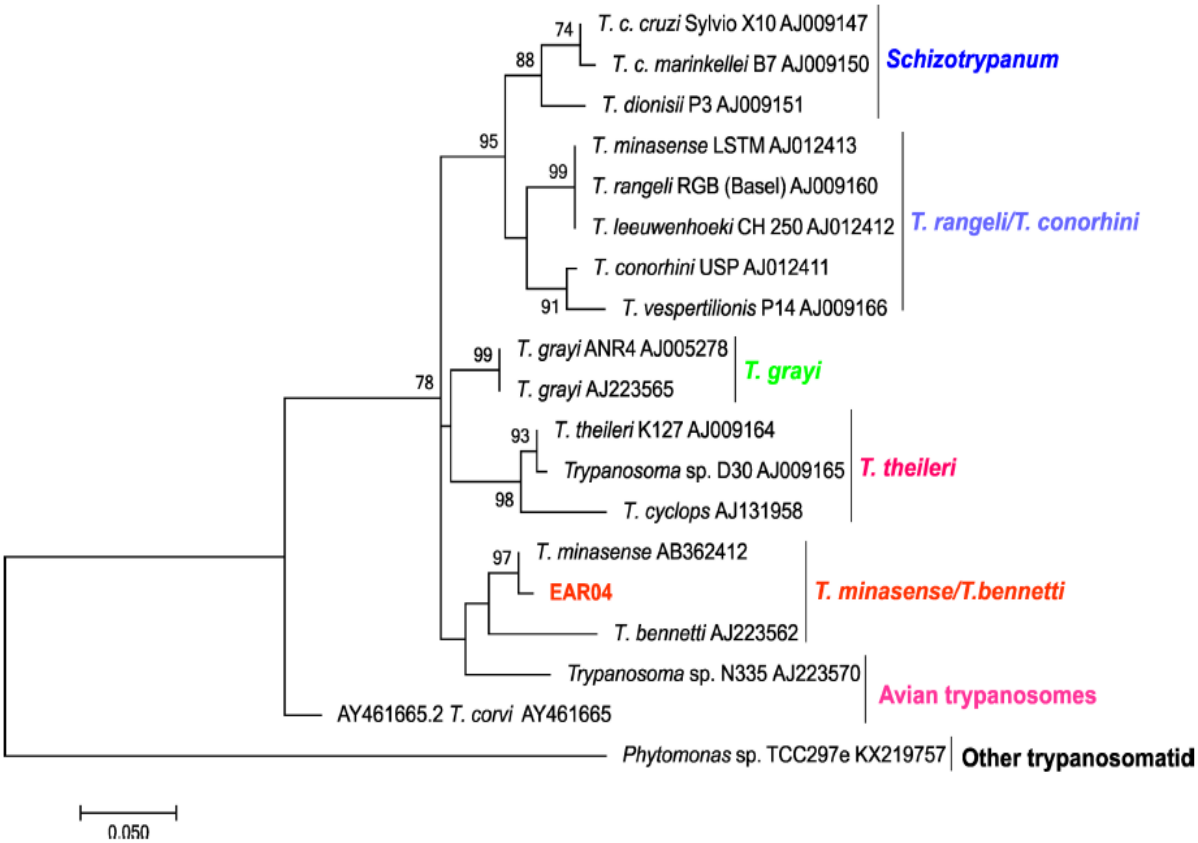

Fig 3. Phylogenetic placement of SSU rRNA sequences from *Callithrix geoffroyi* hemocultures. The tree was inferred by maximum likelihood using the Kimura 2-parameter model plus a gamma-distributed rate of variation among sites (K2P + G). The numbers at the nodes indicate support from 1000 bootstrap pseudoreplicates. The sample clustered with *T. minasense* from the red-handed tamarin in the same branch as *T. bennettii*.

https://doi.org/10.1371/journal.pone.0188412.g003

marinkellei was identified in bats collected in Buenos Aires and Rio da Prata sites. *Trypanosoma rangeli* lineages B and D were found to infect bats at the Amarelos and Rio da Prata sites, and a *Trypanosoma* sp. similar to a species from Neotropical bats was observed in Buenos Aires and Amarelos (Table 3). *Carollia perspicillata* specimens had the highest number of *Trypanosoma* species, but this bat species was not found to be infected by *T. c. marinkellei* (Table 3). The gGAPDH and SSU rRNA results differed in terms of the *Trypanosoma* spp. identified in three samples (Fig 4B, Table 3). Two samples identified as *T. c. cruzi* TcIII/V based on SSU rRNA were classified as *Trypanosoma* sp. from Neotropical bats, and one sample identified as *T. dionisii* based on SSU rRNA was classified as *T. c. cruzi* TcI; we confirmed that these samples had mixed infections by *T. c. cruzi* TcIII/V-*Trypanosoma* sp. and *T. dionisii*-*T c. cruzi* TcI. The sample LBT 7053 was also confirmed to be infected by *Trypanosoma* sp. from Neotropical bats. DNA from the hemocultures of eight bat samples did not amplify, and the sequences from sample c683 were ambiguous, likely due to a mixed infection.

Trypanosoma spp. identification by deep sequencing

Based on deep sequencing, *M. americana* isolate c624 exhibited a mixed infection with three OTUs: *Trypanosoma* sp. (OTU 1), *T. cascavelli* (OTU 2) and *T. dionisii* (OTU 3). In the phylogenetic analysis, OTU 1 clustered with the *Trypanosoma* species from Neotropical bats (*T.*

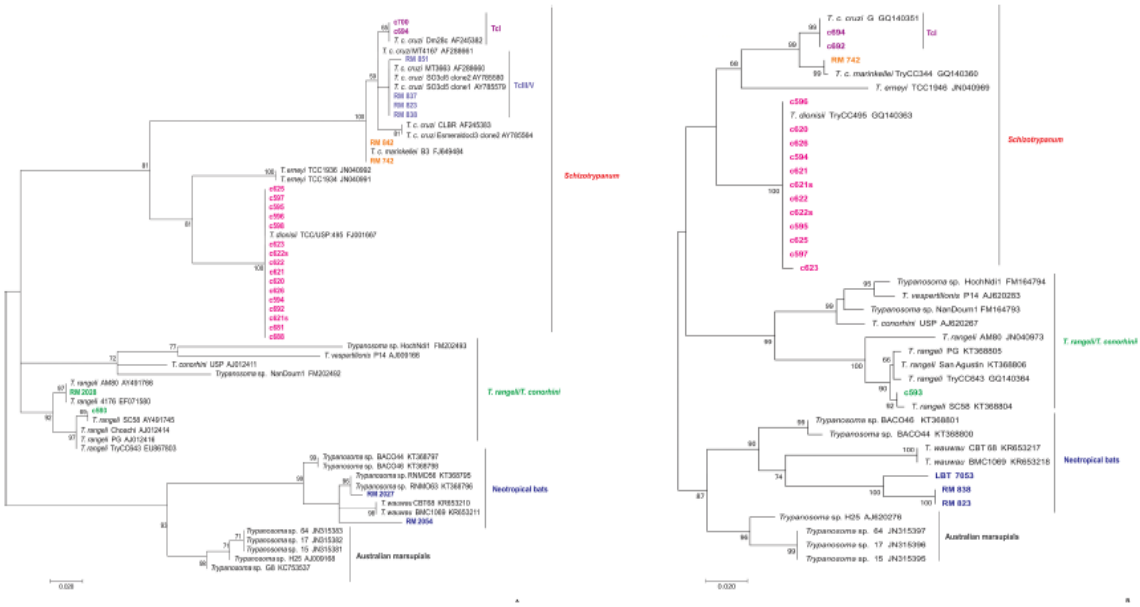

Fig 4. Phylogenetic placements of SSU rRNA and gGAPDH sequences from bat hemocultures. The tree was inferred by maximum likelihood using the Kimura 2-parameter model with a gamma-distributed rate with invariant sites (K2P + G + I) for SSU rRNA and the Tamura 3-parameter model and gamma-distributed variation among sites (T92P + G) for gGAPDH. The numbers at the nodes indicate support from 1000 bootstrap pseudoreplicates. (A) The samples clustered within the *T. cruzi* clade, in the *Schizotrypanum* group (*T. c. cruzi*, *T. c. marinkellei*, and *T. dionisii*), in the *T. rangeli*/*conorhini* group (*T. rangeli*), and with *Trypanosoma* species from Neotropical bats. Two *T. c. cruzi* genotypes and two *T. rangeli* lineages were identified as TcI, TcIII/V, and lineages B and D, respectively. (B) Samples RM 823, RM 838, and LBT 7053 clustered with *Trypanosoma* species from Neotropical bats. Sample c692 clustered within the *Schizotrypanum* group (*T. c. cruzi*).

https://doi.org/10.1371/journal.pone.0188412.g004

wauwau and *Trypanosoma* sp. RNMO and BACO); OTU 2 clustered within the reptile clade in the same branch as *T. cascavelli* 632; and OTU 3 clustered within the *Schizotrypanum* subgenus in the same branch as *T. dionisii* TCC/USP:495 (Fig 6).

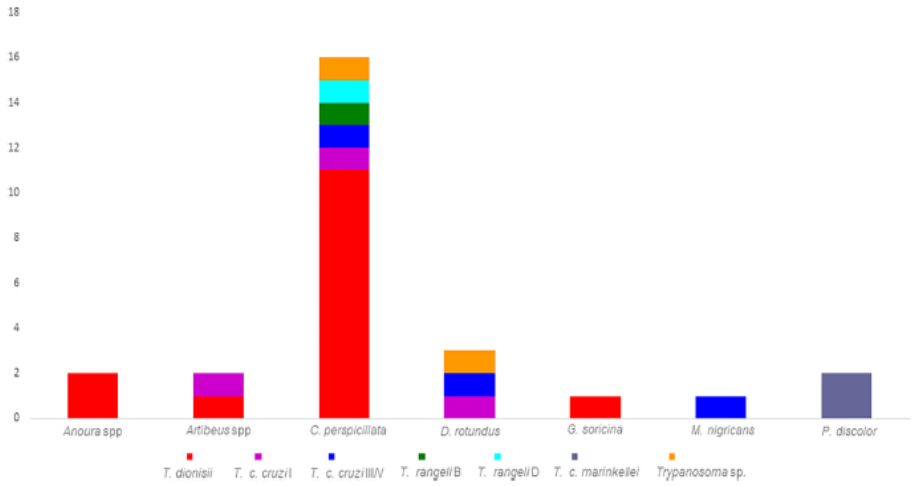

Fig 5. *Trypanosoma* spp. infection in bats captured in Guarapari municipality, ES state, Brazil. The column represents the *Trypanosoma* infection profile of each bat species. The colors represent each *Trypanosoma* species identified in bats.

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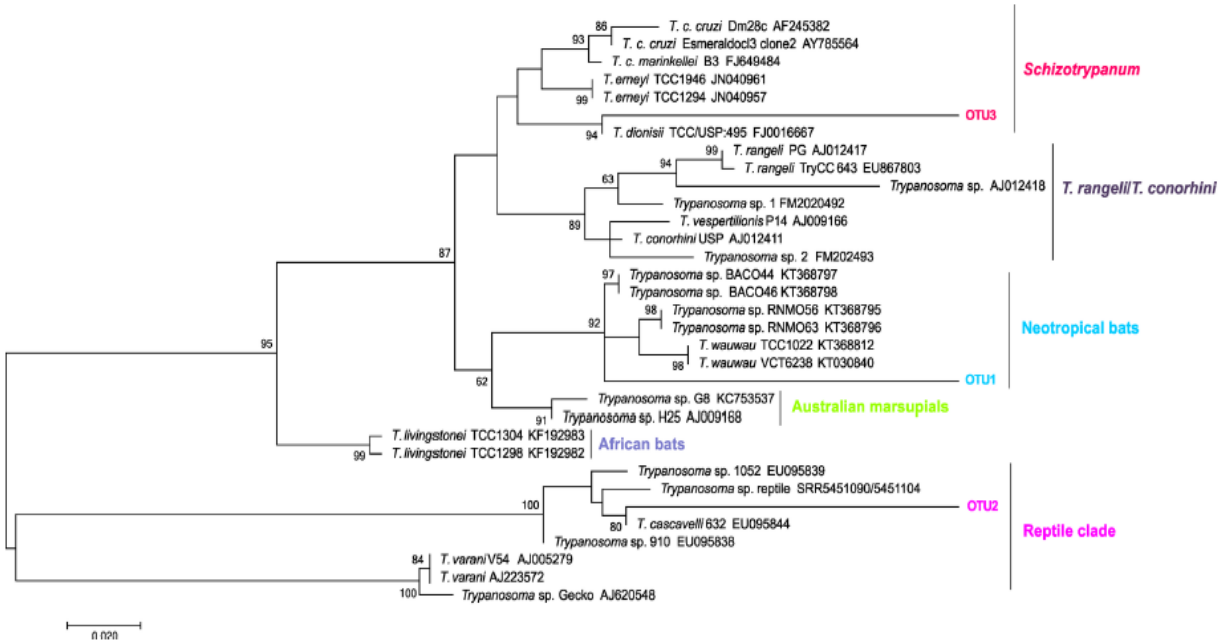

Fig 6. Phylogenetic placement of *Trypanosoma* OTUs detected in *Monodelphis americana* from the Guarapari municipality, ES state, Brazil. The tree was constructed based on 18S rRNA using the maximum likelihood method with Kimura's 2-parameter model and gamma-distributed variation among sites (K2P + G). The numbers at the nodes indicate support from 1000 bootstrap pseudoreplicates. Two OTUs clustered within the *T. cruzi* clade (OTUs 1 and 3), and one OTU clustered within the reptile clade (OTU 2).

https://doi.org/10.1371/journal.pone.0188412.g006

Trypanosoma cruzi survey in dogs

Fifty-five dogs were examined during the four surveys, 18 from Amarelos, 17 from Buenos Aires and 20 from Rio da Prata. Among the serological tests performed on the dog samples, nine presented borderline titers (serological titers = 1:40), and four dogs from Amarelos, two from Buenos Aires and one from Rio da Prata presented positive titers for *T. cruzi* (Table 4). None of the dogs presented positive fresh blood smears or hemocultures.

Trypanosoma spp. infection in triatomines

We received 79 adult triatomine specimens between 2014 and 2015 from different rural areas in the Guarapari municipality (Fig 1). Seventy-three specimens were identified as *T. vitticeps* (92.40%), and six were identified as *P. geniculatus* (7.60%). The *Trypanosoma* infection rates observed via the intestinal content examinations with optical microscopy were high for both: 52% in the former and 50% in the latter.

Forty-seven DNA samples were extracted to directly identify *Trypanosoma* species from the intestinal content: 37 from positive samples and eight from negative samples, which were

Table 4. Serological survey of dogs in Amarelos, Buenos Aires and Rio da Prata, Guarapari municipality, ES state, Brazil.

Location	IFAT	ELISA
Amarelos (n = 6)	1:40 (n = 2); 1:80 (n = 2); 1:160 (n = 1); 1:320 (n = 1)	Positive
Buenos Aires (n = 4)	1:40 (n = 2); 1:80 (n = 2)	Positive
Rio da Prata (n = 6)	1:40 (n = 5); 1:80 (n = 1)	Positive

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randomly selected. DNA from five samples, four from *T. vitticeps* and one from *P. geniculatus*, could not be extracted. Forty-two samples were PCR-positive (~650 bp), and they were sequenced. Seven samples that were negative based on the examination of intestinal content using optical microscopy were positive according to the PCR analysis, increasing the *Trypanosoma* species infection rates. Four samples that were positive for *Trypanosoma* sp. based on optical microscopy did not amplify in the PCR. Twenty-five samples (59.52%) had single infections, and 17 samples (40.48%) had sequences with ambiguities, likely due to mixed infections. Phylogenetic analysis (Fig 7) revealed that *T. c. cruzi* was the predominant species. We observed four circulating DTUs: TcI, TcII, TcIII/V and TcIV. TcII was the most prevalent DTU (Table 5). We found *T. vitticeps* specimens infected with *T. c. marinkellei* and *T. dionisii* (Table 5).

Discussion

The epizootic scenario observed in 2012 in the area of a fatal aCD case in Guarapari municipality, ES state [22], was currently the same approximately two years later, showing that these *Trypanosoma* spp. transmission cycles are stable and well-established in the study region. A reservoir is defined as a system formed by a group of species capable of maintaining a certain parasite in nature [41]. In this study, although we focused on reasonably preserved areas and used a higher number of captured mammals than in the previous study [22], we found only bats infected with *T. cruzi*. Thus, we can confirm that bats are the reservoir system in this Atlantic Forest area.

We observed a diversity of bat species at the three study sites, demonstrating that environmental changes in the examined Atlantic rainforest fragment did not drastically impoverish the local bat biodiversity. In terms of bat identification, we found a fairly representative number of species. Of the 42 species of Phyllostomidae and ten species of Vespertilionidae that have already been described in ES state [42], we found 38% and 10%, respectively. We observed that environmental variables interfered with the distributions of the bats, as only 1/3 of the bat species were found at the three study sites. The predominance of phyllostomids, especially the generalists *C. perspicillata* and *A. lituratus*, was expected because Phyllostomidae is the most common family in the Neotropical region [43].

Our data confirm that bats are suitable reservoir hosts for several *T. c. cruzi* DTUs as well as *Trypanosoma* species of the *T. cruzi* clade, which may occur in single or mixed infections. Because of the diversity of the *T. cruzi* clade observed among bats captured in the Atlantic Forest, these data support the bat seeding hypothesis. We also confirm that bats are the primary reservoir hosts of *T. c. cruzi* in this area and have increased our knowledge regarding the range of bat species that harbor *T. c. cruzi* genotypes, having observed TcI and TcIII/V infections in *Artibeus* spp., *D. rotundus* and *M. nigricans*. Dario and coworkers [23] previously observed TcI and TcIII/V in *Anoura* spp., *C. perspicillata*, and *R. pumilio*. Here, we once again observed *T. c. cruzi* infecting the generalist *C. perspicillata* as well as the hematophagous bat species *D. rotundus*. Additionally, the presence of *T. dionisii* and *T. c. marinkellei* has already been reported in Atlantic rainforest in northern ES state. Our results show that the occurrence of *T. c. marinkellei* in the Atlantic rainforest biome is not occasional and that its occurrence is not restricted to the Amazon and Pantanal regions [44–45].

We observed a broader lineage and host species distribution for *T. rangeli*, since only lineages A and E have been previously reported in bats [46]. *Trypanosoma rangeli* lineage B has been described as being exclusive to the Amazon region, infecting primates and humans [47–49]. In addition, this parasite has been transmitted to *Rhodnius* species [47–48, 50]. Here, we confirmed that other triatomine species are responsible for its transmission, as other *Rhodnius*

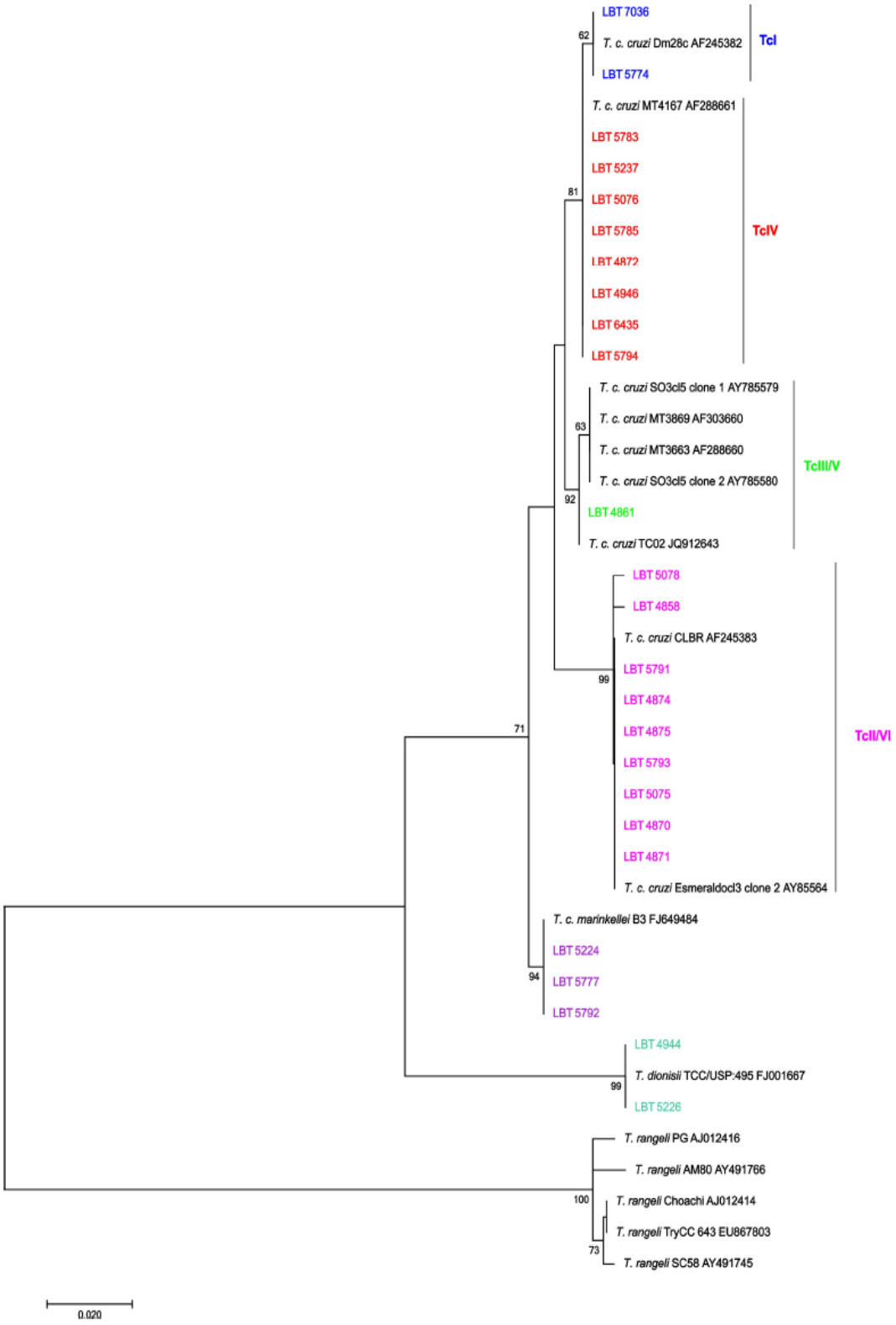
LBT 7036
T. c. cruzi Dm28c AF245382
LBT 5774
TcI
T. c. cruzi MT4167 AF288661
LBT 5783
LBT 5237
LBT 5076
LBT 5785
LBT 4872
LBT 4946
LBT 6435
LBT 5794
TcIV
T. c. cruzi SO3cl5 clone 1 AY785579
T. c. cruzi MT3869 AF303660
T. c. cruzi MT3663 AF288660
T. c. cruzi SO3cl5 clone 2 AY785580
LBT 4861
T. c. cruzi TC02 JQ912643
TcIII/V
LBT 5078
LBT 4858
T. c. cruzi CLBR AF245383
LBT 5791
LBT 4874
LBT 4875
LBT 5793
LBT 5075
LBT 4870
LBT 4871
T. c. cruzi Esmeraldocl3 clone 2 AY85564
TcII/VI
T. c. marinkellei B3 FJ649484
LBT 5224
LBT 5777
LBT 5792
LBT 4944
T. dionisii TCC/USP:495 FJ001667
LBT 5226
T. rangeli PG AJ012416
T. rangeli AM80 AY491766
T. rangeli Choachi AJ012414
T. rangeli TryCC 643 EU867803
T. rangeli SC58 AY491745
62
81
63
92
99
71
94
99
100
73
0.020

Fig 7. Phylogenetic placement of SSU rRNA sequences from the intestinal contents of *Triatoma vitticeps*. The tree was inferred by maximum likelihood using the Kimura 2-parameter model plus a gamma-distributed rate of variation among sites (K2P + G). The numbers at the nodes indicate support from 1000 bootstrap pseudoreplicates. The samples were clustered into three distinct groups: *T. c. cruzi*, *T. c. marinkellei* and *T. dionisii*. Four *T. c. cruzi* DTUs were identified: TcI, TcII, TcIII/V and TcIV.

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spp. have not been reported in this area. Other authors, including Steindel and coworkers [51], have reported the isolation of *T. rangeli* from *P. megistus* in Santa Catarina state.

Bat trypanosomes are morphologically identical, as observed in the *T. cruzi* clade in the subgenus *Schizotrypanum*, species that were all considered *T. cruzi*-like in the past [3]. Perhaps for these reasons, bat trypanosomatids still represent an undiscovered world. The use of powerful analytical methodologies, such as molecular tools with high discriminatory power, has enabled the identification of several new trypanosome species. Recently, many research groups worldwide have reported new *Trypanosoma* species that infect bats and are associated with the *T. cruzi* clade [7, 10, 14, 52], increasing the likelihood of understanding the role played by bats in the origin, diversity, and ecology of trypanosomatids. We encountered two trypanosome samples that clustered into the Neotropical bat groups, with one in the same branch as a *Trypanosoma* sp. from Neotropical bats [10] and the other likely a new species. These trypanosomes from Neotropical bats have been reported in Brazil, Bolivia, Ecuador and Panama [10, 53–55]. Thus, by detecting *Trypanosoma* spp. in *C. perspicillata*, *D. rotundus*, and *M. nigricans* (a bat species from the Vespertilionidae family), in the Atlantic rainforest, we have extended the host range of *Trypanosoma* spp. in Neotropical bats.

Bats are known to host different trypanosome species and have been suggested to be the ancestral hosts of the *T. cruzi* clade [15]. A high trypanosome infection rate was observed in the study areas for *C. perspicillata*, which was the primary captured/analyzed species. In addition, we found a lower species diversity due to the isolation method (hemoculture) that was used. The ability of bats to host so many distinct species could be explained by their diverse behavior, which could facilitate the transmission/dispersal of trypanosomes. Bats are generalists in terms of their feeding habits, which include the consumption of insects, and they have a long lifespan [56–57], which may increase their chances of acquiring trypanosome infections. In addition, some chiropteran species are capable of living in small or large colonies [58–61], and they habitually groom and regurgitate for one another [62–64]. These behavioral traits may enhance *Trypanosoma* spp. transmission.

Triatoma vitticeps, the primary vector found in this Atlantic rainforest area, was observed to maintain single and mixed infections with four *T. c. cruzi* DTUs. The same DTUs were described in a human infection in the same area [22]. *Trypanosoma cruzi cruzi* TcI is considered the most frequent *T. c. cruzi* DTU circulating on the American continent [65], but it was not observed frequently in the study area, where the predominant type was TcII. The

Table 5. *Trypanosoma* ssp. occurrence and identification in *Triatoma vitticeps* collected from distinct locations in Guarapari municipality, ES state, Brazil.

Species	DTU	Number of samples
Trypanosoma cruzi cruzi	TcI	01
	TcII	09
	TcIII/V	01
	TcIV	07
Trypanosoma cruzi marinkellei	NA	03
Trypanosoma dionisii	NA	02

NA: not applicable

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predominance of TcII corroborates the notion that this DTU is maintained successfully by several host species, including bats, in the sylvatic environment of the Atlantic rainforest, as has already been shown among other wild mammal taxa [66–67].

We found six *T. vitticeps* specimens infected by *T. c. marinkellei* and *T. dionisii,* and this is the first time these species have been observed in the *Triatoma* genus. *Trypanosoma cruzi marinkellei* is known to be transmitted by triatomines of the *Cavernicola* genus, and *Rhodnius* spp. have been infected experimentally, as shown by xenodiagnosis [68–69]. This study is the first report of *T. dionisii* infection in triatomines. *Trypanosoma dionisii* transmission is associated with cimicid bugs [70], but there have been no reports of this bug taxon in ES state. Importantly, because they are members of the same subgenus (*Schizotrypanum*), *T. c. cruzi* and *T. dionisii* can likely share the same invertebrate hosts, and infection of cimicid bugs by *T. c. cruzi* has previously been reported [71]. We do not know whether it is possible for *T. dionisii* to be transmitted to mammalian hosts through vectorial contamination, but human infections by *T. dionisii* via the oral route have already been described [22].

In our study, we observed 12 species of small, sylvatic non-volant mammals (rodents and marsupials) in three Guarapari Atlantic rainforest fragments; this figure corresponds to 25.5% of the marsupial and rodent species that have been described in ES state [72] and is therefore a reflection of an environmental disturbance. We observed *D. aurita* in all of the sites, but it was not the most abundant species in any of the areas. Some species, such as *M. americana*, *G. microtarsus*, *R. mastacalis*, and *T. paratus*, which were recorded in the Buenos Aires location, are typical of less disturbed environments, indicating that this is a reasonably preserved area.

One *M. americana* specimen presented mixed infection by *T. cascavelli*, *T. dionisii* and *Trypanosoma* sp. The observation of *T. dionisii* infecting a marsupial reinforces that this parasite is not restricted to infecting only bats. *Trypanosoma dionisii* has already found in a human infection [22], but the unexpected finding of *T. cascavelli* infections in mammals is intriguing, as this species was described in *Crotalus durissus*, a species of snake [73–75]. The current study is not the first to find trypanosomes from the reptile clade infecting sylvatic mammals, as this occurrence has already been observed in *C. perspicillata* and *D. rotundus* bats [23]. Little is known about how *T. cascavelli* is maintained in nature. Sand flies have been hypothesized to be involved in its transmission cycle, given that the transmission of anuran and reptilian trypanosomes by sandflies has previously been described [76–79], and this trypanosome has been isolated from these insects [74]. The infection of mammals by this trypanosome could also be occurring via sandflies in this area.

Analysis of this situation raises questions regarding the ancestral and secondary hosts of *T. cascavelli* in nature. We can hypothesize that marsupials are the ancestral hosts of this trypanosome species and that snakes are accidental hosts. *Monodelphis americana* presents insectivorous-omnivorous feeding habits [80] and might contract infections by *T. cascavelli* via the oral route (predation of insects). Therefore, snakes could be infected through their predation of small mammals, including small marsupials. Marsupials have a lower body temperature than placental mammals [81], and this condition could have facilitated the adaptation of this trypanosome to cold-blooded animals. In fact, mammals from the *Monodelphis* genus have low body temperatures, between 32 and 34˚C, and they can attain much lower body temperatures [82–83]. In addition, other trypanosome species infecting members of the lizard/snake clade have been isolated from marsupials. *Trypanosoma gennarii* [84] was isolated from the marsupial *M. domestica* in the Cerrado biome, while *T. freitasi* was isolated for the first time in 1957 from *Didelphis albiventris* and later from *D. marsupialis* [85–86]. The hypothesis that marsupials were the first hosts seems to be the most parsimonious, since the converse, i.e., snakes being the original hosts of *T. cascavelli*, would necessarily imply vectorial transmission because *M. americana* does share habitats with or feed on snakes.

Trypanosoma minasense is a trypanosomatid that infects various monkey families [86–90]. According to optical microscopy, it is morphologically similar to *T. rangeli* [90] and was considered to be a variant of this species [8]. *Trypanosoma minasense* is distributed from Central America to Argentina [87–88, 91–95], and little is known about its transmission in nature [3, 95–96]. Our results show that the *T. minasense* isolated from the *C. geoffroyi* monkey clustered with *T. bennetti* in the *Megatrypanum* clade in a branch close to *T. theileri* [97], reinforcing that at least this *T. minasense* sample is not related to *T. rangeli.* One possible explanation is that *T. minasense* is a diverse taxon or includes more than one species. In fact, there are many remaining open questions concerning *Trypanosoma* species in wild animals.

In conclusion, we observed a unique enzootic scenario in an area with aCD occurrence in the municipality of Guarapari. Here, we also observed that aCD cases can occur even without an enzootic cycle occurring near residential areas. The high trypanosome diversity that exists in such a small, fragmented region of the Atlantic rainforest may be due to the high capacity of bats and *T. vitticeps* to act as bioaccumulators of trypanosomes. Even two years after an aCD case occurred, the enzootic scenario did not change. Moreover, *T. vitticeps* maintained its vectorial capacity in terms of *T. c. marinkellei* and *T. dionisii*, in addition to four *T. c. cruzi* DTUs. Understanding this unique scenario will require multidisciplinary foci that include abiotic factors. Ultimately, our study reinforces the plasticity/complexity of the *Trypanosoma* species transmission cycle in nature.

Supporting information

S1 Table. SSU rRNA and gGAPDH GenBank reference sequences used in the phylogenetic analyses of *Trypanosoma* spp.
(DOCX)

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S1 Table. SSU rRNA and gGAPDH GenBank reference sequences used in phylogenetic analyses of *Trypanosoma* spp.

Isolate	Host Origin	GenBank Accession No.	
		SSU rRNA	gGAPDH
T. c. cruzi Dm28c	*Didelphis marsupialis*	AF245382	-
T. c. cruzi G	*Didelphis marsupialis*	-	GQ140351
T. c. cruzi Y	*Homo sapiens*	-	AB362560
T. c. cruzi Sylvio X10	*Homo sapiens*	AJ009147	-
T. c. cruzi Esmeraldocl3 clone2	*Homo sapiens*	AY785564	-
T. c. cruzi MT3663	*Panstrongylus geniculatus*	AF288660	JN040971
T. c. cruzi MT3869	*Homo sapiens*	AF303660	-
T. c. cruzi TC02	*Canis familiaris*	JQ912643	-
T. c. cruzi CanIII	*Homo sapiens*	-	KT305818
T. c. cruzi MT4167	*Rhodnius brethesi*	AF288661	-
T. c. cruzi SO3cl5 clone1	*Triatoma infestans*	AY785579	-
T. c. cruzi SO3cl5 clone 2	*Triatoma infestans*	AY785580	-
T. c. cruzi Nrcl3	*Homo sapiens*	-	GQ140357
T. cruzi CLBR	*Triatoma infestans*	AF245383	-
T. c. marinkellei B3	*Phyllostomus discolor*	FJ649484	FJ649495
T. c. marinkellei B7	*Phyllostomus discolor*	AJ009150	-
T. c. marinkellei TryCC 344	*Carollia perspicillata*	-	GQ140360
T. c. marinkellei TryCC 501	*Carollia perspicillata*	-	GQ140361
T. erneyi TCC1294	*Tadarida* sp.	JN040957	-
T. erneyi TCC1946	*Mops condylurus*	JN040961	JN040969
T. erneyi TCC1934	*Mops condylurus*	JN040991	JN040967
T. erneyi TCC1936	*Mops condylurus*	JN040992	JN040968
T. dionisii TCC/USP495	*Carollia perspicillata*	FJ0016667	GQ140363
T. dionisii P3	*Pipistrellus pipistrellus*	AJ009151	FJ649494
T. dionisii TryCC211	*Eptesicus brasiliensis*	-	GQ140362
T. rangeli PG	*Homo sapiens*	AJ012417	KT368805
T. rangeli AM80	*Homo sapiens*	AY491766	JN040973
T. rangeli San Augustin	*Homo sapiens*	-	KT368806
T. rangeli 4176	*Rhodnius brethesi*	EF071580	-
T. rangeli Choachi	*Rhodnius prolixus*	AJ012414	-
T. rangeli SC58	*Echimys dasythrix*	AY491745	KT368804
T. rangeli TryCC643	*Platyrrinus lineatus*	EU867803	GQ140364
T. rangeli RGB (Basel)	*Canis familiaris*	AJ009160	-
Trypanosoma sp. bat	*Rousettus aegyptiacus*	AJ012418	-
T. vespertilioni P14	*Pipistrellus pipistrellus*	AJ009166	AJ620283
Trypanosoma sp.	*Cercopithecus nictitans*	FM202493	FM164794
T. conorhini USP	*Rattus rattus*	AJ012411	AJ620267
Trypanosoma sp.	*Nandinia binotata*	FM2020492	FM164793
T. minasense LSTM	*Saimiri boliviensis*	AJ012413	-
T. leeuwenhoeki CH 250	*Choloepus hoffmanni*	AJ012412	-
Trypanosoma sp. BACO44	*Artibeus lituratus*	KT368797	KT368800
Trypanosoma sp BACO46	*Artibeus lituratus*	KT368798	KT368801
T. wauwau CBT68	*Pteronotus parnellii*	KR653210	KR653217
T. wauwau BMC1069	*Pteronotus parnellii*	KR653211	KR653218

T. wauwau TCC1022	*Pteronotus* sp.	KT368812	-
T. wauwau VCT6238	*Pteronotus gymnonotus*	KT030840	-
Trypanosoma sp. RNMO56	*Trachops cirrhosis*	KT368795	-
Trypanosoma sp. RNMO63	*Trachops cirrhosus*	KT368796	-
Trypanosoma sp. 64	*Trichosurus vulpecula*	JN315383	-
Trypanosoma sp. 17	*Trichosurus vulpecula*	JN315382	-
Trypanosoma sp. 15	*Trichosurus vulpecula*	JN315381	-
Trypanosoma sp. G8	*Bettongia penicillata*	KC753537	-
Trypanosoma sp. H25	*Macropus giganteus*	AJ009168	-
T. livingstonei 1298	*Rhinolophus landeri*	KF192982	-
T. livingstonei 1304	*Rhinolophus landeri*	KF192983	-
T. cascavelli 693	*Crotalus durissus*	EU095845	-
T. cascavelli 632	*Crotalus durissus*	EU095844	-
T. gennarii	*Monodelphis domestica*	KT343360	-
T. freitasi	*Didelphis brevicaudata*	MF401951	
Trypanosoma sp. 1052	*Pseudoboa nigra*	EU095839	-
Trypanosoma sp. 910	*Viannamyia tuberculata*	EU095838	-
Trypanosoma sp. Gecko	*Tarentola annularis*	AJ620548	-
T. varani	*Varanus exanthematicus*	AJ223572	-
T. cf. varani	*Python reginus*	AB447493	AB362559
T. varani V54	*Varanus exanthematicus*	AJ005279	-
T. scelopori	*Sceloporus occidentalis*	U67182	-
T. lewisi	*Rattus rattus*	-	AJ620272
T. evansi E 110	*Hydrochoerus hydrochaeris*	AJ009154	-
T. b. gambiense Tsuaa (clone G)	*Homo sapiens*	AJ009141	-
Trypanosoma sp. D30	*Dama dama*	AJ009165	-
T. theileri K127	*Bos taurus*	AJ009164	-
T. cyclops	*Macaca nemestrina*	AJ131958	-
Trypanosoma sp. wallaby ABF	*Wallabia bicolor*	AJ620564	-
T. grayi Crocamp1	*Crocodylus niloticus*	KF546526	-
T. grayi ANR4	*Glossina palpalis gambiensis*	AJ005278	-
T. grayi	*Glossina gambiensis*	AJ223565	-
T. minasense	*Saguinus midas*	AB362412	-
T. bennetti	*Falco sparverius*	AJ223562	-
Trypanosoma sp. N335	*Padda aryzivora*	AJ223570	-
T. corvi	*Corvus frugilegus frugilegus*	AY461665	-
T. terrestris CBT61	*Tapirus terrestris*	KF586848	-
Leishmania sp. MHOM/MQ/92/MAR1	n/a	AF303938	-
L. tarentolae	n/a	M84225	-
C. fasciculata	n/a	Y00055	-
H. samuelpessoai	n/a	U01016	-
H. muscarum	n/a	L18872	-
H. megaseliae	n/a	U01014	-
Phytomonas sp. TCC297e	n/a	KX219757	-

Artigo 3. Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats.

Maria Augusta Dario, Ricardo Moratelli, Philipp Schwabl, Ana Maria Jansen, Martin S. Llewellyn.

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No trabalho anterior, observamos que os morcegos são os principais hospedeiros de tripanosomatídeos nessa região da Mata Atlântica. Além disso, uma diversidade de espécies de tripanosomatídeos foram identificadas por PCR em amostras de DNA de hemocultivo desses animais. Decidimos avaliar a infecção e a diversidade de cinetoplastídeos, a partir de sangue total dos animais capturados no ano de 2015, nas mesmas áreas descritas no artigo 2. Para identificação das diferentes espécies de cinetoplastídeos, muitos dos quais não são cultiváveis, e determinação da diversidade e abundância das mesmas em uma única amostra, usamos o sequenciamento de nova geração.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats

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Abstract

Background

Bats are a highly successful, globally dispersed order of mammals that occupy a wide array of ecological niches. They are also intensely parasitized and implicated in multiple viral, bacterial and parasitic zoonoses. Trypanosomes are thought to be especially abundant and diverse in bats. In this study, we used 18S ribosomal RNA metabarcoding to probe bat trypanosome diversity in unprecedented detail.

Methodology/Principal Findings

Total DNA was extracted from the blood of 90 bat individuals (17 species) captured along Atlantic Forest fragments of Espírito Santo state, southeast Brazil. 18S ribosomal RNA was amplified by standard and/or nested PCR, then deep sequenced to recover and identify Operational Taxonomic Units (OTUs) for phylogenetic analysis. Blood samples from 34 bat individuals (13 species) tested positive for infection by 18S rRNA amplification. Amplicon sequences clustered to 14 OTUs, of which five were identified as *Trypanosoma cruzi* I, *T. cruzi* III/V, *Trypanosoma cruzi marinkellei*, *Trypanosoma rangeli*, and *Trypanosoma dionisii*, and seven were identified as novel genotypes monophyletic to basal *T. cruzi* clade types of the New World. Another OTU was identified as a trypanosome like those found in reptiles. Surprisingly, the remaining OTU was identified as *Bodo saltans*–closest non-parasitic relative of the trypanosomatid order. While three blood samples featured just one OTU (*T. dionisii*), all others resolved as mixed infections of up to eight OTUs.

Conclusions/Significance

This study demonstrates the utility of next-generation barcoding methods to screen parasite diversity in mammalian reservoir hosts. We exposed high rates of local bat parasitism by multiple trypanosome species, some known to cause fatal human disease, others non-pathogenic, novel or yet little understood. Our results highlight bats as a long-standing nexus

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among host-parasite interactions of multiple niches, sustained in part by opportunistic and incidental infections of consequence to evolutionary theory as much as to public health.

Author summary

Bats make up a mega-diverse, intensely parasitized order of volant mammals whose unique behavioural and physiological adaptations promote infection by a vast array of microorganisms. Trypanosomes stand out as ancient protozoan parasites of bats. As cryptic morphology, low parasitaemia and selective growth in culture have recurrently biased survey, we used 18S ribosomal RNA metabarcoding to resolve bat trypanosomatid diversity in Atlantic Forest fragments of southeast Brazil. Next to several unknown species, our deep sequence-based detection and assignment protocol recognized multiple known human-pathogenic trypanosomes, another linked to reptile hosts as well as a non-parasitic kinetoplastid in the blood of various phyllostomid bats. The striking permissivity exposed here, in a region where bat trypanosomes recently featured in a fatal case of Chagas disease, compels further research on bats' role in the dispersal and spill-over of various microorganisms among humans and wildlife.

Introduction

Trypanosoma cruzi is the etiological agent of Chagas disease, a complex zoonosis that continues to take dozens of human lives each day [1]. Alongside its close relative *Trypanosoma cruzi marinkellei* in the *Schizotrypanum* subgenus, this important protozoan flagellate belongs to a broader, inter-continental group (the "*T. cruzi* clade") of ancient endoparasites found to infect the mammalian fauna far and wide [2–3]. Infections have been reported in primates of Africa [4], marsupials of Australia [5] and a multitude of terrestrial mammals across the Americas [6], but most of this striking spread in host diversity tallies to few taxa within the clade (above all to *T. cruzi sensu stricto*, i.e., *T. cruzi*, and to *T. rangeli*).

The majority of *T. cruzi* clade diversity is found in bats. Chiroptera are known to carry both generalists such as *T. cruzi* and *T. rangeli* as well as multiple bat-restricted species—some abundant (e.g., *T. c. marinkellei*, *T. dionisii* and *T. erneyi*), others rare (e.g. *T. livingstonei* and *T. wauwau*) [3, 7–8]. Chiropteran immunity is unique with respect to other mammalian genera, coincident perhaps with physiological adaptations to flying [9]. Several features of bat immunity may predispose bats to long-term asymptomatic infections [10] with viruses [11–12], bacteria [13–14], fungi [15–16], protozoa [17–18] and helminths [19–20], several of which cause disease in humans and animals [21].

Given the diversity of bat-infecting *T. cruzi*-clade trypanosomes throughout the New and Old Worlds, many now accredit the Chiroptera with a fundamental role in the evolution of this parasite group [22]. In fact, the most parsimonious explanation to date for the origin and past expansion of the *T. cruzi* clade suggests a common ancestral lineage of bat-restricted trypanosomes that diversified into several independent lineages that on rare occasion switched into other terrestrial mammal hosts [17]. Bats' recurrent interaction with other mammals and their various ectoparasites are thought to have afforded enough opportunity for at least five such switching or "seeding" events, likely since the early Eocene (54 to 48 million years ago) [7].

Many trypanosomes from bats are morphologically indistinguishable, often described simply as "*T. cruzi*-like" in the past [23]. As mixed species/genotype infections are probably common but overlooked or mistaken, molecular barcoding presents expedient recourse in resolving intricate trypanosomatid taxonomy and ecology. Metabarcoding couples classic molecular barcoding with next generation sequencing techniques [24–25] to generate thousands of sequence reads from a single sample [26–27]. These reads correspond to the diversity and abundance of organisms infecting the host individual [28–30].

In this study, we applied next-generation metabarcoding methods to the most bat-diverse (per area) biome of Brazil [31]. We focused on a degraded section of Atlantic Forest in Espírito Santo (ES) state where terrestrial mammals appear reduced in abundance as well as in *T. cruzi* infection. A fatal case of human *T. cruzi* (I-IV) and *T. dionisii* coinfection [32] immediately predated the bat trypanosome survey by 18S ribosomal RNA deep sequencing in this region.

Methods

Ethical statement

The sampling procedures reported herein were authorized by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) under license no. 19037–1 (23-05-2009). Euthanasia and blood collection met guidelines set by the Federal Council of Veterinary Medicine, Resolution 1000 (11-05-2012), in accordance to Federal Law 11.794/2008. All procedures followed protocols approved by the Oswaldo Cruz Foundation (Fiocruz) Ethics Committee for Animal Research (L0015-07).

Study area, bat capture and sampling

Bat captures were carried out in two periods of 2015: June (dry season) and November (rainy season). Mist nets were opened upon sunset for four hours on two consecutive nights at each study location. A total of 108 bats were captured using ten mist nets (3 x 9 m, 35 mm mesh) placed along forest edges near banana and coffee crops at three different rural locations in Guarapari municipality, ES state, southeast Brazil: Rio da Prata (350 m a.s.l.), where a fatal case of Chagas disease occurred in 2012; Buenos Aires (250 m a.s.l.), where reports of triatomine invasion have increased in recent years; and Amarelos (at sea level), where triatomines have not been reported from the domestic zone (based on records by the Zoonosis Control Center, Guarapari municipality, ES) (S1 Fig).

Taxonomic identification by morphology followed [33] and a maximum of ten individuals per species (per site) were kept for further sampling, as specified by law. Once anesthetized with acepromazine (2%) in 9:1 ketamine hydrochloride (10%), these individuals were cleared of fur in the pectoral region (by scalpel) and sterilized with antiseptic soap and iodinated ethanol (70%) for blood withdrawal by cardiac puncture. Within the safety area of a flame, 300 μl blood was collected into sterile 1.5 ml vials and stabilized in two parts (i.e., 600 μl) 6 M Guanidine-HCl, 0.2 M EDTA solution for storage at -20˚C. All bats used in these analyses received a collection number with the initials of the collector (RM) and were prepared for fluid preservation. This material will be subsequently deposited at the mammal collection of Museu Nacional, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

18S rRNA amplification and deep sequencing

DNA was purified from 90 guanidine-EDTA blood lysates in DNeasy mini spin columns (Qiagen), with each of nine extraction rounds including one negative control. Purified DNA samples were then PCR-amplified with primers 5'-TGGGATAACAAAGGAGCA-3' (forward)

and 5'-CTGAGACTGTAACCTCAAAGC-3' (reverse) for 30 cycles of 94˚C (30 s), 55˚C (60 s) and 72˚C (90 s) to target a trypanosome-specific, ~556 bp region of the 18S rRNA gene as established in [5]. For a subset of samples, a wider, ~927 bp region (encompassing the ~556 bp above) was first targeted with external primers 5'-CAGAAACGAAACACGGGAG-3' (forward) and 5'-CCTACTGGGCAGCTTGGA-3' (reverse) at equivalent cycling conditions to form a nested (two-round) PCR amplification procedure following [34]. Sterile water (2x) and sample-free eluate from prior DNA purification (1x) were used to provide three negative controls per 20-sample PCR reaction. Amplicons were single-end barcoded [35], purified by agarose gel electrophoresis (PureLink Quick Gel Extraction Kit, Invitrogen), quantified by fluorometric assay (Qubit 2.0, Thermo Fisher Scientific) and pooled to equimolar concentration for multiplexed, paired-end (2 x 300 bp) sequencing on the Illumina MiSeq platform (Reagent Kit v2).

Species delimitation and phylogenetic analysis

Amplicon sequences were filtered to retain only full-length reads of $\geq$ 99.9% base call accuracy by windowed trimming in Sickle [36], verified for quality in FastQC [37] and mapped against a *Trypanosoma* spp. reference collection from SILVA v119 [38] using Bowtie 2 [39]. Operational Taxonomic Unit (OTU) construction proceeded by UPARSE algorithm in USEARCH [40] and BLAST-based taxonomic assignment in the QIIME environment [41], with run parameters established during prior *in silico* testing on trypanosomatid 18S rRNA sequences from NCBI. Samples were clustered to OTUs *de novo* at 98% sequence similarity and assigned to extant species with a confidence threshold of 80%. Unassigned clusters were considered valid OTUs only if present at $>$ 300 reads in any single sample and present at $>$ 600 reads across all samples of the dataset.

Following OTU establishment, sequence read pairs from one representative per OTU were merged and aligned in Clustal W (with manual refinement of misplaced reads). Phylogenies were inferred in Mega 6 [42] by maximum likelihood (ML) tree construction under Kimura's two-parameter model of nucleotide substitution with gamma-distributed variation among sites (K2 + G). One thousand bootstrap replicates were run to establish nodal support. The 50 18S rRNA reference sequences applied in phylogenetic analyses are listed with accession numbers in S1 Table. All sequences have been deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SRR5451077-SRR5451120.

Results

Bat abundance and diversity

Of the 108 bats captured at Amarelos, Buenos Aires and Rio da Prata study sites, 105 individuals represent 16 species in the Phyllostomidae family, and three individuals represent one species (*Myotis nigricans*) in the Vespertilionidae family. Species and their abundances are listed in Table 1.

Trypanosomatid abundance, diversity and distribution in bats

Standard and/or nested PCR amplified 18S rRNA gene fragments from 34 of 90 (38%) bat blood samples. The 34 positive samples derived from 13 bat species (of 17 species analysed) and comprised 14 distinct kinetoplastid OTUs. Five OTUs were assigned to *T. cruzi* I (OTU 3), *T. cruzi* III/IV (OTU 5), *T. c. marinkellei* (OTU 6), *T. rangeli* lineage D (OTU 10) and *T. dionisii* (OTU 2). A further seven OTUs did not assign to any known species of the *T. cruzi* clade. Phylogenetic analyses placed these seven OTUs (1, 7, 8, 11, 12, 13 and 14) within a

Table 1. Bat species captured in Guarapari municipality, ES state, Brazil.

Bat species	Capture sites		
	Amarelos	Buenos Aires	Rio da Prata
Anoura geoffroyi	-	-	3
Anoura caudifer	1	-	4
Artibeus fimbriatus	-	2	1
Artibeus lituratus	9	3	4
Carollia perspicillata	17	10	12
Desmodus rotundus	9	-	1
Glossophaga soricina	3	-	-
Micronycteris sp.	2	-	-
Myotis nigricans	2	1	-
Phyllostomus discolor	2	-	2
Phyllostomus hastatus	1	-	-
Platyrrhinus lineatus	-	-	2
Platyrrhinus recifinus	-	1	3
Rhinophylla pumilio	-	-	6
Sturnira lilium	2	1	2
Tonatia bidens	1	-	-
Trachops cirrhosus	1	-	-
Total	**50**	**18**	**40**

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monophyletic group that includes trypanosome species from bats of the New World. Finally, two OTUs showed greater homology outside of the *T. cruzi* clade—OTU 4, similar to a trypanosomatid species found in reptiles, and OTU 9, nearly identical to the eubodonid *Bodo saltans* (Figs 1 and 2, S1 Table).

Most trypanosome-infected bats presented mixed infections by two to eight OTUs. Only three positive blood samples (from *D. rotundus*, *G. soricina* and *R. pumilio*) contained a single OTU (*T. dionisii*; OTU 2). The bat species *A. lituratus*, *C. perspicillata*, *D. rotundus* and *P. recifinus* presented greatest trypanosome diversity, with seven to eight OTUs per species (Fig 3). Across the three study sites, trypanosomatid diversity and abundance broadly reflected bat capture success rather than any feature of the capture environment (Table 1 and Fig 4).

Standard vs. nested PCR sensitivity

Nested PCR detected between one and six more OTUs than standard PCR in eight of ten samples subjected to both procedures, showing less sensitivity only in samples RM 847 and RM 2009—one and two less OTUs amplified, respectively (Fig 3).

Discussion

In this study, we exposed unforeseen bat trypanosome 18S rRNA diversity from standard capture effort in Atlantic Forest fragments of Guarapari municipality, ES, southeast Brazil. Our metabarcoding approach identified a preponderance of coinfection, involving several human-pathogenic and bat-associated types of the *T. cruzi* clade, as well as a swathe of yet undescribed diversity closer to its base. Furthermore, we identified sequences from two divergent kinetoplastid taxa—one similar to trypanosomatid isolates from reptiles, another matching the non-parasitic *B. saltans*.

Unprecedented as they may be as complex co-infections, the diversity of individual kinetoplastids we report is not unexpected. Every recent trypanosome survey of bats has revealed

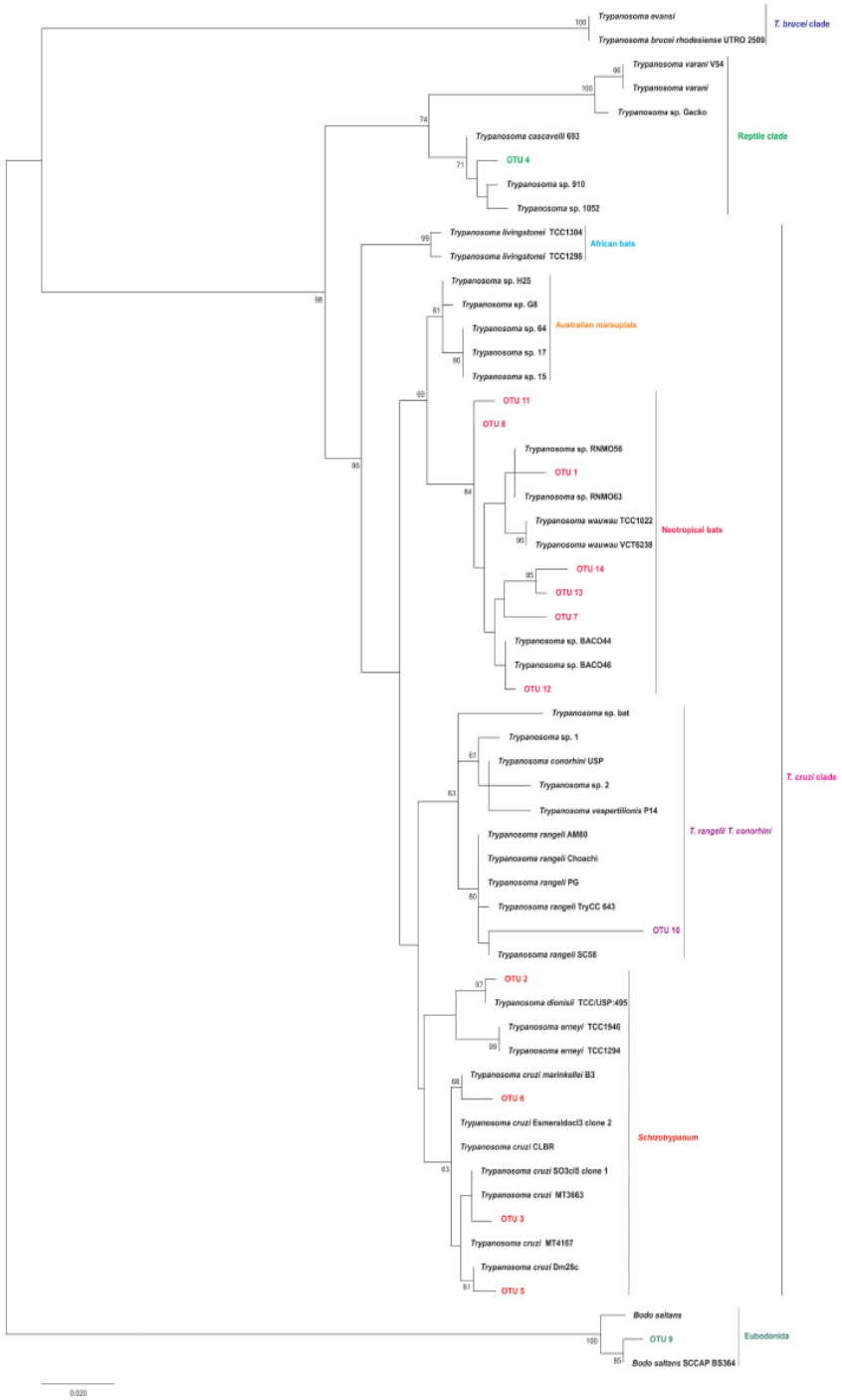

Fig 1. Phylogenetic placement of kinetoplastid OTUs detected in bats of Guarapari municipality, ES state, Brazil. Tree construction from 18S rRNA followed the maximum likelihood (ML) method under Kimura's two-parameter model and gamma-distributed variation among sites (K2 + G). Numbers at nodes indicate support from 1000 bootstrap replicates. The 14 OTUs clustered into the *T. cruzi* clade (OTUs 1, 2, 3, 5, 6, 7, 8, 10, 11, 12, 13 and 14), a reptile-associated region (OTU 4) and the *B. saltans* outgroup (OTU 9).

https://doi.org/10.1371/journal.pntd.0005790.g001

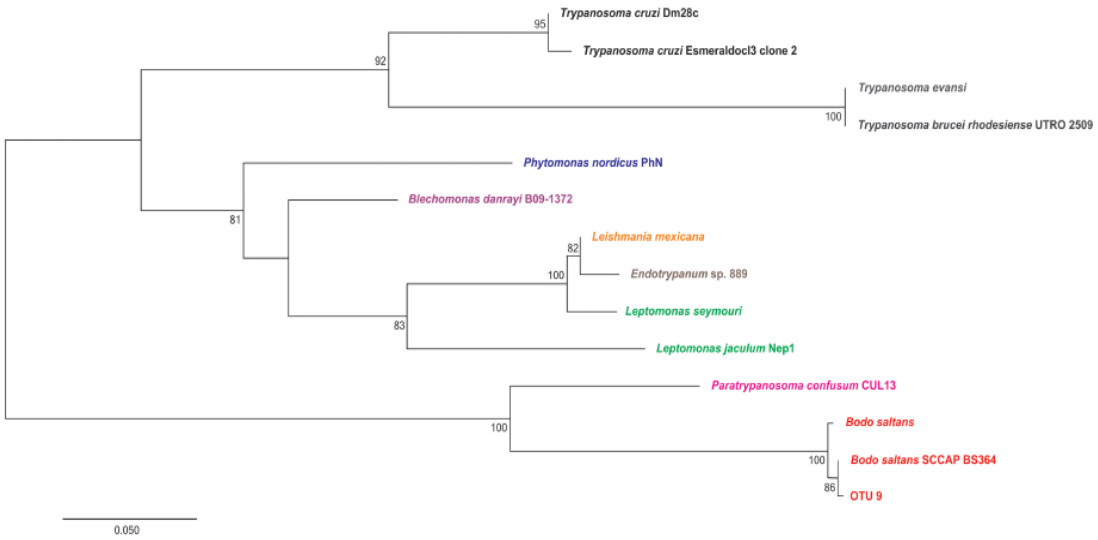

Fig 2. Phylogenetic placement of OTU 9 with *Bodo saltans* among a wider set of trypanosomatid genera. Tree construction from 18S rRNA followed the maximum likelihood (ML) method under Kimura's two-parameter model and gamma-distributed variation among sites (K2 + G). Numbers at nodes indicate support from 1000 bootstrap replicates.

https://doi.org/10.1371/journal.pntd.0005790.g002

novel parasite genotypes, host- and/or geographic range [8, 43–50], with particular surges in discovery following intensified sampling (e.g., transcontinental archival analysis) [8] or innovative approach (e.g., coalescent species delimitation) [43]. The 18S rRNA deep sequencing in bats here identifies further diversity around the most basal *T. cruzi* clade trypanosomes of the

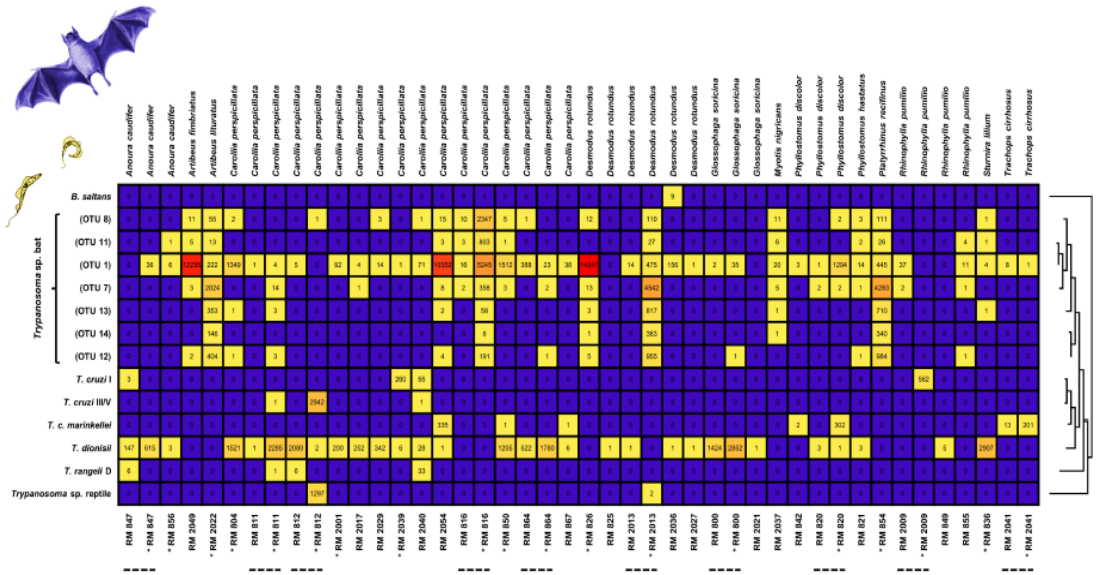

Fig 3. Heatmap of kinetoplastid OTU distribution among bats captured in Guarapari municipality, ES state, Brazil. Each column represents the infection profile of one infected bat individual. Cell colour denotes the sequence read intensity attributed to each OTU (left), increasing from purple (zero reads) through yellow into red. Bat species and sample IDs are given above/below. Asterisks indicate samples subjected to nested PCR, of which ten also underwent standard PCR (dashed lines). Phylogenetic relationships inferred from 18S rRNA by maximum likelihood (ML) tree construction are plotted at right.

https://doi.org/10.1371/journal.pntd.0005790.g003

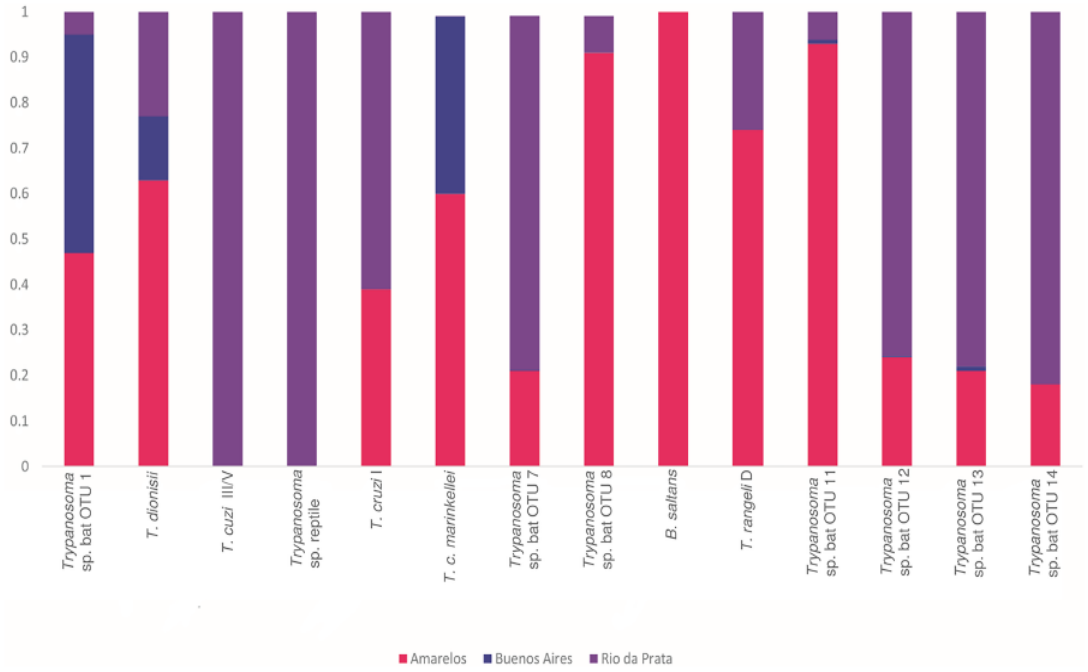

Fig 4. Kinetoplastid OTU distribution among study locations in Guarapari municipality, ES state, Brazil. Sequence reads attributed to each OTU are color-coded by proportions obtained from bats captured at Amarelos (magenta), Buenos Aires (blue) and Rio da Prata (violet) study sites.

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New World, with seven independent and novel taxonomic units forming sister groups to *T. wauwau* and *Neobat* species found in mormoopid and phyllostomid bats [8]. This expansion of a group related more closely to trypanosomatids detected in Australian marsupials than to those known from other neotropical mammals' points to the Chiroptera as an ancient, perhaps original host order of the *T. cruzi* clade. Our data reinforce the bat host range of *T. cruzi*-clade trypanosomes across frugivorous, nectarivorous, carnivorous, generalist and hematophagous phyllostomid genera (*Anoura*, *Artibeus*, *Carollia*, *Desmodus*, *Glossophaga*, *Platyrrhinus*, *Phyllostomus*, *Rhinophylla*, *Sturnira*, *Trachops*) and into the (primarily insectivorous) Vespertilionidae.

Our study provides strong, if circumstantial, evidence for the role of bats as *T. cruzi* reservoirs in ES state. *Trypanosoma cruzi* I and III/V found in bats of this study correspond to Discrete Typing Units (DTUs) associated with a recent fatal *T. cruzi*–*T. dionisii* mixed infection and occur in *Triatoma vitticeps* at the study site [32]. These DTUs were not detected in parasitological or serological tests on local rodents and marsupials [32]. *Triatoma vitticeps* is thought to have poor stercocarian vector competence [51] and oral transmission via insectivory may be one of the few ways in which this species propagates disease. The apparent transfer of trypanosome diversity *en masse* from bat to human host via ingestion of the vector [32] supports transmission efficiency reported elsewhere in oral outbreaks [52]. Furthermore, given the low terrestrial mammal abundance in the heavily fragmented region where the samples were collected [32], bats may function here as principal reservoirs of parasites. There is growing evidence of bats' potential in the maintenance of zoonotic *T. cruzi* transmission elsewhere in South America. For example, recent molecular surveys rank bats as top feeding sources of synanthropic *T. cruzi*-infected triatomines throughout Colombia, emphasize bats' bridging of

domestic and sylvatic transmission cycles in rural areas of Ecuador [45] (where non-volant hosts have shown limited infection [53–54]) and implicate bats as long-term refuges for parasites in areas subject to transmission interventions in Argentina [46]. Evidence of a new *T. cruzi* genotype associated with anthropogenic bats (TcBat) is also accumulating from around the continent [45, 55–58]. TcBat was not, however, observed in this study.

Here, we also provide first report of *T. rangeli* lineage D in bats, a strain initially isolated from *Phyllomys dasythrix* in southern Brazil [59]. As the ecogeographical structure of the *Rhodnius* spp. complex is thought to drive lineage divergence in *T. rangeli* [60–61], an efficiently transmitted salivarian parasite, our detection of lineage D further north and beyond the Rodentia serves well to confirm theory. Its putative vector *R. domesticus* [62] occurs throughout the Atlantic Forest, often in bromeliads [63] that rely on nectarivorous bats (e.g., the specialist flower-feeder *A. caudifer*) for pollination [64].

Whilst the expansion of the range of *T. rangeli* comes as little surprise, the presence of trypanosomes (OTU 4) with reptilian affinities in our study population is perhaps more intriguing. Nonetheless, bats and reptiles do commonly co-occur in an arboreal niche. Ecological host-fitting, involving opportunistic host switching mediated by vectors' feeding patterns within an ecological niche, is thought to be a prevailing mode of trypanosome evolution [65]. Reptilian trypanosomes are transmitted by sand fly vectors [65–66], with reports from Amazonia (*Viannamyia tuberculate* [67]) as well as central Brazil (*Evandromyia evandroi* [68]). Shared microhabitat use among bats, reptiles and sand flies potentiates spill-over of the parasite.

Most trypanosomatid diversity observed in this study was associated with complex mixed infections, a likely consequence of bats' gregarious way of life. Tolerance of intracellular pathogens in the Chiroptera [21] suggests that multiple subclinical/asymptomatic infections may well accumulate in these hosts before triggering pathology linked to adaptive immune reactions in other non-volant mammals [69–72]. Frequent mixed infections, often coupled with low parasitaemia, have impeded bat trypanosome surveys in the past, both in genotyping from primary samples (e.g., low sensitivity in classic barcoding) [73] and on cultured cells (e.g., growth bias) [55, 61, 74]. The data presented here suggest that deep sequencing can resolve both infection identity and complexity.

Although our study demonstrates the power of the metabarcoding approach, several caveats are relevant. Sensitivity to contamination and errors from amplification and sequencing are of foremost concern [27, 75]. We employed a variety of cautionary measures during sample processing (e.g., flame-sterilized blood withdrawal, multiple negative DNA extraction/amplification controls) and in the bioinformatic phase: prior to taxonomic inference, we sent sequenced amplicons through a severe quality filter (99.9% base call accuracy), absorbed potential artefactual variance into broad 98% similarity clusters and rejected unassigned OTUs present at low to moderate depth (< 300/600 reads). Nevertheless, our study would have benefited from the inclusion of traditional methods (e.g., microscopy, ex and in vivo culture) for validation and follow-up. Based on subunit rRNA, OTU 9, isolated from a single bat (*D. rotundus*), was assigned to *B. saltans*, considered the closest free-living relative of the parasitic trypanosomatids. This observation joins others in unsettling assumptions about putatively free-living, yet seldom studied protist taxa. For example, 18S rRNA analysis (complemented by microscopy and serological testing) found an apparent case of babesiosis in China to involve erythrocytic colpodellids, the closest "free-living" relatives of the parasitic Apicomplexa [76].

Regrettably, our field-based study passed over visual and biochemical tests that could have established the occurrence and viability of OTU 9 in mammalian tissue and we hesitate to entirely rule out environmental contamination as its source. *Bodo saltans* belongs to the most

widely adapted, physiologically tolerant zooflagellates on Earth [77]. It abounds in soil and water and can also spread in aerosolized forms. As such, this eubodonid may in rare cases happen upon sampling equipment as well as resist certain antiseptic measures taken in the field. On account of its exceptional halotolerance [78], for example, *B. saltans* may withstand some iodine-based disinfection (as do other protozoans—e.g., *Cryptosporidium* and *Giardia* [79]), though very unlikely as performed in this study (i.e., with ethanol). More importantly, however, OTU detection does not require a living organism, only its DNA. Severe contamination from the "dead" DNA of protist flagellates has indeed preoccupied past rRNA sequence analysis (e.g., see methods in [80]). In any case, we suggest additional (environmental) control samples (e.g., vials opened in the field, topical swabs around the site of cardiac puncture) and laboratory efforts that distinguish DNA from viable cells (e.g., separation of lysed and non-lysed cells, RNA/DNA comparisons) to help test for such possibilities in future research.

In this section of Atlantic Forest, where a rural Chagas disease fatality in all likelihood involved a bat-feeding triatomine [32], our deep sequencing study highlights the role of the Chiroptera as a reservoir for trypanosomiases. Furthermore, the unprecedented transfer of *T. dionisii* to a human from a bat, as well as the presence of reptile-infecting and putatively non-parasitic kinetoplastids in the same bat population, highlights the role of bats as keystone species in parasite spill-over events. Many questions remain on how the role of sylvatic hosts in pathogen dispersal varies in space and time, upon change to environment and at the evolutionary scale. Research into these intricacies of complex zoonosis will require much further innovation with high-sensitivity, high-throughput tools. We point to the power of next-generation metabarcoding strategies in studies of trypanosomatid ecology and evolution and strongly commend their future complementation with non-molecular methods.

Supporting information

S1 Fig. Representative map of bat capture locations in Atlantic Forest of Guarapari municipality, ES state, Brazil.
(TIF)

S1 Table. GenBank reference sequences used in phylogenetic analyses of kinetoplastid 18S rRNA.
(DOCX)

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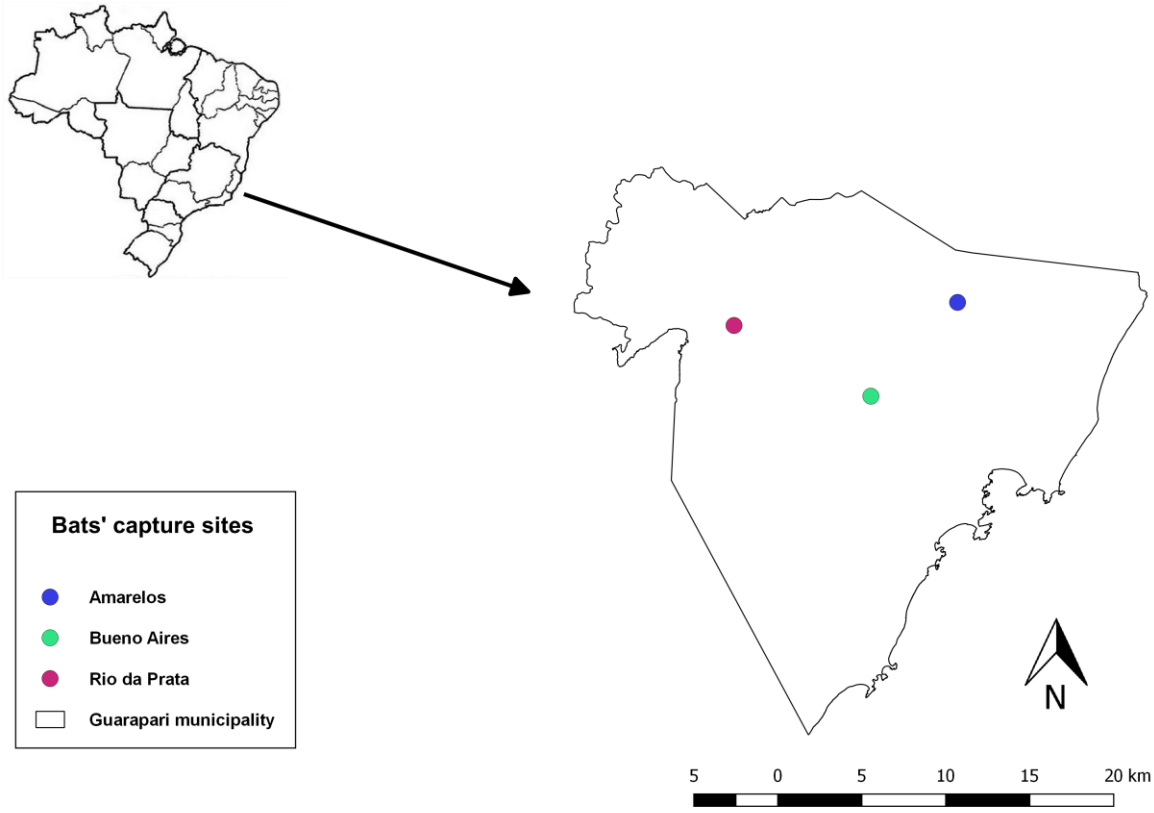

S1 Fig: Representative map of bat capture locations in Atlantic Forest of Guarapari municipality, ES state, Brazil.

S1 Table: GenBank reference sequences used in phylogenetic analyses of trypanosomatid 18S rRNA.

Isolate	Host Origin	GenBank Accession No.
T. cruzi Dm28c	*Didelphis marsupialis*	AF245382
T. cruzi Esmeraldocl3 cl 2	*Homo sapiens*	AY785564
T. cruzi MT3663	*Panstrongylus geniculatus*	AF288660
T. cruzi MT4167	*Rhodnius brethesi*	AF288661
T. cruzi SO3 cl5	*Triatoma infestans*	AY785579
T. cruzi CLBR	*Triatoma infestans*	AF245383
T. c. marinkellei B3	*Phyllostomus discolour*	FJ649484
T. erneyi TCC1294	*Tadarida sp.*	JN040988
T. erneyi TCC1946	*Mops condylurus*	JN040989
T. dionisii TCC/USP495	*Carollia perspicillata*	FJ016667
T. rangeli PG	*Homo sapiens*	AJ012417
T. rangeli AM80	*Homo sapiens*	AY491766
T. rangeli Choachi	*Rhodnius prolixus*	AJ012414
T. rangeli SC58	*Echimys dasythrix*	AY491745
T. rangeli TryCC643	*Platyrrinus lineatus*	EU867803
Trypanosoma sp bat	*Rousettus aegyptiacus*	AJ012418
T. vespertilioni P14	*Pipistrellus pipistrellus*	AJ009166
Trypanosoma sp 2	*Cercopithecus nictitans*	FM202493
T. conorhini USP	*Rattus rattus*	AJ012411
Trypanosoma sp 1	*Nandinia binotata*	FM202492
Trypanosoma sp BACO44	*Artibeus lituratus*	KT368797
Trypanosoma sp BACO46	*Artibeus lituratus*	KT368798
T. wauwau VCT6238	*Pteronotus gymnonotus*	KT030840
T. wauwau TCC1022	*Pteronotus parnellii*	KT030830
Trypanosoma sp RNMO56	*Trachops cirrhosis*	KT368795
Trypanosoma sp RNMO63	*Trachops cirrhosis*	KT368796
Trypanosoma sp 64	*Trichosurus Vulpecula*	JN315383
Trypanosoma sp 17	*Trichosurus Vulpecula*	JN315382
Trypanosoma sp 15	*Trichosurus Vulpecula*	JN315381
Trypanosoma sp G8	*Bettongia penicillate*	KC753537

Trypanosoma sp H25	*Macropus giganteus*	AJ009168
T. livingstonei TCC1298	*Rhinolophus landeri*	KF192982
T. livingstonei TCC1304	*Rhinolophus landeri*	KF192983
Trypanosoma sp 1052	*Pseudoboa nigra*	EU095839
T. cascavelli 693	*Crotalus durissus*	EU095845
Trypanosoma sp 910	*Viannamyia tuberculate*	EU095838
Trypanosoma sp Gecko	*Tarentola annularis*	AJ620548
T. varani	*Varanus exanthematicus*	AJ223572
T. varani V54	*Varanus exanthematicus*	AJ005279
T. evansi	*Tabanus rubidus*	AY904050
T. brucei rhodesiense UTRO 2509	*Homo sapiens*	AJ009142
Leishmania mexicana	*Homo sapiens*	GQ332360
Endotrypanum sp 889	*Psathyromyia dendrophyla*	EU021240
Leptomonas seymouri	*Dysdercus suturellus*	AF153040
Leptomonas jaculum Nep1	Nepa cinereal	EF184218
Phytomonas nordicus PhN	*Troilus luridus*	KT223609
Blechomonas danrayi B09-1372	*Chaetopsylla globiceps*	KF054137
Paratrypanosoma confusum CUL13	*Culex pipiens*	KF963538
Bodo saltans SCCAP BS364	n/a	AY998648
B. saltans	n/a	JF693632

Artigo 4. Identification of novel mammalian hosts and Brazilian biome geographic distribution of *Trypanosoma cruzi* TcIII and TcIV.

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Na Mata Atlântica do estado do Espírito Santo e no paciente que veio a óbito por DCA observamos a ocorrência das DTUs de *T. cruzi* TcIII e TcIV. Essas DTUs já foram observadas em diversos hospedeiros em outros biomas brasileiros. Esse trabalho teve como finalidades recaracterizar isolados antes descritos como zimodema 3 (TcIII/IV) e compreender as associações dessas DTUs com hospedeiros mamíferos, vetores e distribuição geográfica. Verificamos se as DTUs de *T. cruzi* TcIII e TcIV apresentam uma maior distribuição e diversidade de hospedeiros do que já foi descrito na literatura.

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Identification of novel mammalian hosts and Brazilian biome geographic distribution of *Trypanosoma cruzi* TcIII and TcIV

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ABSTRACT

Trypanosoma cruzi is a parasitic protozoan responsible for Chagas disease. Seven different Discrete Typing Units (DTUs) of *T. cruzi* are currently identified in nature: TcI – TcVI, and TcBat whose distribution patterns in nature, hosts/reservoirs and eco-epidemiological importance are still little known. Here, we present novel data on the geographic distribution and diversity of mammalian hosts and vectors of *T. cruzi* DTUs TcIII and TcIV. In this study, we analyzed 61 *T. cruzi* isolates obtained from 18 species of mammals (five orders) and two Hemiptera genera. Samples were collected from five Brazilian biomes (Pantanal, Caatinga, Cerrado, Atlantic Rainforest, and Amazon) previously characterized as Z3 or mixed infection (TcI-Z3) by mini-exon gene PCR. To identify TcIII and TcIV genotypes, we applied restriction fragment length polymorphism analysis to the PCR-amplified histone 3 gene. DTUs TcIII and TcIV were identified in single and mixed infections from wide dispersion throughout five Brazilian biomes studied, with TcIV being the most common. Pantanal was the biome that displayed the largest number of samples characterized as TcIII and TcIV in single and mixed infections, followed by Atlantic Rainforest and Amazon. Species from the Didelphimorphia order displayed the highest frequency of infection and were found in all five biomes. We report, for the first time, the infection of a species of the Artiodactyla order by DTU TcIII. In addition, we describe new host species: five mammals (marsupials and rodents) and two genera of Hemiptera. Our data indicate that DTUs TcIII and TcIV are more widespread and infect a larger number of mammalian species than previously thought. In addition, they are transmitted in restricted foci and cycles, but in different microhabitats and areas with distinct ecological profiles. Finally, we show that DTUs TcIII and TcIV do not present any specific association with biomes or host species.

1. Introduction

Trypanosoma cruzi is a digenetic parasite that maintained an enzootic cycle until the arrival of humans to the Americas (Guhl et al., 2000). The geographic distribution of sylvatic *T. cruzi* stretches from the southern United States to southern Argentina and Chile, including Brazil. *T. cruzi* is considered an eclectic parasite since it inhabits domestic, peridomestic, and sylvatic habitats (Guhl et al., 2000; Jansen et al., 2015). In the latter, the parasite can establish transmission cycles through different ecological niches and forest strata that may or not overlap even within the same forest fragment. In addition, each particular habitat in a biome exhibits quantitatively and qualitatively different species of infected animals, which currently include eight orders of mammals (Artiodactyla, Carnivora, Chiroptera, Didelphimorphia, Perissodactyla, Primates, Rodentia, and Cingulata) and dozens of triatomine species as insect vectors (Rassi et al., 2010).

The genetic diversity of *T. cruzi*, as well as its biological plasticity, wide geographic distribution, numerous hosts, and the difficulty of obtaining representative samples from hosts and biomes represent additional obstacles to understanding the variables that support the transmission cycles (Lima et al., 2014).

Over the years, numerous approaches have been used to characterize the population structure of *T. cruzi*, with the aim of defining and determining the number and ecology of the relevant subgroups (Andrade 1974; Miles et al., 1981; Morel et al., 1980; Tibayrenc and Ayala 1991; Souto et al., 1996; Tibayrenc et al., 1988; Kawashita et al., 2001; Freitas et al., 2006). Polymerase chain reaction (PCR) amplification of specific genetic markers has proven particularly useful. One such marker is the mini-exon intergenic region, which permits the identification of three *T. cruzi* discrete typing unit (DTU) groups: Tc1 (DTU TcI), Tc2 (DTUs TcII/TcV/TcVI), and Zymodeme 3 (Z3; DTUs TcIII/TcIV), as well as *Trypanosoma rangeli* and mixed infections of different DTUs

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(Fernandes et al., 1999; Aliaga et al., 2011). Currently, there are seven recognized *T. cruzi* DTUs: TcI-TcVI and Tcbat (Lima et al., 2014; Zingales et al., 2012). These DTUs have distinct profiles that can be characterized by performing restriction fragment length polymorphism (RFLP) analyses of different nuclear genes. This technique allows for rapid, easy, and reliable identification of the isolates belonging to specific DTUs and mixed infections (Rozas et al., 2007).

Whereas all DTUs can be found in Brazil, the most prevalent are TcI and TcII. To date, no specific associations have been found between DTUs and mammalian host species. Indeed, both DTUs are frequently found infecting different host species in transmission cycles with diverse ecological and geographic distribution patterns (Zingales et al., 2012; Lima et al., 2014; Lisboa et al., 2006; Xavier et al., 2007; Araujo et al., 2014).

DTUs TcIII and TcIV were previously classified as a single group named Z3 or as two groups named TcIIc and TcIIa, respectively. Though far less studied than TcI and TcII, these DTUs have been detected in a variety of sylvatic cycles throughout South America (Zingales et al., 2009).

The terrestrial transmission cycle of DTU TcIII in the Amazon biome has been associated to fossorial mammals such as armadillos, and the so-called terrestrial marsupials (Miles et al., 1981; Povoa et al., 1984; Llewellyn et al., 2009). Recent studies have shown that TcIII has a vast distribution in humans, sylvatic reservoirs, and triatomines across central, northern, and southern Brazil (Marcili et al., 2009; Câmara et al., 2010; Monteiro et al., 2010; Abolis et al., 2011); and from western Venezuela to the Argentine Chaco (Llewellyn et al., 2009; Brisse et al., 2000). In Brazil, this DTU has been described in the Atlantic Rainforest, Caatinga, Pantanal, and Amazon biomes; however, it is extremely dispersed and occurs only in small and focal transmission cycles. It infects a wide range of hosts: armadillos, caviomorph rodents, bats, coatis, opossums, and carnivores (Lisboa et al., 2009; Rocha et al., 2013). TcIII is occasionally isolated from domestic dogs in Brazil and the Chaco region of Paraguay and Argentina (Marcili et al., 2009; Cardinal et al., 2008). So far, few vectors, such as *Panstrongylus* and *Triatoma* genera (e.g., *Triatoma rubrovaria* in Rio Grande do Sul), have been implicated in the wild transmission cycle of TcIII (Câmara et al., 2010; Martins et al., 2008). Although TcI and TcII are more abundant than TcIII in Brazil, the latter was described to infect humans. Thus, in 2007 the TcIII genotype was identified in an orally transmitted Chagas disease outbreak in Coari, a rural area in Amazon State (Monteiro et al., 2010). This DTU was thought to be predominant in wild areas and was rarely associated with acute Chagas disease. Nevertheless, it was isolated from a case of chronic human disease in northeastern Brazil (Martins et al., 2015).

TcIV has been found in primates and *Rhodnius* spp. (e.g., *Rhodnius brethesi*) in the Amazon basin, as well as in humans with orally transmitted Chagas disease (Marcili et al., 2009), confirming an earlier report by Yeo et al., 2005. TcIV was proposed to be associated to the arboreal ecotope, where it was linked to sylvatic cycles. Moreover, TcIV has been reported in the context of acute oral outbreaks in the Brazilian Amazon and tropical wet areas of Colombia (Monteiro et al., 2012; Ramírez et al., 2013), as well as in Bolivia (Santalla Vargas et al., 2012). Finally, TcIV has recently been detected in dogs, suggesting a potential adaptation to a domestic transmission cycle (Ramírez et al., 2013).

Acute Chagas disease caused by both TcIII and TcIV DTUs has been described in outbreaks in the Amazon and in cases of single or mixed infections in Brazil and neighboring countries (Valente et al., 2009; Marcili et al., 2009; Monteiro et al., 2012). In Madrid, *T. cruzi* strains isolated from a population of chronically infected Bolivian migrants belonged mainly to TcV, TcIV, and TcI, with TcIV being the second most frequent (Perez-Molina et al., 2014). Similar observations were reported in Venezuela by Carrasco et al. (2012).

Altogether, the history and complex ecological associations of DTUs TcIII and TcIV remain little understood. Here we present new information about the geographic distribution and diversity of mammalian hosts and vectors of TcIII and TcIV in Brazilian biomes.

2. Materials and methods

2.1. Parasite samples

Sixty-one *T. cruzi* isolates were obtained in a cryopreserved form from the "Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, FIOCRUZ – COLTRYP" (http://coltryp.fiocruz.br) which comprises a total of 714 isolates of *Trypanosoma* genus. The capture of mammals and the sample collection were performed as Xavier et al. (2014, 2012), Roque et al. (2013). They were characterized by multiplex PCR amplification of the mini-exon intergenic region in *T. cruzi* zymodeme 3 (Z3) or in mixed infections with TcI (Fernandes et al., 2001). Samples were isolated from five orders of mammals (Artiodactyla, Carnivora, Cingulata, Didelphimorphia, and Rodentia) and triatomines of the Reduviidae family of the genera *Triatoma* e *Rhodnius* between 1995 and 2016. They were collected from five geographic biomes: Amazon Forest, Atlantic Rainforest, Cerrado, Caatinga, and Pantanal.

2.2. DNA extraction

Trypanosome isolates were removed from liquid nitrogen and expanded in biphasic Novy-Mc-Neal-Nicole (NNN) medium with Liver Infusion Tryptose (LIT). Epimastigote forms at the end of the log growth phase were washed and then lysed with proteinase K and Sodium Dodecyl Sulfate (SDS). Genomic DNA was extracted using a standard phenol-chloroform protocol (Sambrook et al., 1989).

2.3. PCR-RFLP for T. cruzi DTU characterization

To differentiate DTUs TcIII and TcIV, and mixed TcI-TcIII and TcI-TcIV infections, the histone 3 gene was PCR-amplified and the products digested with AluI according to Westenberger et al. (2005). Each PCR reaction included water as a negative control and *T. cruzi* DTUs (TcI-TcIV) as positive controls. PCR products and RFLP fragments were electrophoresed on a 3% agarose gel, stained with 0.5 g/mL ethidium bromide, and visualized under ultraviolet light. Size was estimated by comparison with a molecular weight marker (100-bp DNA ladder).

2.4. Statistical analysis

The number of *T. cruzi* Z3 isolates (TcIII and TcIV DTUs) characterized in this study was compared among mammalian and triatomine hosts and the biomes of origin using Chi-square test. All statistical analyses were performed in program R – version 2.11.1 (R Development Core Team, 2010). The significance level of 5% was adopted, and the normality and homoscedasticity of the data were tested.

3. Results

The *T. cruzi* isolates previously typed as Z3 and re-characterized by PCR-RFLP (n = 61) showed that DTUs TcIII and TcIV were widely distributed, infecting mammalian and triatomine species without any specific association to biomes or hosts. These DTUs were identified in single and mixed infections throughout the five Brazilian biomes.

The wide range of mammalian host species (n = 18) infected with DTUs TcIII and TcIV included five mammalian orders: Artiodactyla, Carnivora, Cingulata, Didelphimorphia, and Rodentia; and two genera of Hemiptera: *Triatoma* and *Rhodnius* (Fig. 1).

The 61 *T. cruzi* Z3 isolates comprised 8,5% of the total number of *T. cruzi* isolates (n = 714). This is a noticeable lower number than TcI and TcII and shows that Z3 (TcIII and TcIV) is less present in free-ranging wild mammals than TcI (75,3%) and TcII (16,1%). The infection rates

Fig. 1. *Trypanosoma cruzi* DTUs TcIII and TcIV geographic distribution in the Brazilian Biomes and their mammalians and vectors hosts.

by TcIII and TcIV of wild mammals, were similar (p-value = 0,1020). Moreover, the infection rates of triatomines by TcIV was significantly higher than that by TcIII (p-value < 0,0001).

Among all *T. cruzi* Z3 characterized isolates (n = 61), 44 demonstrated to be DTU TcIV and 17 were DTU TcIII. DTU TCIV was observed in single infections (32/44) or in concomitant infections with DTU TcI (12/44). This DTU was observed infecting eight mammals species and triatomine genera (Table 1). The majority of the single infection by DTU TcIV was observed in rodents (n = 6), while mixed infections by TcI-TcIV (n = 6) occurred mainly in Didelphimorphia (Table 2).

TcIII was characterized in 28% (17/61) of the T. cruzi Z3 isolates. Single TcII infections were observed in 82% (14/17) of mammalian and triatomine (*Triatoma* and *Rhodnius* genera) isolates, most often in the Cingulata order (n = 4). Mixed TcI-TcIII infections were found in (3/17) of isolates, one in Didelphimorphia and two in *Triatoma* genera (Table 1).

T. cruzi DTU TcIV was more frequent that DTU TcIII in isolates obtained from infected mammals and triatomines of, Pantanal (p = 0.0002), Atlantic Rainforest (p < 0.0001) and Caatinga (p = 0.0289) biomes. Pantanal was the biome that displayed the highest diversity of mammal species infected by *T. cruzi* DTU TcIII and TcIV (Cingulata, Rodentia, Artiodactyla, Didelphimorphia, and Carnivora), followed by Amazon and Atlantic Rainforest (Table 1).

Among all the studied biomes, Didelphimorphia species were the most frequently infected by DTUs TcIV, TcIII, or mixed infections, i.e., *Monodelphis domestica* in Caatinga (by TcIV), Cerrado (by TcIII), and Amazon (by TcIV/TcI); and *Philander frenata* in Pantanal (by TcI-TcIV) and Atlantic Rainforest (by TcIV). The latter was the biome with the highest diversity of species from the Didelphimorphia order. In addition, the species of this order were the only ones infected with both

Table 1
Trypanosoma cruzi discrete typing units (DTUs) TcIII and TcIV in wild and domestic hosts from Brazilian biomes.

Biome	Orders	Species	Locality	Year	TcIII (n)	TcIII/TcI (n)	TcIV (n)	TcIV/TcI (n)
Pantanal	Artiodactyla	*Sus scrofa*	Aquidauana/MS	2001	–	–	1	–
	Carnivora	*Canis familiaris*	Corumbá/MS	2015	3	–	–	–
	Cingulata	*Euphractus sexcinctus*	Corumbá/MS	2006	1	–	–	–
		Dasypus novemcinctus	Aquidauana/MS	2013	1	–	–	–
			Aquidauana/MS	2014	1	–	–	–
	Didelphimorphia	*Philander frenata*	Aquidauana/MS	2002	–	–	–	1
	Rodentia	*Oecomys mamorae*	Aquidauana/MS	2002	–	–	3	–
		Trichomys fosteri	Aquidauana/MS	2002	–	–	1	–
	Hemiptera	*Triatoma* sp.	Corumbá/MS	2013	–	1	11	3
Atlantic Rainforest	Carnivora	*Galictis vittata*	Teresópolis/RJ	2003	1	–	–	–
	Didelphimorphia	*Didelphis aurita*	Rio de Janeiro/RJ	2014	–	–	–	4
		Philander sp.	Sumidouro/RJ	2006	–	–	1	–
		Micoreus paraguayanus	Rio de Janeiro/RJ	2014	–	–	1	–
	Rodentia	*Trinomys* sp.	Paraty/RJ	2016	1	–	–	–
	Hemiptera	*Triatoma vitticeps*	Guarapari/ES	2012	–	1	8	1
			Guarapari/ES	2014	–	–	2	
Amazon Forest	Cingulata	*Dasypus novencinctus*	Cachoeira do Arari/PA	2006	–	–	1	–
	Didelphimorphia	*Monodelphis domestica*	Araguatins/TO	2010	–	–	–	1
		Didelphis marsupialis	Araguatins/TO	2010	–	1	–	–
	Rodentia	*Proechimys* sp.	Rio Branco/AC	2014	–	–	–	1
	Hemiptera	*Rhodnius pictipes*	Abaetetuba	2008	1	–	–	–
			Castanhal/PA	2010	1	–	–	–
Caatinga	Cingulata	*Dasypus novencinctus*	Curaçá/BA	2000	1	–	–	–
	Didelphimorphia	*Monodelphis domestica*	Redenção/CE	2006	–	–	1	–
	Rodentia	*Trichomys laurentius*	Coronel José Dias/PI	2001	–	–	–	1
			Jurubeba/PI	2006	–	–	2	–
Cerrado	Carnivora	*Lycalopex vetulus*	Araguari/GO	2013	2	–	–	–
	Didelphimorphia	*Monodelphis domestica*	Luziânia/GO	2005	1	–	–	–
Total				Year	14	3	32	12

Table 2
Trypanosoma cruzi discrete typing units (DTUs) TcIII and TcIV in wild and domestic host's orders.

Host orders	TcIII (n)	TcIII/TcI (n)	TcIV (n)	TcIV/TcI (n)
Artiodactyla	–	–	1	–
Carnivora	6	–	–	–
Cingulata	4	–	1	–
Didelphimorphia	1	1	3	6
Rodentia	1	–	6	2
Hemiptera	2	2	21	4
Total	14	3	32	12

TcIII and TcIV DTUs. The Hemiptera order was found infected with TcIV in Pantanal and Atlantic Rainforest biomes. Ifs necessary to note that the majority of collected triatomines derived mostly from a simple sampling locality – Corumbá (Pantanal) (Table 1).

4. Discussion

The ecology of *T. cruzi* DTUs remains challenging owing to the inherent difficulties of performing fieldwork, the extent and diversity of biomes and habitats in Brazil, and the wide range of mammalian hosts. In this study, we found that mammals and triatomines in five out of six Brazilian biomes were infected with DTUs TcIII and TcIV. Considering the broad differences in microhabitats and eco-epidemiological characteristics of the biomes, these DTUs appear widely distributed in nature. Indeed, we found TcIII and TcIV to be more widespread geographically and concerning host species than formerly thought (Valente et al., 2009; Miles et al., 1978). Actually, TcIII has never been described before in Brazilian Savannah biome. Additionally this DTU has been classically associated to mammals of the order Cingulata; moreover, herein, we are demonstrating that wild rodents, marsupials and carnivores as well domestic dogs, may also act as hosts of this DTU. The 61 samples studied here were obtained from a study in which 7285 specimens of wild mammals and triatomines were examined for *T. cruzi* infection (Jansen et al., 2015). These findings demonstrated the low prevalence of DTUs TcIII and TcIV (0,8%) despite of its wide geographic distribution.

Pantanal was the biome that displayed the largest number of samples characterized as TcIII or TcIV in single and mixed infections, including the highest number of infected mammalian orders (n = 5) and species (n = 7), followed by Atlantic Rainforest and Amazon. These biomes are well known for their abundance and diversity of wildlife species. A direct association between higher biodiversity of hosts and parasites species was already been proposed by Hechinger and Lafferty (2005), Hechinger et al. (2007), Xavier et al. (2012), Lafferty (2012). Pantanal, Atlantic Rainforest and Amazon Rainforest biomes are well known for their abundance and diversity of wildlife species. In contrast, in the Caatinga and Cerrado that are respectively, the semiarid and savannah biome and that display lower mammal species biodiversity, infection rates of mammals by TcIII and TcIV were also lower. Moreover, Lisboa et al. (2009) described a similar infection rate of by Z3 (TcIII or TcIV DTUs) in the Caatinga biome, respectively in Cingulata and Rodentia. The same authors described Z3 infection *Nasua nasua* (Carnivora). All these data together demonstrate the biological flexibility of this *T. cruzi* DTU, derived from its ability to infect a broad spectrum of mammal species from biomes with contrasting characteristics. Besides, the TcIII and TcIV *T. cruzi* DTUs isolates, were derived from *Didelphis aurita*, *Micoreus paraguayanus*, and *Trichomys laurentius*, which are respectively, generalist, arboreal and terrestrial mammal species demonstrating that TcIII and TcIV DTUs may be transmitted in all forest strata.

Wild mammals are constantly exposed to infection by single, mixed, or multiple *T. cruzi* DTUs, either once or several times. TcIII and TcIV occur at significantly lower rates than TcI and TcII in Brazilian isolates (Jansen et al., 2015). Furthermore, TcIII and TcIV are isolated from a smaller number of animals and focal sites, suggesting that they present a peculiar transmission and maintenance strategy in the wild. Here we also show that the overall infection rate of the assemblage of wild mammals and triatomines collected in the wild by DTU TcIV is higher than by DTU TcIII ($p < 0.0001$). The lower prevalence of mammal

infection by DTU TcIII in comparison to TcIV, may be due to a temporal feature or methodological bias. In this sense, TcIV was isolated from mammals of the Pantanal biome, respectively in 2001 and 2002 but in contrast, DTU TcIII was isolated only in 2006. The different distribution pattern in nature raises the following question, what is the transmission and maintenance strategy of a DTU that displays scattered distribution and low prevalence? Clearly, DTUs TcIII and TcIV like TcI and TcII, can infect a wide variety of mammals. Two hypothesis may explain our findings: a) the infection of wild mammals with TcIII and TcIV tends to result in lower parasitemia than those infections with the DTUs TcI and TcII and b) different transmission strategies in restricted foci and cycles of TcIII and TcIV DTUs in nature.

Our results show that TcIII and TcIV can infect a variety of wild host species. i.e., at least five of the eight mammalian orders, including 15 different species, and two important genera of insects, *Triatoma* and *Rhodnius*. Marcili et al. (2009) proposed that the lower prevalence of TcIIc (TcIII) might be due to undersampling. This is plausible, due to the difficulties involved in the capture and transport of biological materials, and the isolation of the parasite from wild hosts and vectors. Therefore, existing data on the distribution of *T. cruzi* genotypes are aggregated and do not reflect all the habitats where they may occur Lima et al. (2014).

Generalist mammals of the Didelphimorphia order were the most frequent hosts infected by TcIII, TcIV, or mixed DTUs. *Monodelphis domestica* was found infected in more biomes (Amazon, Caatinga, and Cerrado) than the genus *Didelphis*, which is considered one of the most important *T. cruzi* reservoirs (Jansen et al., 2015). In addition, *Didelphis spp*. presented only mixed infections with TcI-TcIII and TcI-TcIV in the Amazon and Atlantic Rainorest biomes, respectively. The second most infected order was Rodentia. In this case, TcIII and TcIV isolates were obtained from arboreal *Oecomys spp.*, and terrestrial *Trichomys*, *Trinomys*, and *Proechimys* genera in Pantanal, Caatinga, Atlantic Rainforest, and Amazon biomes. Our results are in agreement with the fact that marsupials are the animals most frequently infected by *T. cruzi* in all biomes. Marsupial species are nomadic, and generalists in habitat and diet, therefore more exposed to *T. cruzi* infection. In fact, these ancient mammals may act as bioaccumulators of Trypanosomatids, including subpopulations of *T. cruzi* (Jansen et al., 2015).

It has been proposed that TcIIc (TcIII) DTUs are restricted to the Cingulata order (Yeo et al., 2005), or that they are associated almost exclusively with terrestrial transmission cycles and fossorial mammalian genera, such as Cingulata and terrestrial marsupials (Llewellyn et al., 2009). Here, we found that the Cingulata order was not the most infected mammal by TcIII and TcIV DTUs. TcIII was found to infect *Euphractus* and *Dasypus* in Pantanal and Caatinga, and TcIV infected *Dasypus* in the Amazon. In the Cerrado, foxes (*Lycalopex vetulus*) were found infected only by TcIII, although they are considered on top of the food chain and consequently potential bioaccumulators of the several DTUs (Rocha et al., 2013). The DTU TcIII, previously associated with *Panstrongylus* genus (Zingales et al., 2012) probably due to under sampling, was found infecting *Triatoma* and *Rhodnius* genus in Pantanal, Atlantic Rainforest and Amazon, reinforcing absence of association of *T. cruzi* DTU and a specific genus of triatomine (Jansen et al., 2015).

Here, we describe for the first time the infection of an Artiodactyla (*Sus scrofa*) with DTU TcIV. As domestic animals, pigs are probably dead-end hosts of marginal importance in the transmission cycle of *T. cruzi*. In Pantanal, pigs often return to a wild state, so-called feral pigs. These animals may display high seroprevalence but isolation of the parasite is rare, indicating low infectivity competence (Roque et al., 2008; Cominetti et al., 2011; Bezerra et al., 2014). In addition, we uncovered new hosts of *T. cruzi* DTUs TcIII and TcIV, including marsupials (*Monodelphis domestica, Micoreus paraguayanus, and Didelphis marsupialis*), rodents (*Trinomys sp., Oecomys mamorae, Proechimys* sp., *and Trinomys* sp.), and Hempitera (*Triatoma vittceps* and *Rhodnius pictipes*).

In this study, we identified mixed infections (TcI-TcIII and TcI-TcIV) in mammals and triatomines in different ecotopes of Brazilian biomes. Thus, the same host or vector can simultaneously accommodate different parasite subpopulations. These can compete with each other and can be selected over others in niches, hosts, and specific geographic areas (Macedo et al., 2004). A limitation of this study is that we were restricted to the parasites that could be isolated from the blood. In fact, mixed infections should be much more common in nature than we have been able to detect using cultures, a technique with high specificity but low sensitivity. Each host species acts as a unique biological filter positively selecting different parasite clones. Therefore, the clones that may be found in a host species and even in each specimen vary according to the nature and kinetics of the selection process during infection (Jansen et al., 2015).

In this study, *T. cruzi* DTU TcIII and TcIV did not demonstrate any specific association to a biome, habitat, or mammalian host species. This way, in Brazilian biomes we could not observe an "strong association" of TcIII with armadillo species as proposed by Acosta et al. (2017). Other studies have also been unable to find an association between TcIII or TcIV and preferred vertebrate hosts and vectors, ecological niches, and domestic or sylvatic environments (Rocha et al., 2013; Herrera et al., 2015). At present, we cannot exclude that mammals and vectors other than the ones identified in this study are natural hosts of TcIII and TcIV. Given the natural distribution of mammals and triatomines in their biomes, there is no doubt that the genetic diversity characterizing *T. cruzi* isolates is more complex. Taken together, the present findings improve our knowledge of the geographic and host range, as well as distribution pattern, of *T. cruzi* DTUs TcIII and TcIV.

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5 DISCUSSÃO

O número de casos e surtos de DC por via oral tem aumentado nos últimos anos, tanto na região amazônica (Pinto *et al.*, 2008; Valente *et al.*, 2009; Souza-Lima *et al.*, 2013; Xavier *et al.*, 2014; Coura e Junqueira, 2015), quanto em outras regiões do país. Os primeiros relatos de DC por via oral no Brasil foram registrados no município de Teutônia, no estado do Rio Grande do Sul (Nery-Guimarães *et al.*, 1968; Silva *et al.*, 1968). Shikanai-Yassuda *et al.* (1987, 1991) descreveram um surto ocorrido pela ingestão de caldo-de-cana contaminado, no município de Catolé do Rocha, no estado da Paraíba em 1986. No ano de 2005, no município de Santa Catarina, 19 pessoas adquiriram a doença também pela ingestão de caldo-de-cana contaminado (Roque *et al.*, 2008; Steindel *et al.*, 2008) e na Bahia, no ano de 2006, 13 pessoas adquiriram a doença (Bastos *et al.*, 2010). Outros países da América do Sul também têm enfrentado casos e surtos de DC por via oral, como a Venezuela, Chile e Colômbia (Alarcon de Noya *et al.*, 2010; Toso *et al.*, 2011; Rueda *et al.*, 2014). Esse novo cenário epidemiológico da DC deve ser tratado de maneira única, pois as medidas de controle antes aplicadas para eliminação do principal vetor da doença, o *T. infestans*, uma espécie domiciliada e que no Brasil não foi capaz de estabelecer um ciclo de transmissão silvestre, não se adequam ao atual cenário. De fato, a borrifação no atual cenário é inócua, além de levar a uma sensação equivocada de proteção pela população. Cada caso e/ou surto deve ser tratado de forma singular, no qual é preciso conhecer o perfil epienzoótico da região e o modo como os habitantes interagem com a natureza.

No caso do ES, num primeiro momento já se tinha o conhecimento da invasão de triatomíneos infectados por *T. cruzi* nas residências. Uma medida que poderia ter sido aplicada é o uso de telas nas janelas, assim evitando essa invasão e por consequência, o contato dos moradores com os triatomíneos. No entanto, até o caso que levou a óbito uma criança de dois anos, nenhuma providência foi tomada. Depois do caso de DCA, as autoridades sanitárias locais estavam já planejando a borrifação das casas na área.

No primeiro artigo, nós tivemos como objetivo caracterizar as DTUs de *T. cruzi* do paciente que veio a óbito de DCA por via oral, na zona rural do município de Guarapari, estado do ES. Obtivemos o tecido cardíaco incluído em um bloco de parafina. Por meio desse material, foi possível identificar que o tecido cardíaco desse paciente apresentava infecção mista por quatro DTUs de *T. cruzi* e também

por outra espécie de tripanosomatídeo do subgênero *Schizotrypanum*, *T. dionisii*, que até o momento era considerado um tripanosomatídeo exclusivo de morcegos (Gardner e Molyneux, 1988a; Molyneux, 1991). Assim, relatamos o primeiro caso de infecção mista por quatro DTUs de *T. cruzi*, além da infecção por *T. dionisii* em humanos. Provavelmente esse encontro só foi possível devido ao paciente estar em fase aguda da infecção (segunda semana da infecção) e porque a identificação foi realizada diretamente em tecido cardíaco. Em geral, quando o indivíduo humano chega ao serviço de saúde, ele está em uma fase menos precoce da infecção e o isolamento do parasito acontece por meio de hemoculturas ou xenodiagnóstico. É possivel, portanto, hipotetizar que a infecção por *T. dionisii* no homem seja autolimitante e que as demais DTUs não resultem em parasitemias expressivas. Esta hipótese explicaria porque, em geral, a DTU TcII de *T. cruzi* tem sido classicamente associada as infecções no homem.

Outro ponto que merece destaque é a infecção mista por várias DTUs de *T. cruzi*. Não se tem conhecimento do grau de envolvimento das infecções mistas na patogenicidade ou do impacto que podem causar no curso da doença. Porém, este é um aspecto que deve ser levado em consideração. Infecções mistas por duas ou três DTUs em humanos já foram registradas na Colômbia (Mantilla *et al.*, 2010; Ramírez *et al.*, 2010), Chile (Apt *et al.*, 2015), Argentina (Monje-Rumi *et al.*, 2014) e em pacientes bolivianos na Espanha (Martinez-Peres *et al.*, 2016). No entanto, o impacto dessas infecções mistas é pouco estudado. Infecções mistas por diferentes subpopulações ou espécies de parasitos deve ser o padrão mais comum em mamiferos de vida livre (Jansen *et al.*, 2015). Infecções mistas por DTUs de *T. cruzi* já foram relatadas em triatomíneos (Lima *et al.*, 2014; Cura *et al.*, 2015). Esse conjunto de resultados mostra que as infecções mistas são muito mais frequentes do que se tem reportado.

Esse é o primeiro caso de infecção em humano pelas DTUs TcIII e IV no estado do ES, sendo as mesmas já descritas em casos de DC na região amazônica (Monteiro *et al.*, 2010, 2012), no sul (Abolis *et al.*, 2011), sudeste (D'Ávila *et al.*, 2009) e nordeste (Martins *et al.*, 2015) do Brasil e em pacientes bolivianos na Espanha (Martinez-Peres *et al.*, 2016). Esses achados mostram que a distribuição de TcIII e em especial, TcIV é maior do que foi assumida até agora e confirma que essas duas DTUs estão envolvidas em infecções humanas. Os resultados mostram que nós estamos longe de contarmos com dados suficientemente robustos para estabelecer associações entre uma DTU de *T. cruzi* e patogenicidade, curso da

infecção ou epidemiologia da DC. De fato, uma doença é o resultado da interação de várias variáveis, incluindo as peculiaridades do hospedeiro e sua interação com o ambiente.

Para a nossa surpresa, nós conseguimos identificar em tecido cardíaco humano um tripanosomatídeo de morcego, a espécie *T. dionisii*. Sabe-se que *T. dionisii* é capaz de invadir, assim como *T. cruzi*, células de mamíferos e de formar cistos em tecidos cardíacos (Gardner e Molyneux, 1988a; Oliveira *et al.*, 2009; Maeda *et al.*, 2011, 2012). Devido ao tempo de detecção dessa espécie, diretamente de tecido cardíaco de um paciente recém-infectado (duas semanas de infecção), podemos dizer que *T. dionisii* é capaz de invadir e se diferenciar em célula humana *in vivo* mas não sabemos se a infecção é passivel de ser mantida a longo prazo.

Não é a primeira vez que espécies de tripanosomatídeos consideradas não-patogênicas são encontradas infectando humanos. Na Ásia e África, existem relatos de casos humanos de infecção por *T. lewisi*, *T. b. brucei*, *T. congolense*, *T. vivax, T. evansi* e de *Leptomonas seymouri* (Joshi *et al.*, 2005; Verma *et al.*, 2011; Gosh *et al.*, 2012; Truc *et al.*, 2013). *Leishmania tarentolae*, espécie tipicamente isolada de largatos, já foi descrita em múmias humanas (Novo *et al.*, 2015). TcBat, DTU de *T. cruzi* até há pouco tempo considerada como restrita a morcegos (Marcili *et al.*, 2009a; Zingales *et al.*, 2012) já foi descrita em um caso de DC em uma criança na Colômbia (Ramírez *et al.*, 2014a) e em múmias humanas (Guhl *et al.*, 2014). Esses resultados mostram que os tripanosomatídeos apresentam uma expressiva plasticidade biológica e que podem ser parasitos generalistas.

A identificação de parasitos a partir de material biológico, nesse caso em tecido, é uma fonte importante para investigação e entendimento da epidemiologia de uma doença e o material recuperado desse tipo de amostra é extremamente informativo (Gilbert *et al.*, 2007). O nosso resultado reforça essa importância, uma vez que provavelmente o hemocultivo desse paciente resultaria em pressão seletiva, podendo afetar o crescimento de determinada população parasitária (Bosseno *et al.*, 2000) e com isso, nós não teriamos detectado essa diversidade de DTUs de *T. cruzi* e a espécie *T. dionisii*.

O ciclo enzoótico de *T. cruzi* ocorre em cenários distintos, nos quais sua transmissão acontece associada a uma rede trófica, em que cada animal apresenta um papel diferente, no espaço e no tempo, quanto a sua competência mantenedora e infectiva (Roelling *et al.*, 2009). Isso resulta em que surtos e/ou casos de DCA ocorram em cenários enzoóticos distintos, no qual cada região é peculiar e

apresenta uma rede de transmissão específica, tornando necessário entendê-la e conhecê-la, para que se possa orientar de forma correta os moradores das áreas de risco, evitando novos surtos da doença (Roque *et al.*, 2008). Na região da Mata Atlântica no município de Guarapari, estado do ES, observou-se um ciclo enzoótico peculiar de transmissão de *T. cruzi*, jamais visto em outras regiões do país. O mesmo ocorre longe das residências, no qual os triatomíneos se infectam nas regiões mais distantes e altas, e esses vem voando junto com as correntes de vento ou apresentam uma capacidade de voo muito maior do que se tem relatado para o genêro *Triatoma* (*T. infestans* é capaz de voar por aproximadamente 200 metros) (Schweigmann *et al.*, 1988; Schofield *et al.*, 1992).

No ano de 2012, poucos meses depois da morte do paciente por DCA, foi realizada a captura de pequenos mamíferos silvestres não-voadores e coleta de sangue de cães na área onde ocorreu o caso e suas adjacências para o estudo do ciclo enzoótico de *T. cruzi*. Foram amostrados animais domésticos (cães) e pequenos mamíferos silvestres não-voadores. Apesar do esforço de captura alto (840 armadilhas-noite), o sucesso de captura de pequenos mamíferos não-voadores foi baixo (apenas seis espécies), mostrando apenas moderada diversidade de espécies de mamíferos não-voadores. Nenhum dos animais examinados, tanto os pequenos mamíferos silvestres não-voadores, como os cães apresentaram resultados de hemocultivo positivos e em relação aos exames sorológicos, somente cinco animais apresentaram títulos sorológicos no ponto de corte, ou seja, não sendo capaz de determinar a infecção desses animais por *T. cruzi*. Vale mencionar que todos os exames de sangue a fresco estavam negativos. Esses resultados nos sugeriram que o ciclo de transmissão de *T. cruzi* estaria ocorrendo em remanescentes de floresta distantes. Essa hipótese foi proposta pelo fato do elevado número de triatomíneos, em especial a espécie *T. vitticeps*, apresentar altas taxas de infecção por *T. cruzi* e continuarem invadindo as residências.

Dois anos após a primeira excursão realizada em Guarapari, nós decidimos expandir o estudo realizado no ano de 2012 para identificar os possíveis reservatórios de *T. cruzi* na região. Investigamos o ciclo enzoótico de *T. cruzi* na área onde ocorreu o caso de DCA (Rio da Prata) e em duas áreas distantes de onde houve o surto e que apresentam como característica diferentes níveis de preservação ambiental (Amarelos e Buenos Aires). Além da captura de pequenos mamíferos silvestres não-voadores e coleta de sangue dos cães, nós realizamos a captura de morcegos e recebemos exemplares de triatomíneos de diferentes

localidades do município de Guarapari. Os resultados obtidos durante esse trabalho demonstraram que o cenário do ciclo enzoótico de *T. cruzi* na região não sofreu alteração: os triatomíneos continuavam apresentando altas taxas de infecção por *T. cruzi,* os pequenos mamíferos silvestres não-voadores e os cães igualmente demonstraram baixa taxa de infecção pelo parasito, mostrando que esses não estão atuando como reservatório de *T. cruzi* na área. Porém, durante esse estudo nós identificamos morcegos infectados por *T. cruzi*, levando-nos a conclusão que os morcegos são os reservatórios desse flagelado naquela região da Mata Atlântica. Além disso, observamos uma diversidade de tripanosomatídeos e de DTUs de *T. cruzi*, nos morcegos, como em triatomíneos da espécie *T. vitticeps*.

Foram capturadas 12 espécies de pequenos mamíferos silvestres não-voadores, entre marsupiais e roedores, que correspondem a 25,5% do total de espécies desses animais descritas no estado do ES (Moreira *et al.*, 2008). Não esperaríamos encontrar todas as espécies de pequenos mamíferos descritas no ES na nossa região de estudo – no entanto, 25% sugere que tenha ocorrido uma perda de diversidade de pequenos mamíferos na área. A presença do *Didelphis aurita* foi recorrente em todas as áreas estudadas, porém não foi a espécie mais abundante em nenhuma das mesmas. Algumas espécies que foram capturadas em nosso trabalho, como *Monodelphis americana*, *Gracilinanus microtarsus*, *Rhipidomys mastacalis* e *Trinomys paratus* são típicas de áreas que sofreram menos perturbação (dados não publicados), mostrando que estas regiões estudadas são um pouco melhor preservadas, principalmente a localidade de Buenos Aires, onde todas essas espécies foram capturadas. Chamou a atenção o fato de que também ali a enzootia por *T. cruzi* está sendo mantida basicamente por morcegos.

Em relação aos morcegos, foram capturados 186 espécimes, sendo 16 espécies da família Phyllostomidae e uma espécie da família Vespertilionidae. A predominância de morcegos da família Phyllostomidae é esperada, especialmente das espécies generalistas *C. perspicillata* e *A. lituratus*, pois essa é a família de morcegos mais comum nas regiões neotropicais (Bernard, 2002). A presença de espécies de morcegos da subfamília Phyllostominae é um indicativo que a área examinada seria mais preservada ou então que as alterações ambientais não afetaram os habitats dos morcegos da área (Fenton *et al.*, 1982; Clarke *et al.*, 2005; Medellín *et al.*, 2010). Nós observamos cinco espécies dessa subfamília, principalmente na localidade de Amarelos e isso mostra que esse fragmento de

Mata Atlântica, como já foi visto no caso dos pequenos mamíferos não-voadores, ainda apresenta moderado grau de preservação.

O nosso estudo demonstrou que os morcegos estavam atuando como reservatório de *T. cruzi* nas três regiões estudadas na Mata Atlântica do município de Guarapari, estado do ES. *Trypanosoma cruzi* TcI e TcIII/V foram encontrados em morcegos das famílias Phyllostomidae e Vespertilionidae (*Anoura* spp., *C. perspicillata*, *Artibeus* spp., *D. rotundus*, *R. pulimio* e *M. nigricans*), sendo essas as mesmas DTUs identificadas no caso de DCA e em triatomíneos na região. Não é a primeira vez que morcegos são considerados reservatórios de *T. cruzi* na América do Sul: na Colômbia, uma das principais fontes alimentares detectadas em triatomíneos infectados por *T. cruzi* foram os morcegos (Hernández *et al.*, 2016); no leste da Venezuela, morcegos e triatomíneos foram encontrados infectados com *T. cruzi*, sendo prováveis dispersores do parasito (Añez *et al.*, 2009); no Equador, esses animais foram considerados responsáveis pela ligação do ciclo de transmissão domiciliar e silvestre (Pinto *et al.*, 2015), uma vez que os pequenos mamíferos não-voadores não apresentavam infecções por *T. cruzi*; e na Argentina, morcegos da espécie *D. rotundus* foram encontrados infectados por *T. cruzi* (Argibay *et al.*, 2016).

Nós evidenciamos que morcegos são bioacumuladores de espécies de tripanosomatídeos dentro do clado *T. cruzi*. Além de *T. cruzi*, esses mamíferos também foram encontrados infectados por outros tripanosomatídeos do subgênero *Schizotrypanum* - *T. c. marinkellei* e *T. dionisii*; *T. rangeli* e por *Trypanosoma* spp. de morcegos neotropicais, de espécie ainda não identificada ou descrita. A presença de *T. c. marinkellei* e *T. dionisii* já foi relatada no estado do ES (Acosta *et al.*, 2014), na Mata Atlântica da região norte do estado. O nosso resultado mostra que a ocorrência de *T. c. marinkellei* na Mata Atlântica não foi acidental e que sua distribuição é mais ampla do que se tem admitido até o momento, não ocorrendo somente nos biomas Amazônia e Pantanal (Cavazzana Jr *et al.*, 2010; Marcili *et al.*, 2013; da Costa *et al.*, 2016). Em relação a *T. dionisii*, nós expandimos o conhecimento sobre a área de ocorrência dessa espécie no Brasil. A mesma já foi encontrada na Mata Atlântica de outros estados e outros biomas brasileiros (Szpeiter *et al.*, 2017) e também em outros países do continente sulamericano, como Bolívia (García *et al.*, 2012) e Colômbia (Ramírez *et al.*, 2014b), além dos Estados Unidos (Hodo *et al.*, 2016).

Em relação ao *T. rangeli*, nós identificamos pela primeira vez a infecção de morcegos com as linhagens B e D. *Trypanosoma rangeli* B até o momento foi

descrito na região Amazônica e só havia sido encontrado em primatas humanos e não-humanos (Maia da Silva *et al.*, 2004b, 2007). Já a linhagem D foi primeiramente identificada na Mata Atlântica do estado de Santa Catarina, na espécie de roedor *Phyllomys dasythrix* (Steindel *et al.*, 1991). Nossos resultados ampliam as espécies de hospedeiros de *T. rangeli*, uma vez que somente as linhagens A e E haviam sido descritas em morcegos (Maia da Silva *et al.*, 2009). Embora a transmissão de *T. rangeli* esteja correlacionada a triatomíneos do gênero *Rhodnius* spp. (Guhl *et al.*, 2003; Maia da Silva *et al.*, 2004a, 2004b, 2007, 2009), outras espécies de triatomíneos demonstram ser responsáveis pela transmissão do parasito nessa região da Mata Atlântica. Embora haja relatos da presença de *R. domesticus* no estado do ES (Galvão *et al.*, 2003; Gurgel-Gonçalves *et al.*, 2012), nós não encontramos nenhum, ou seja, a espécie deve apresentar baixa abundância relativa. Adicionalmente, já foi relatado por Steindel *et al.* (1994), o isolamento de *T. rangeli* a partir de *P. megistus* no estado de Santa Catarina. Esses dados sugerem que no estado do ES, outras espécies de triatomíneos, como a espécie *T. vitticeps*, pode ser considerada vetor de *T. rangeli*.

Os tripanosomatídeos de morcegos, até há pouco tempo representavam um mundo desconhecido, pois os mesmos são morfologicamente idênticos, dificultando a sua identificação por espécie. O uso de ferramentas moleculares com maior poder de discriminação vem possibilitando a descoberta de novas espécies de tripanosomatídeos e aos poucos, algumas peças desse mundo desconhecido vêm sendo desvendadas. Na Mata Atlântica do estado do ES, observamos uma diversidade de tripanosomatídeos que se agruparam no clado composto por espécies isoladas de morcegos neotropicais nos estados de Rondônia, Pará, Rio Grande do Norte, Sergipe, Mato Grosso e Tocantins, além da Colômbia, Guiana, Guatemala e Panamá (Lima *et al.*, 2015b; da Costa *et al.*, 2016). Essas espécies formaram grupos irmãos com as espécies descritas em morcegos da família Mormoopidae e Phyllostomidae: *Trypanosoma wauwau* (*Pteronotus* spp.) e *Trypanosoma* sp. de morcegos neotropicais (*Artibeus* spp. e *T. cirrhosis*) (Lima *et al.*, 2015b). É a primeira vez que tripanosomatídeos desse clado são descritos na região da Mata Atlântica do sudeste brasileiro, assim como em outras espécies de morcegos da família Phyllostomidae (*Anoura* spp., *C. perspicillata*, *D rotundus*, *G. soricina*, *Phyllostomus* spp., *Platyrrhinus recifinus*, *Rhinophylla pumilio* e *S. lilium*) e um morcego da família Vespertilionidae (*M. nigricans*).

Os nossos resultados reforçam a ampla distribuição dos tripanosomatídeos do clado *T. cruzi* em morcegos que apresentam características variadas, desde espécies de hábitos generalistas até espécies mais restritas. Morcegos são conhecidos por serem hospedeiros de diferentes espécies de tripanosomatídeos do clado *T. cruzi* e são considerados os hospedeiros ancestrais dessas espécies, a partir da hipótese de *bat seeding* (Hamilton *et al.*, 2012). A habilidade dos morcegos em manter infecções por diferentes espécies de tripanosomatídeos pode ser justificada por seu ecletismo ecológico. Nesse caso, podemos incluir os diferentes hábitos alimentares (Findley, 1993), a sua capacidade de voo (Constantine, 2003), o seu longo tempo de vida (Kalko, 2002), a capacidade de viver em colônias onde interagem muito estreitamente entre si (Constantine, 1967; Greenhall *et al.*, 1983; Nowak, 1994; McCracken, 2003) e o fato de algumas espécies terem o hábito de se lamber e regurgitar entre eles (Schmidt e Manske, 1973; Wilkinson, 1986; Wilkinson *et al.*, 2016). Todas essas características facilitam a dispersão e transmissão desses parasitos.

Trypanosoma minasense é um tripanosomatídeo morfologicamente semelhante ao *T. rangeli* (Ziccardi e Lourenço-de-Oliveira, 1999), sendo supostamente considerado uma variante dessa espécie (Stevens *et al.*, 1999). *Trypanosoma minasense* ocorre da América Central até a Argentina (Sousa *et al.*, 1974; Sousa e Dawson, 1976; de Resende *et al.*, 1994; Ziccardi *et al.*, 2000; Chinchilla *et al.*, 2005; Martínez *et al.*, 2016) e foi descrita infectando diferentes espécies de macacos (Deane *et al.*, 1974; Sousa e Dawson, 1976; de Resende *et al.*, 1994; Ziccardi e Lourenço-de-Oliveira, 1999). Pouco se sabe do seu ciclo de transmissão na natureza (Dunn *et al.*, 1963; Hoare, 1972; Martínez *et al.*, 2016). Os nossos resultados demonstraram que o *T. minasense* isolado de *Callithrix geoffroyi* está posicionado junto ao *T. bennetti*, como foi descrito por Sato *et al.* (2008). Esse resultado mostra que *T. minasense* é, em si, uma espécie e reforça que esse isolado não está correlacionado ao grupo do *T. rangeli*.

Pela primeira vez observamos o encontro de tripanosomatídeos de répteis em mamíferos. Por caracterização molecular direta em amostras de sangue de morcegos das espécies *C. perspicillata* e *D. rotundus* identificamos um *Trypanosoma* sp. réptil-*like*. Pouco se sabe como ocorre o ciclo de transmissão de tripanosomatídeos de répteis. Há relatos que flebotomíneos possam estar envolvidos nessa transmissão (Adler e Theodor, 1957; Anderson e Ayala, 1963; Minter-Goedbloed *et al.*, 1993; Telford, 1995), uma vez que alguns

tripanosomatídeos que se agrupam junto aos tripanosomatídeos de répteis já foram isolados de flebotomíneos na Amazônia e na região centro-oeste do Brasil (Viola *et al.*, 2008; Ferreira *et al.*, 2015). Nesse caso, a transmissão pode ter ocorrido devido a um *spillover* do parasito, uma vez que os répteis, os flebotomíneos e os morcegos podem ocupar um mesmo habitat (copas de árvores, grutas entre outros). O *spillover* de um parasito para uma nova espécie de hospedeiro é explicado pelo conceito de e*cological host-fitting* (Agosta e Klemens, 2008): a troca de hospedeiro é possível devido a competência do parasito de colonizar o novo hospedeiro por habilidades que foram adquiridas durante o seu processo evolutivo, independente do hospedeiro em questão.

Nós também identificamos um isolado de *T. cascavelli* obtido de cultura de sangue de marsupial da espécie *M. americana. Trypanosoma cascavelli* foi descrito pela primeira vez em cobras da espécie *Crotalus durissus* (Pessôa e de Biasi, 1972; Viola *et al.*, 2008, 2009b). Em relação ao encontro de *T. cascavelli* infectando um marsupial da espécie *M. americana*, nos fez pensar sobre qual teria sido o hospedeiro ancestral e qual o hospedeiro secundário desse parasito. Nós levantamos a hipótese de que os marsupiais seriam os hospedeiros ancestrais dessa espécie de tripanosomatídeo, enquanto os répteis seriam os hospedeiros subsequentes. A justificativa para essa hipótese é que *M. americana* apresenta hábito alimentar insetívoro-omnívoro (Paglia *et al.*, 2012) e com isso, esses animais estariam se infectando por via oral pela predação de insetos, inclusive o inseto vetor de *T. cascavelli*. Os répteis, nesse caso as cobras, teriam se infectado pela predação desses marsupiais. Como os marsupiais apresentam temperatura corpórea baixa comparado a outros mamíferos (Jansen, 2002), no caso do gênero *Monodelphis*, essa temperatura é ainda mais baixa (Dawson e Olson, 1988; Busse *et al.*, 2014), entre 32-34°C. Esta condição poderia facilitar a adaptação dessa espécie de tripanosomatídeo em animais de sangue frio. Além disso, outros trypanosomatídeos pertencentes ao clado cobra/lagarto foram isolados de marsupiais. *Trypanosoma gennarii* foi isolado do marsupial da espécie *M. domestica*, no bioma Cerrado (Ferreira *et al*., 2017); *T. freitasi* foi isolado pela primeira vez em 1957, de marsupiais da espécie *D. albiventris* e mais tarde da espécie marsupial *D. marsupialis* (Rego *et al.*, 1957; Deane e Jansen, 1986). Com isso, a hipótese de os marsupiais serem os hospedeiros ancestrais ganha força, já que para as cobras serem esses hospedeiros, a transmissão se daria pela via vetorial, através dos

flebotomíneos, uma vez que *M. americana* não compartilha de habitats e nem se alimenta de cobras.

Para nossa surpresa, encontramos um morcego da espécie *D. rotundus* infectado com o cinetoplastídeo de vida-livre *B. saltans*. Esse protozoário é considerado o cinetoplastideo de vida-livre mais próximo dos tripanosomatídeos parasitas obrigatórios, sendo muito utilizado em estudos sobre o parasitismo nesses microrganismos (Simpson *et al.*, 2002, 2004, 2006; Stevens, 2008; Deschamp *et al.*, 2011; Lukes *et al.*, 2014). Esse achado nos faz pensar como um protozoário de vida-livre conseguiu se manter em um hospedeiro vertebrado mamífero e nos levar a questionar essa espécie como exclusivamente de vida-livre. Podemos levantar a hipótese que a espécie *B. saltans* possa estar adquirindo a capacidade de parasitar ou, ao contrário, abandonando o parasitismo e se tornando um eucarioto de vida-livre, uma vez que poucos estudos são realizados para identificação de espécies de vida-livre. Um caso semelhante aconteceu na China, onde infecção por *Colpodella* spp. foi registrada em humano, sendo esse um protozoário de vida-livre próximo dos protozoários parasitas obrigatórios do filo Apicomplexa (Yuan *et al.*, 2012). Embora *B. saltans* seja considerada uma espécie de ampla adaptação no ambiente (Lee e Patterson, 1998) e capaz de resistir a algumas técnicas de assepsia (McDonnell e Russell, 1999; Hauer e Rogerson, 2005), nós não consideramos que nesse caso teria acontecido uma contaminação. O local onde é realizada a punção cardíaca é lavado com sabão, tricotomizado, higienizado com álcool iodado e álcool 70%, além da punção ser realizada perto do fogo e com materiais estéreis. Outro ponto que salientamos é que morcegos não se banham e em nenhuma outra amostra de morcego foi identificada essa espécie.

A detecção de infecções mistas é muitas vezes difícil de ser realizada. Muitos microrganismos são difíceis ou mesmo impossíveis de serem cultivados. De fato, somente 10% dos microrganismos crescem em meios de cultura (Lima *et al.*, 2013; Lima, 2014), o que resulta na não identificação de 90% destes, em se tratando tanto de amostras ambientais como biológicas. Além disso, a própria técnica de cultivo de microrganismos leva a seleção de populações mais bem adaptadas àquele ambiente (Bosseno *et al.*, 2000; Maia da Silva *et al.*, 2009; Marcili *et al.*, 2009; Cavanzza Jr *et al.*, 2010). O uso de métodos moleculares com maior poder analítico, como é o sequenciamento de nova geração (*next generation sequencing* – NGS) permite a identificação de espécies de microrganismos que poderiam passar despercebidas. No nosso caso, o uso dessa metodologia em amostras de sangue total, mostrou que

a maioria dos morcegos examinados apresentava infecção de no mínimo duas, até oito espécies de cinetoplastideos. Essa mesma metodologia, quando empregada na amostra de hemocultivo do didelphídeo *M. americana*, indicou que o mesmo apresentava infecção mista por três espécies de tripanosomatídeos. Os resultados obtidos mostram que, as infecções concomitantes por vários organismos ou unidades taxonômicas são muito frequentes, além de revelar organismos ainda não identificados. Foi visto também, em um estudo similar realizado na Austrália (Barbosa *et al.*, 2017), a presença de infecções mistas por tripanosomatídeos em diferentes espécies de marsupiais. Esses resultados contribuem com maiores esclarecimentos sobre infecção mistas e demonstram que o NGS é uma ferramenta valiosa para estudos de espécies de microrganismos não-cultiváveis, ajudando na identificação das infecções, das espécies envolvidas e sua complexidade.

Triatoma vitticeps é a principal espécie de triatomíneo encontrada invadindo as residências na região da Mata Atlântica do estado do ES. Observamos que essa espécie é capaz de manter infecções simples e mistas até por quatro DTUs de *T. cruzi*, sendo as mesmas encontradas no tecido cardíaco do paciente que veio a óbito. A DTU TcI considerada a mais frequente no continente americano (Brenière *et al.*, 2016), foi a menos encontrada na região Mata Atlântica do estado do ES, onde a DTU predominante foi TcII. Esse resultado corrobora que TcII é mantida na natureza por diversas espécies de hospedeiros silvestres na Mata Atlântica (Jansen *et al.*, 2015; Lisboa *et al.*, 2015) e que no estado do ES o ciclo de transmissão de TcII é disperso na natureza e não é focal.

Nós encontramos seis espécimes de *T. vitticeps* infectados com *T. c. marinkellei* e *T. dionisii*. Esta é o primeiro relato de *T. c. marinkellei* infectando triatomíneos do gênero *Triatoma*. *Trypanosoma cruzi marinkellei* é conhecido por sua transmissão ser realizada por triatomíneos do gênero *Cavernicola* (Marinkelle, 1982) e sua competência em infectar triatomíneos do gênero *Rhodnius* já foi relatada pela execução do xenodiagnóstico (García *et al.*, 2012). Nossos resultados mostram que triatomíneos do gênero *Triatoma* também são capazes de se infectar por essa espécie. Esta é a primeira vez que *T. dionisii* é descrito infectando triatomíneos, uma vez que a sua transmissão está correlacionada com insetos cimicídeos (Gardner e Molyneux, 1988b). Como *T. dionisii* e *T. cruzi* pertencem ao mesmo subgênero (*Schizotrypanum*), os mesmos podem compartilhar seu hospedeiro invertebrado. Esse fato já foi demonstrado por Salazar *et al.* (2015), que relatam a infecção de cimicídeos por *T. cruzi*. Nós não sabemos se a transmissão de

T. dionisii ao hospedeiro mamífero seria possível pela via vetorial contaminativa, porém a transmissão por via oral é possível, já que esse parasito foi encontrado no paciente com DCA.

Uma questão ainda não respondida se refere à ecologia das DTUs TcIII e TcIV. Assim sendo, o estudo da eco-epidemiologia dessas DTUs é importante, pois assim é possível entender sua ecologia e confirmar ou não supostas associações com hospedeiros e sua distribuição geográfica. O pouco conhecimento da ecologia das DTUs de *T. cruzi*, deve-se as dificuldades inerentes ao trabalho de campo, a diversidade de biomas e habitats que o Brasil possui e pela quantidade de espécies de hospedeiros mamíferos que o *T. cruzi* é capaz de infectar. As DTUs de *T. cruzi* TcIII e TcIV foram observadas circulando entre morcegos e triatomíneos da espécie *T. vitticeps* na Mata Atlântica do município de Guarapari, estado do ES. As mesmas já foram observadas em *T. vitticeps* e em outra espécie de triatomíneo, *P. geniculatus* (TcIII), em diversos municípios do estado do ES (Dario, 2013). Nós observamos que além do bioma Mata Atlântica, as DTUs TcIII e TcIV foram encontradas também no Cerrado, Caatinga, Pantanal e Amazônia, mostrando que essas apresentam uma ampla distribuição na natureza. Além disso, podemos confirmar que o número de hospedeiros mamíferos que se infectam por essas DTUs é muito maior do que se tem relatado (Miles, 1978; Valente *et al.*, 2009), sendo encontradas em hospedeiros mamíferos de cinco diferentes ordens, totalizando 15 espécies e três gêneros de insetos vetores: *Triatoma*, *Rhodnius* e *Panstrongylus*.

O bioma Pantanal apresentou o maior número de amostras identificadas como TcIII e TcIV e também o maior número de espécies de mamíferos infectadas, seguido pelo bioma Mata Atlântica e pelo bioma Amazônia. Esses biomas apresentam uma elevada diversidade de hospedeiros mamíferos e uma associação direta entre diversidade de espécies hospedeiras e espécies de parasitos já foi proposta (Hechinger e Lafferty, 2005; Hechinger *et al.*, 2007; Lafferty, 2012; Xavier *et al.*, 2012). Os biomas Cerrado e Caatinga, nós observamos uma baixa diversidade de mamíferos e também uma baixa taxa de infecção pelas DTUs TcIII e TcIV. Essa baixa taxa de infecção por TcIII e/ou TcIV já havia sido observada no bioma Caatinga (Lisboa *et al.*, 2009), em hospedeiros da ordem Cingulata e Rodentia. Como se trata de estudo de uma área restrita, os nossos resultados podem estar refletindo uma característica local, já que o estudo foi realizado em um fragmento pequeno desse bioma. Essas DTUs foram encontradas em mamíferos

generalistas e que frequentam diferentes habitats, mostrando que essas DTUs são capazes de ser transmitidas em todos os estratos florestais.

A ocorrência das DTUs TcIII e TcIV é menor do que as DTUs TcI e TcII nas amostras de isolados brasileiros (Jansen *et al.*, 2015). Isso sugere que a estratégia de transmissão e a manutenção dessas DTUs na natureza possam ser peculiares. Nós observamos um número significativamente maior da DTU TcIV do que TcIII. A menor taxa de infecção de mamíferos por TcIII pode ser talvez explicada, por essa DTU resultar em baixa parasitemia e os animais infectados com essa DTU apresentarem resultados positivos para *T. cruzi* apenas por sorologia. Vale mencionar que em média, apenas 10% dos mamíferos examinados apresentam parasitemia suficientemente alta para ser detectada por hemocultura. Outro fator que deve ser considerado é a questão temporal, como pode ser exemplificado pelos isolados de *T. cruzi* obtidos de mamíferos silvestres no Pantanal: nos anos de 2001 e 2002, esses animais apresentavam perfil TcIV, mas quatro anos depois, passaram a apresentar perfil TcIII. Esse padrão aparentemente temporal de distribuição de DTUs na natureza, mostra a importância de se ter extremo cuidado com o uso de dados secundários. É fato que TcIII e TcIV são capazes de infectar diversas espécies de mamíferos silvestres, assim como TcI e TcII. Os nossos achados possibilitam levantar duas hipóteses para a “temporalidade” da distribuição de DTUs na natureza: a) ocorrência de diferentes estratégias de transmissão e; b) a infecção de mamíferos silvestres pelas DTUs TcIII e TcIV tende a apresentar baixas parasitemias, quando comparadas com infecções pelas DTUs TcI e TcII. Marcilli *et al.* (2009) propuseram que a baixa ocorrência da DTU TcIII possa ser justificada pela subamostragem. Com isso, os dados existentes sobre a distribuição das DTUs de *T. cruzi* são agregados e não reflete todos os seus habitats de ocorrência nos biomas (Lima *et al.*, 2014). Esta é uma hipótese muito provável.

A DTU TcIII foi proposta como sendo relacionada a ordem Cingulata, mais especificamente a espécie *Dasypus novemcinctus*, como também a espécies de triatomíneos do gênero *Panstrongylus* (Yeo *et al.*, 2005; Llewellyn *et al.*, 2009; Zingales *et al.*, 2012). Já a DTU TcIV foi proposta como sendo relacionada a ordem Primata, especificamente aos gêneros *Alaouta* e *Cebus*, e a espécies de triatomíneos do gênero *Rhodnius* (Marcili *et al.*, 2009c; Zingales *et al.*, 2012). Os nossos resultados não confirmam essa associação, na medida em que mostram a diversidade de hospedeiros mamíferos que as DTUs apresentam. Esse aspecto fica exemplificado pelos marsupiais, os quais foram os hospedeiros que mais

apresentaram infecções pelas DTUs TcIII e TcIV. A espécie *M. domestica* foi encontrada infectada por essas DTUs nos biomas Amazônia, Cerrado e Caatinga. Marsupiais do gênero *Didelphis* apresentaram TcIII e TcIV com infecção simples e infecções mistas por TcI-TcIII e TcI-IV, nos biomas Amazônia e Mata Atlântica. Esse trabalho confima os marsupiais como sendo os mamíferos mais frequentemente encontrados infectados por *T. cruzi* e isso provavelmente se deve a esses animais apresentarem hábitos alimentares generalistas, serem nômades e frequentarem todos os estratos florestais. A diversidade de espécies de *Trypanosoma* spp. e DTUs de *T. cruzi* encontrada infectando marsupiais permite considerar que os marsupiais são bioacumuladores de tripanosomatídeos.

A ordem Rodentia também apresentou um número elevado de espécies infectadas pelas DTUs TcIII e TcIV. As mesmas foram isoladas de roedor arbóreo do gênero *Oecomys* e dos roedores terrestres do gênero *Trichomys*, *Trinomys* e *Proechimys*, nos biomas Pantanal, Caatinga e Mata Atlântica. Um fato interessante foi o isolamento pela primeira vez da DTU TcIV em um mamífero da ordem Artiodactyla, o porco (*Sus scrofa*). Por serem animais que vivem em liberdade, são considerados asselvajados. Esses animais apresentam alta soroprevalência para *T. cruzi*, mas o isolamento do parasito a partir desses animais é raro e isso mostra sua baixa competência infectiva, ou seja, baixa competência como reservatório (Roque *et al.*, 2008; Cominetti *et al.*, 2011; Bezerra *et al.*, 2014).

Em relação aos triatomíneos, nós também observamos que as DTUs TcIII e TcIV são bastante generalistas em relação a gênero e espécies vetoras. As DTUs TcIII e TcIV foram encontradas em triatomíneos do gênero *Triatoma* e *Rhodnius* nos biomas Pantanal, Mata Atlântica e Amazônia. Dario (2013) já havia observado a ocorrência dessas DTUs em *T. vitticeps* e *P. geniculatus* (TcIII) no bioma Mata Atlântica. Esses resultados reforçam a ausência de associação entre DTUs e um gênero específico de triatomíneos.

Infecções mistas, TcI-TcIII e TcI-TcIV, foram observadas em mamíferos e triatomíneos nos diferentes biomas brasileiros. Esse resultado mostra mais uma vez que infecções mistas são muito frequentes na natureza. Uma limitação dessa parte do estudo foi que usamos somente amostras isoladas de cultivo, restringindo possíveis populações que não puderam ser isoladas. O cultivo de parasitos em meio de cultura para posterior isolamento é uma técnica específica, porém com baixa sensibilidade.

6 CONCLUSÕES

- O ciclo enzoótico das espécies de *Trypanosoma* spp. na natureza é complexo e diverso, na medida em que pode ocorrer em infecções simples, duplas ou múltiplas, que incluem todas as DTUs de *T. cruzi* e outras espécies de tripanosomatídeos;
- Casos de DC podem acontecer independente do ciclo de transmissão enzoótica próximo das casas;
- No município de Guarapari, o sistema-reservatório de *T. cruzi* incluiu espécies diferentes de Chiroptera;
- Associações entre as DTUs de *T. cruzi* e espécies de hospedeiros, área geográfica e patogenicidade ainda não foram claramente estabelecidas;
- Em humanos, a infecção mista por diversas espécies e DTUs de *T. cruzi* é provavelmente muito mais frequente do que relatado e pode eventualmente estar relacionada com a patogenia;
- *Trypanosoma dionisii* é capaz de infectar diferentes espécies de mamíferos além de quiróptera;
- *Triatoma vitticeps* é um vetor generalista;
- A distribuição dos tripanosomatídeos do clado *T. cruzi* na natureza é mais ampla do que se tem relatado até o presente;
- Morcegos e marsupiais são bioacumuladores de diferentes espécies de tripanosomatídeos;
- *Trypanosoma* spp. e mesmo o gênero *Bodo* são aparentemente muito menos específicos do que admitido até o momento;
- A ocorrência e a transmissão de diferentes DTUs de *T. cruzi*, e espécies do clado *T. cruzi* podem acontecer simultaneamente em um mesmo fragmento florestal;
- O acesso a parasitos não cultiváveis pelo NGS resultará na descoberta de novos aspectos do fenômeno parasitismo;
- O NGS reforçou o papel dos morcegos como hospedeiros/reservatórios de diferentes espécies da classe Kinetoplastea;
- O encontro do um táxon de protozoário de vida-livre, *B. saltans*, infectando um mamífero demonstrou plasticidade biológica dessa espécie;

- As DTUs TcIII e TcIV apresentam uma distribuição e espectro de hospedeiros no território brasileiro mais amplos do que conhecido até o presente;
- As DTUs TcIII e TcIV não apresentaram associação com espécie de hospedeiro mamífero ou bioma;
- A maioria dos estudos sobre a ecologia de tripanosomatídeos de mamíferos ainda é baseada em subamostragem, o que resulta em lacunas no conhecimento da ecologia desses e torna pouco robusta qualquer afirmação sobre associação destes organismos com espécies de hospedeiro ou bioma.

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8 ANEXO

Parasitology

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Research Article

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Trypanosoma sp. diversity in Amazonian bats (Chiroptera; Mammalia) from Acre State, Brazil

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Abstract

Bats are ancient hosts of *Trypanosoma* species and their flying ability, longevity and adaptability to distinct environments indicate that they are efficient dispersers of parasites. Bats from Acre state (Amazon Biome) were collected in four expeditions conducted in an urban forest (Parque Zoobotânico) and one relatively more preserved area (Seringal Cahoeira) in Rio Branco and Xapuri municipalities. *Trypanosoma* sp. infection was detected by hemoculture and fresh blood examination. Isolated parasite species were identified by the similarity of the obtained DNA sequence from 18S rDNA polymerase chain reaction and reference strains. Overall, 367 bats from 23 genera and 32 species were examined. Chiropterofauna composition was specific to each municipality, although *Artibeus* sp. and *Carollia* sp. prevailed throughout. *Trypanosoma* sp. infection was detected in 85 bats (23·2%). The most widely distributed and prevalent genotypes were (in order) *Trypanosoma cruzi* TcI, *T. cruzi marinkellei*, *Trypanosoma dionisii*, *T. cruzi* TcIV and *Trypanosoma rangeli*. At least one still-undescribed *Trypanosoma* species was also detected in this study. The detection of *T. cruzi* TcI and TcIV (the ones associated with Chagas disease in Amazon biome) demonstrates the putative importance of these mammal hosts in the epidemiology of the disease in the Acre State.

Introduction

Chiropterans are nocturnal and widely dispersed mammals, representing approximately 20% of the recognized mammalian species in the world (Fenton and Simmons, 2015). These flying mammals display long life spans and can occupy diverse and numerous habitats in both natural and urban environments. These characteristics result in their high capacity as seed dispersers as well as parasite dispersion, making these animals important contributors to biodiversity (Luis *et al.* 2013).

Bats are ancient hosts of *Trypanosoma* sp. (Trypanosomatida; Protozoa), a parasite genus transmitted between several vertebrate species and blood-sucking invertebrate vector worldwide. Bat trypanosomes included in the *Trypanosoma cruzi* clade were already described in Asia, Africa, the Americas and Europe (Lima *et al.* 2013, 2015; Barbosa *et al.* 2016). Within this clade, *T. cruzi*, a zoonotic parasite responsible for Chagas disease that is currently considered a worldwide problem (Coura *et al.* 2014). This taxon displays marked heterogeneity, and six discrete typing units (DTUs) are currently recognized (TcI–TcVI) in addition to a putative TcVII DTU, which was previously associated with bats (Tcbat) and has already been observed infecting humans (Zingales *et al.* 2012; Ramírez *et al.* 2014). Other than *T. cruzi*, all species from the subgenus *T.* (*Schizotrypanum*) are described to be restricted to bats, although *Trypanosoma dionisii* was recently described in cardiac tissue in one human (Dario *et al.* 2016).

The most accepted theory to explain the origin of the trypanosomatids from cruzi clade indicates bats are the ancestral hosts (the bat seeding hypothesis), and their flying capacity is responsible for the dispersal of some species, such as *T. dionisii*, between the Old and New Worlds (Hamilton *et al.* 2012). Recent molecular and phylogenetic studies have corroborated this theory, describing new species of bat trypanosomes within the *T. cruzi* clade, including *Trypanosoma erneyi* and *Trypanosoma livingstonei* in African bats (Lima *et al.* 2012, 2013), *Trypanosoma wauwau* in South and Central America bats (Lima *et al.* 2015) and *Trypanosoma teixeirae* in an Australian little red flying fox (Barbosa *et al.* 2016). In Brazil, bats have been found to be infected by *T. cruzi* (TcI, TcII and Tcbat), *T. rangeli*, *T. c. marinkellei*, *T. dionisii* and *T. wauwau* (Lima *et al.* 2015; da Costa *et al.* 2016).

The Acre state is in the Amazon basin, the biome that contains the greatest biodiversity in the world (Hoorn *et al.* 2010). The Amazonian region that previously was free from Chagas disease and where only the enzootic transmission cycle of *T. cruzi* existed, started to be considered as endemic for this disease (Coura and Junqueira, 2015). In the state of Acre, 13 autochthonous cases were reported between 1988 and 2015, which contrasts the 25 cases reported in 2016 (data from the Secretary of Health from Acre State). Considering the recent higher effort for

case identification and the dispersion of the municipalities that reported cases in a huge area of the state, this historical report of cases is underestimated. There are an estimated 59 species of bats in Acre State in both forest fragments and urban areas. The most abundant species are those from the genera *Artibeus, Carollia* and *Phyllostomus* (Bernard *et al.* 2011). Despite their abundance and dispersion within a biome characterized by its biodiversity, the diversity of trypanosomes infecting bats from Acre state is completely unknown. The aim of this study was to describe the chiropterofauna and their associated trypanosomes in areas with different ecological landscapes and degrees of human disturbance in one urbanized and one rural municipality from Acre State in the Brazilian Amazon.

Materials and methods

Study areas

Bats were captured in two municipalities from Acre State, north Brazil: Rio Branco, the capital of the state (09°58′29″S/67°48′36″W), and Xapuri, 175 km away from the former (10°10′95″S/68°30′16″W). Characteristic of Amazonia, the climate is tropical-humid, displaying high levels of regular rainfalls and elevated temperatures. The rainy period is from October to March, whereas April to September has the lowest rain volumes. In Rio Branco, captures were conducted in Parque Zoobotânico (PZ), which is the highest vegetation area of the municipality; it belongs to the Acre Federal University and consists of 150 ha of secondary vegetation that has been preserved since 1983. In Xapuri, captures occurred in Seringal Cahoeira (SC), a well-preserved area of almost 25 thousand ha of pristine vegetation that is 30 km from the centre of the municipality alongside management areas of *Hevea brasiliensis*. Expeditions were conducted four times to each locality during the wet and dry seasons, in March and August 2014 and June and November 2015.

In each area (PZ and SC), the following three localities were selected according to a gradient of preservation and vegetation characteristics (Fig. 1): Solid Ground (A1 and A4), areas consisting of dense forest with native (A4) or secondary (A1) vegetation with palm trees and bamboo as well as lower stratum with clean aspect; Sandbank (A2 and A5), a less dense vegetation area mainly consisting of palm trees near the floodplains of rivers with high flow during the wet season and characterized by an open canopy forest and spots of dense forest with emergent trees; and Open areas (A3 and A6) near dwellings of human activity (A3) or consisting of rubber trees associated with banana and various timber species (A6).

Sample collection

Bats were captured using 10 mist nets (12 m × 3 m, 35 mm mesh) per night, which were arranged approximately 50 cm from the ground. The nets were installed in the early evening, maintained over 6 h from 6:00 to 12:00 pm (two nights in each area), and checked every 30 min. The total effort was the same for the six areas, which was 2,160 m^2-h per night; 4320 m^2-h per expedition in each area; 12 960 m^2-h per municipality in each expedition and 51 840 m^2-h in the four expeditions conducted to each municipality.

Pregnant and/or lactating bat females were removed from the net and immediately released. The others were individually placed in cloth bags and transported to the field laboratory where they stayed until the next morning when the collection procedures were performed. For all collected specimens, the morphological characteristics and body measurements were recorded for age estimations and taxonomic identification, which was determined using the available bibliography (Gardner, 2007; Díaz *et al.* 2011).

In the field laboratory that was established exclusively for this purpose, bats were anesthetized (9:1, ketamine chloridrate 10% and acepromazine 2%), and their blood was collected by cardiac puncture. The collected blood was processed as follows: (i) for fresh blood examination, one drop was set between a glass slide and coverslip and observed in an optical microscope at 400× magnification; and (ii) for hemoculture, approximately 0·6 mL of blood was cultured in tubes containing Novy-Mc Neal-Nicole medium with either a liver infusion tryptose medium or Schneider's Insect Medium overlay (0·3 mL in each one).

Parasitologic diagnostic procedures and DNA extraction

Fresh blood examination was performed in the field laboratory and considered positive when at least one parasite or a flagellar movement beyond blood cells was observed. Hemocultures were transported to LABTRIP and analysed every other week for up to 5 months (usually three because of bacterial contamination). The positive hemocultures were amplified, cryopreserved and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, COLTRYP/FIOCRUZ (www.coltryp.fiocruz.br). The amplification of the positive hemoculture means that the observed parasites were allowed to multiply spontaneously at 27 °C until the stationary phase (when cells stopped to multiply), and after what, the flagellates were harvested and DNA was extracted as described below.

We sometimes observed in the culture tubes the presence of flagellates morphologically identical to trypanosomatids, but that did not grow, that is, we could not establish a culture of these parasites. In these cases, the liquid phase of the cultures were harvested, centrifuged and the pellet subjected to DNA extraction for further molecular characterization. To this sediment we gave the name 'culture mass'. Positive hemocultures also point to infective potential to vectors since we were able to obtain parasites starting from 300 μL.

Positive cultures and/or culture mass were washed with phosphate-buffered saline solution and incubated with proteinase K (100 μg mL^{-1}) and 0·5% of sodium dodecyl sulphate at 56 °C for 2 h. Then, DNA was extracted by the classical phenol–chloroform method (Vallejo *et al.* 1999) and then quantified in a NanoDrop 1000 Spectrophotometer (Thermo Scientific®), and the final concentration was adjusted to 50 ng μL^{-1}.

Molecular characterization and phylogenetic analyses

A nested polymerase chain reaction (PCR) targeting the 18S rRNA gene (~600–800 bp) was performed as described by Noyes *et al.* (1999) with external primers TRY927F (5′GAAAC AAGAAACACGGGAG3′) and TRY927R (5′CTACTGGGCAGC TTGGA 3′) for 30 cycles at 94 °C for 30 s, 55 °C for 60 s and 72 °C for 90 s. The products from the first amplification were diluted 1:10 in sterile deionized water, and 2 μL was used as template for the second-round PCR with the following internal primers: SSU561F (5′TGGGATAACAAAGGAGCA3′) and SSU561R (5′CTGAGACTGTAACCTCAAAGC3′) using the same cycling conditions. The products derived from the second-round reaction were electrophoresed in a 2% agarose gel run at 90 V for 1·5 h in Tris-acetate EDTA buffer, which was stained with ethidium bromide and visualized by illumination with UV light. Six samples obtained from the first expedition were characterized by other primer pairs from the same 18S rRNA gene (V7V8) using PCR conditions described elsewhere (Borghesan *et al.* 2013).

Amplified PCR products were purified using Illustra GFX PCR DNA and a gel band purification kit (GE Healthcare Life

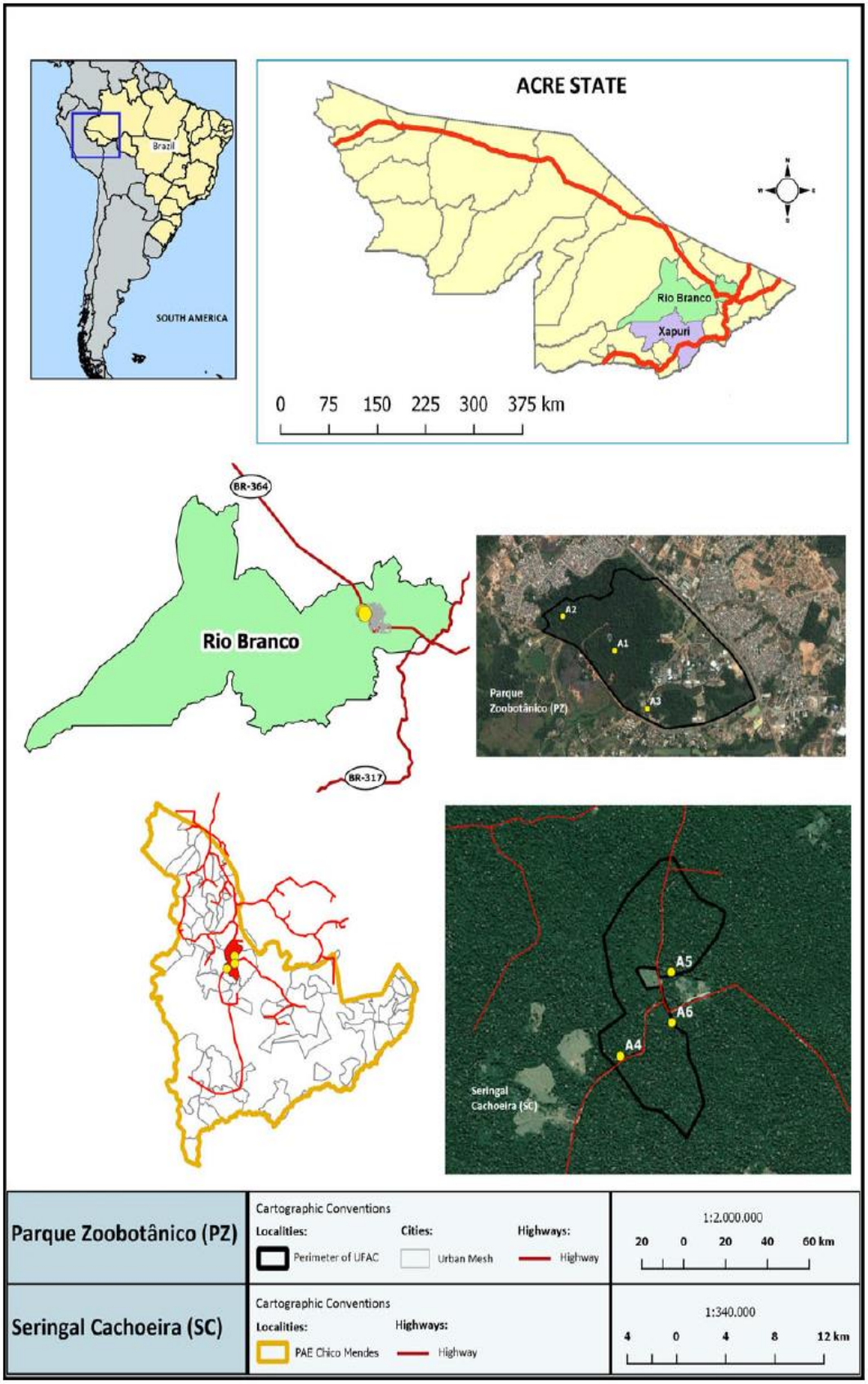

Fig. 1. Geographical location of study area: Mapping of the bat collected areas in Acre state (Amazon Biome). Bats were collected in an urban forest: Parque Zoobotânico – PZ (A1–A3); and one more preserved area: Seringal Cachoeira – SC (A4–A5) in Rio Branco and Xapuri municipalities, respectively. On the left of the figure is the study site in Brazil, highlighting the Acre state and the Brazilian and South America limits.

Sciences, Little Chalfont, Buckinghamshire, UK). The purified PCR products were sequenced using the corresponding internal reverse primers diluted at 3·2 picomoles with ABI 3730 BigDye Terminator (v3·1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystem DNA Analyzer on the PDTIS/FIOCRUZ sequencing platform.

Nucleotide sequences obtained from 18S rRNA were manually edited using the DNASTAR's Lasergene Sequence Analysis Software (Burland, 2000) and aligned using CLUSTALW. The obtained sequences were compared with nucleotide sequences deposited in GenBank using the NCBI BLAST (Basic Local Alignment Search Tool) algorithm to identify *Trypanosoma* species and/or *T. cruzi* DTUs. Identification was confirmed when the obtained sequence had a minimum length of 400 bp, an e-value equal to zero, 98% coverage and a minimum of 97% of identity with reference sequences from GenBank. The evolutionary histories at 18S rRNA gene were inferred in Mega 7 (Kumar *et al.* 2016) by Maximum Likelihood (ML) using Kimura 2-parameter model of nucleotide substitution with gamma-distributed plus invariant sites (K2+G+I), with bootstrapping at 1000 replicates for nodal support. We used in the analysis reference strains from *T. cruzi* clade and *Herpetomonas ztiplika* as outgroup (Table S1).

Statistical analysis

The richness and prevalence of *Trypanosoma* species (and *T. cruzi* DTUs) were compared between species, sex and micro habitats, using the Chi-square statistical analysis. The test was performed using software R (Version 2.11.1, R Development Core Team, 2010) considering the level of significance ($P < 0·05$).

Ethical statements

Bat captures were licensed by the Chico Mendes Institute for Biodiversity Conservation Brazilian (ICMBio – SISBIO), license numbers 44089-1 and 47377-1. All procedures with the bats followed protocols that had been approved by the Ethics Committee of the Fiocruz Animal Use (LW81-12). The carcasses of euthanized animals were deposited as voucher specimens in the collection of the Mammalian Ecology Laboratory of the Acre Federal University (UFAC).

Results

Diversity and abundance of the Chiropteran fauna

The four field excursions resulted in the capture of 367 bat specimens that were included in three families (Phyllostomidae, Emballonuridae and Vespertilionidae), 23 genera and 32 species (Fig. 2). The number of captured bats in the two study areas, PZ and SC, did not differ significantly ($P = 0·08$). Moreover, the species composition of the chiropteran fauna was specific to each of the two areas (Fig. 2). Additionally, the bat species diversity was significantly higher in SC ($n = 29$) compared with PZ ($n = 17$) ($P = 0·016$). The *Artibeus* sp and *Carollia* sp predominated in both areas, and the first was slightly more abundant (35·9% *vs* 34·3%). The relative abundance of the *Artibeus* sp was significantly higher in PZ (49·2%) than in SC (18·1%) ($P < 0·0001$). In contrast, the relative abundance of *Carollia* sp bats was comparable in the two localities, with 32·6% in PZ and 39·7% in SC ($P = 0·10$). Data concerning the abundance of bat species in each area of both municipalities are listed in Table S2.

Distribution of Trypanosoma sp. infection in Chiroptera of Acre State, Amazon Biome

Infection by *Trypanosoma* sp. was observed in 85 bats (23·2%); four of them were only observed by fresh blood examination, while 81 (22%) bats from 15 genera and 22 species displayed positive hemocultures (Table 1 and Table S2). Of these, 12 (14·8%) also displayed positive fresh blood examination. Infection by *Trypanosoma* sp. was three times higher in Chiroptera from SC ($n = 60$; 34·5%) in comparison with Chiroptera from PZ ($n = 21$; 10·9%) ($P < 0·0001$).

In PZ, the infectivity potential for vectors, expressed by positive hemocultures of the examined bats, was restricted to species from the two most abundant genera, *Artibeus* and *Carollia* (85·7%). A distinct enzootical picture was observed in SC, where a higher number of infected bats and overall higher bat species diversity were observed in all collected areas. Overall, the most common *Trypanosoma* sp. infected bat species was the generalist short tailed bat species *Carollia perspicillata*, wherein 16·3% were infected.

Of the 81 bats that were positive in the hemoculture, 68 isolates were obtained from 67 bats (two isolates were obtained from one *C. perspicillata* from PZ – LBT 7063). Three culture mass were

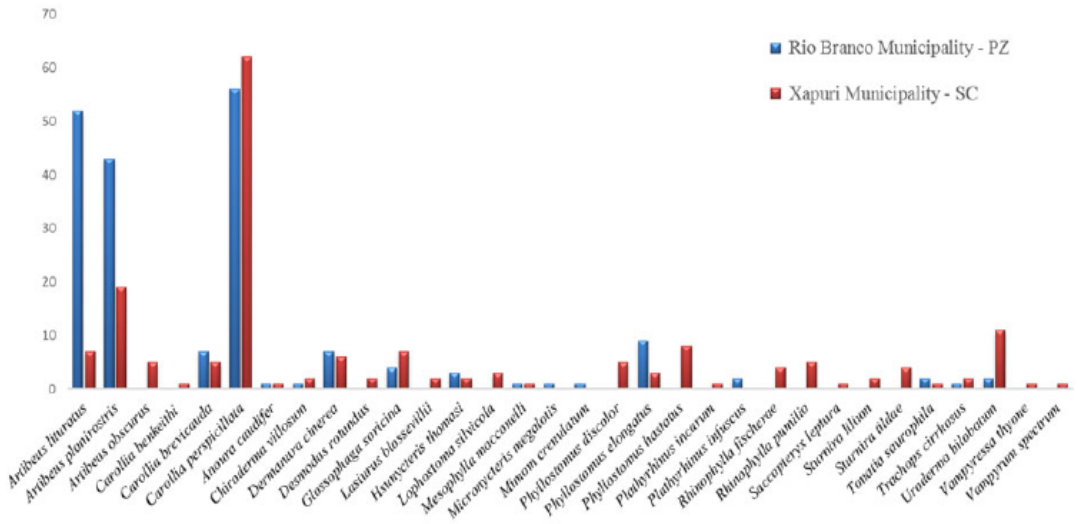

Fig. 2. Diversity and distribution of bats in two municipalities of Acre state, Rio Branco (Parque Zoobotânico –PZ) and Xapuri (Seringal Cachoeira – SC), in the Amazonian region of Brazil. Except for the species from the two most abundant genera (*Artibeus* and *Carollia*), the bat species are presented in alphabetical order.

Table 1. Distribution of *Trypanosoma* sp. infection in Chiroptera from Rio Branco and Xapuri municipalities, Acre State

Genus	Rio Branco municipality (PZ) Positive/Total (%)	Xapuri municipality (SC) Positive/Total (%)
Artibeus	9/95 (9·5)	10/31 (32·3)
Carollia	9/63 (14·3)	24/68 (35·3)
Phyllostomus	0/9	12/16 (75)
Anoura	0/1	1/1 (100)
Dermanura	0/7	1/6 (16·7)
Glossophaga	1/4 (25)	1/6 (16·7)
Lasiurus	–	1/2 (50)
Hsunycteris	0/3	1/2 (50)
Lophostoma	–	2/3 (66·6)
Platyrhinus	1/2 (50)	0/1
Sturnira	–	2/6 (33·3)
Tonatia	0/2	1/1 (100)
Trachops	1/1 (100)	2/2 (100)
Uroderma	0/2	1/11 (9·1)
Vampyressa	–	1/1 (100)
Total	21/193 (10·9)	60/174 (34·5)

also successfully characterized. Infection of the other 11 bats was only diagnosed by the morphological characteristics of the parasites observed in positive cultures.

The diversity of bat species in PZ was higher in area A2, where 12 distinct species could be distinguished among the 86 collected individuals. Additionally, the prevalence of *Trypanosoma* sp. infection was higher in this area (16·3%) compared with A1 (7·6%) and A3 (4·9%), although there was only a significant difference between A2 and A3 ($P = 0·035$). In SC, no differences in the *Trypanosoma* sp. infection were noted among the distinct areas ($P = 0·305$).

Trypanosoma sp. diversity infecting bats from Acre state

At least five different and one unidentified species and/or *T. cruzi* DTUs were observed in bats from Acre. Moreover, two mixed infections were also detected; one was identified in the same hemoculture (LBT 5060), and the other was identified in two different hemocultures from the same bat (LBT 7063). *Trypanosoma cruzi* TcI followed by *T. c. marinkellei* were the most widely distributed and prevalent trypanosomatid species (Tables 2 and 3). However, whereas *T. cruzi* TcI was found infecting bats in all areas, 11 of the 12 *T. c. marinkellei* isolates were derived from bats collected in the three SC areas. A similar feature was observed for *T. dionisii* wherein five of the six infected bats were from the same municipality. *Trypanosoma cruzi* TcIV was only detected in the most preserved areas from PZ (A1 and A2) and in SC, and the latter had co-infection with *T. c. marinkellei*. The two isolates that were characterized as *T. rangeli* belong to the genotype A and were derived from SC bats (*C. perspicillata* and *Artibeus planirostris*). One still-undescribed *Trypanosoma* species was isolated in co-infection with *T. cruzi* TcI in a bat from PZ (Tables 2 and 3). One of those samples clustered in the same branch as trypanosomes described in neotropical bats, between *T. wauwau* and *Trypanosoma* sp RNMO56 and 63 (Fig. 3). Although parasites were observed in another 11 positive hemocultures, those cultures were not established, and the parasites were characterized as *Trypanosoma* sp. based on their morphology in axenic cultures (Table 2).

The most abundant bat genera were *Carollia* sp. and *Artibeus* sp., but *Phyllostomus* sp. also displayed infections with the greatest diversity of *Trypanosoma* species (Table 4). *Trypanosoma cruzi* DTU TcI was the most dispersed *Trypanosoma* species and DTU. Only two of the 15 infected bat genera (*Lophostoma* and *Sturnira*) were not infected by *T. cruzi* TcI. *Trypanosoma dionisii* infected bats from four genera, including the most abundant bat genera, *Carollia* sp. *T. marinkellei* was mainly observed in *Phyllostomus* sp. (including one mixed infection with *T. cruzi* TcIV) as well as in *Lophstoma* sp. and *Artibeus* sp. *Artibeus* sp. was the taxon with the highest *Trypanosoma* sp species diversity, followed by the most abundant bat genera *Carollia* sp. and *Phyllostomus* sp (Table 4).

Discussion

Bats are mammals that live for a long time, and their lifespan may easily reach more than two or even three decades (Wilkinson and South, 2002). Additionally, bats have high displacement capacity covering wide areas, which means that they are highly capable of dispersing parasites for a long time. The majority of the captured bats were included in 21 genera of Phyllostomidae, the 'leaf nosed' bat family that represent the most common and diverse bat taxon in Brazil and have a huge ecological importance.

Table 2. Distribution and diversity of *Trypanosoma* sp. in Chiroptera of Acre State, Amazon Biome according to distinct study areas and habitats

	Rio Branco municipality			Xapuri municipality			Total
Trypanosoma	A1	A2	A3	A4	A5	A6	
Trypanosoma cruzi TcI	1	10	2	9	16	7	45
Trypanosoma cruzi TcIV	2	1	–	–	–	–	3
Trypanosoma cruzi marinkellei	–	1	–	3	4	4	12
Trypanosoma dionisii	–	1	–	3	–	2	6
Trypanosoma rangeli	–	–	–	1		1	2
Trypanosoma cruzi TcIV + *T. cruzi marinkellei*	–	–	–	–	1	–	1
Trypanosoma sp. + *T. cruzi* TcI		1					1
Trypanosoma sp.[a]	2	–	–	5	4	–	11
Total	5	14	2	21	25	14	81

Characterization was performed using 18S SSU primers that were described by Noyes *et al.* (1999).
[a]Morphologically identified.

Table 3. Identification numbers, bat species, locality and molecular characterization of the trypanosomatids using SSUrDNA

Coltryp no[a]	Isolate	Host specie	Area	18S SSU	GenBank accession no
549	LBT 5060	*Phyllostomus hastatus*	A5	*T. cruzi* TcIV[b] + *T. c. marinkellei*[b]	KY748354 KY824655
550	LBT 5004	*Glossophaga soricina*	A2	*T. cruzi* TcIV[b]	KY748355
555	LBT 5009	*Artibeus lituratus*	A1	*T. cruzi* TcIV[b]	KY748356
558	LBT 5040	*Carollia perspicillata*	A4	*T. dionisii*[b]	KY689928
567	LBT 5043	*Sturnira tildae*	A4	*T. dionisii*[b]	KY689929
568	LBT 5042	*Trachops cirrhosus*	A4	*T. dionisii*[b]	KY689930
574	LBT 5469	*Carollia perspicillata*	A6	*T. dionisii*	KY649111
579	LBT 5340	*Artibeus lituratus*	A1	*T. cruzi* TcIV	KY649112
584	LBT 5473	*Carollia perspicillata*	A6	*T. dionisii*	KY649113
589	LBT 5408	*Artibeus planirostris*	A4	*T. cruzi* TcI	KY649114
587	LBT 5472	*Carollia perspicillata*	A6	*T. rangeli A*	KY649115
590	LBT 5439	*Uroderma bilobatum*	A5	*T. cruzi* TcI	KY649116
592	LBT 5427	*Carollia perspicillata*	A4	*T. cruzi* TcI	KY649117
634	LBT 6568	*Artibeus planirostris*	A3	*T. cruzi* TcI	KY649118
640	LBT 7060	*Artibeus planirostris*	A2	*T. dionisii*	KY649119
647S 647L	LBT 7063	*Carollia perspicillata*	A2	*Trypanosoma* sp.+ *T. cruzi* TcI	KY649120 KY649121
648	LBT 7065	*Trachops cirrhosus*	A2	*T. cruzi* TcI	KY649122
650	LBT 7068	*Carollia perspicillata*	A1	*T. cruzi* TcI	KY649123
–	LBT 7058	*Carollia perspicillata*	A2	*T. cruzi* TcI	N.D.
651	LBT 7074	*Artibeus lituratus*	A3	*T. cruzi* TcI	KY649124
652	LBT 7064	*Carollia perspicillata*	A2	*T. cruzi* TcI	KY649125
653	LBT 7056	*Carollia brevicauda*	A2	*T. cruzi* TcI	KY649126
654	LBT 7057	*Carollia perspicillata*	A2	*T. cruzi* TcI	N.D.
655B	LBT 7066	*Carollia perspicillata*	A2	*T. cruzi* TcI[c]	KY649127
656	LBT 7059	*Carollia brevicauda*	A2	*T. cruzi* TcI	KY649128
657	LBT 7080	*Carollia perspicillata*	A4	*T. cruzi* TcI	KY649129
658	LBT 7081	*Carollia perspicillata*	A4	*T. cruzi* TcI	KY649130
659	LBT 7083	*Carollia perspicillata*	A4	*T. cruzi* TcI	N.D.
660	LBT 7084	*Vampyressa thyone*	A4	*T. cruzi* TcI	N.D.
661	LBT 7085	*Carollia perspicillata*	A5	*T. cruzi* TcI	KY649131
662	LBT 7088	*Carollia perspicillata*	A5	*T. cruzi* TcI	N.D.
663	LBT 7089	*Carollia benkeithi*	A5	*T. cruzi* TcI	KY649132
664	LBT 7090	*Carollia perspicillata*	A5	*T. cruzi* TcI	KY649133
665	LBT 7092	*Artibeus lituratus*	A5	*T. cruzi* TcI	KY649134
666	LBT 7094	*Carollia perspicillata*	A5	*T. cruzi* TcI	KY649135
667	LBT 7095	*Carollia perspicillata*	A5	*T. cruzi* TcI	KY649136
668	LBT 7100	*Phyllostomus discolor*	A6	*T. cruzi* TcI	KY649137
669	LBT 7102	*Artibeus lituratus*	A6	*T. cruzi* TcI	KY649138
670	LBT 7078	*Carollia brevicauda*	A4	*T. cruzi* TcI	KY649139
671	LBT 7097	*Phyllostomus discolor*	A6	*T. cruzi* TcI	KY649140
672	LBT 7098	*Hsunycteris thomasi*	A6	*T. cruzi* TcI	KY649141
674	LBT 7104	*Carollia perspicillata*	A5	*T. cruzi* TcI	KY649142
676	LBT 7087	*Carollia perspicillata*	A5	*T. cruzi* TcI	N.D.
677	LBT 7086	*Trachops cirrhosus*	A5	*T. cruzi* TcI	KY649143

(*Continued*)

Table 3. (*Continued.*)

Coltryp no[a]	Isolate	Host specie	Area	18S SSU	GenBank accession no
678	LBT 7105	*Dermanura cinerea*	A5	*T. cruzi* TcI	KY649144
680	LBT 7110	*Lasiurus blossevillii*	A5	*T. cruzi* TcI	KY649145
682	LBT 7067	*Plathyrrhinus infuscus*	A2	*T. cruzi* TcI	KY649146
684	LBT 7061	*Artibeus planirostris*	A2	*T. cruzi* TcI	KY649147
686	LBT 7091	*Tonatia saurophilla*	A5	*T. cruzi* TcI	KY649148
687	LBT 7099	*Anoura caudifer*	A6	*T. cruzi* TcI	KY649149
689	LBT 7108	*Artibeus planirostris*	A5	*T. cruzi* TcI	KY649150
690	LBT 7101	*Carollia perspicillata*	A6	*T. cruzi* TcI	KY649151
691	LBT 7093	*Artibeus obscurus*	A5	*T. cruzi* TcI	KY753877
695B	LBT 7062	*Carollia perspicillata*	A2	*T. cruzi* TcI[c]	KY649152
696	LBT 7079	*Carollia perspicillata*	A4	*T. cruzi* TcI	KY649153
697	LBT 7082	*Carollia perspicillata*	A4	*T. cruzi* TcI	KY649154
–	LBT 5428	*Artibeus planirostris*	A4	*T. rangeli A*	KY649155
–	LBT 7096	*Glossophaga soricina*	A6	*T. cruzi* TcI	KY649156
575	LBT 5416	*Phyllostomus hastatus*	A4	*T. c. marinkellei*	KY649157
576	LBT 5423	*Phyllostomus hastatus*	A4	*T. c. marinkellei*	KY649158
577	LBT 5467	*Phyllostomus hastatus*	A6	*T. c. marinkellei*	KY649159
578	LBT 5466	*Phyllostomus hastatus*	A6	*T. c. marinkellei*	KY649160
580	LBT 5434	*Lophostoma silvicola*	A5	*T. c. marinkellei*	KY689835
581	LBT 5471	*Phyllostomus discolor*	A6	*T. c. marinkellei*	KY689836
582	LBT 5441	*Phyllostomus elongatus*	A5	*T. c. marinkellei*	KY689837
585	LBT 5425	*Artibeus planirostris*	A4	*T. c. marinkellei*	KY689838
586	LBT 5389	*Artibeus planirosrtis*	A2	*T. c. marinkellei*	KY689839
591	LBT 5450	*Lophostoma silvicola*	A5	*T. c. marinkellei*	KY689840
673	LBT 7113	*Phyllostomus discolor*	A6	*T. c. marinkellei*	KY689841
679	LBT 7106	*Phyllostomus hastatus*	A5	*T. c. marinkellei*	KY689842

The last column includes the GenBank accession number of each deposited sequence.
N.D. not deposited in GenBank due to the presence of ambiguous sites.
[a]COLTRYP number codes of cultures deposited and cryopreserved in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores – COLTRYP (www.coltryp.fiocruz.br) of the Laboratório de Biologia de Tripanosomatídeos do Instituto Oswaldo Cruz – FIOCRUZ/RJ/Brazil.
[b]Characterized by V7V8 primer pairs (Borghesan *et al.* 2013).
[c]Parasites isolated from spleen culture.

This family includes the three genera of hematophagous, as well as small predators of vertebrates, and consumers of pollen, fruits, nectar and insects. The latter correspond to an ancestral food item that is kept in the diet of almost all species of bats to a greater or lesser degree (Gardner, 1979; Rojas *et al.* 2011; Carrillo-Araujo *et al.* 2015). Considering that bats are voracious insect predators, it is very likely that bats may infect at least by *T. cruzi*, *T. dionisii* and *T. rangeli* by ingesting infected Triatomine vectors.

Even in the highly preserved SC area, we collected 29 bat species, which is far less than the 59 bat species Bernard *et al.* (2011) described as extant in Acre State. One possible reason may be that, although well preserved, SC has a history of logging, which may reduce roost offerings and adequate shelters for some species of bats. In addition, we collect in the sub-forest, which therefore decreases the chances of capturing the bats that fly in higher forest strata.

The PZ area, as expected, displayed a significantly lower number of bat species ($n = 17$). In fact, although it is an area of urban forest that is not frequented by tourists, it suffers anthropic action because there is a research institute in this area where people and vehicles circulate. *Carollia* sp seem to be well adapted to altered environments, as shown by the similar number of specimens of this taxon collected in both study areas. By contrast, *Phyllostomus* sp. was mainly collected in SC. In PZ, the generalist bat genus *Artibeus* predominated.

There are ecological traits of chiropterans that may enhance their exposure to *Trypanosoma* sp infection. In fact, bats can roost in several natural and artificial habitats and can even modify vegetation to form suitable roosting places as tents that are built by some bat genera. In all these distinct habitats bats may get in contact with triatomines (Stoner, 2000). Palm trees whose leaves are frequently used as refuges by bats, are classical habitat of *Rhodnius* specimens, an efficient *T. cruzi* vector that is very common in the Amazon region (Coura and Junqueira, 2015). Considering that bats are voracious insect predators, it is very likely that tent building bats may be infected at least by *T. cruzi* and *T. rangeli* by ingesting infected vectors.

Bats may have a complex social structure that includes cooperative behaviour even among distinct groups. Cooperation includes roosting, taking care of offspring, grooming, foraging and feeding (Gardner, 2007). Food sharing is especially evident in the three hematophagous bat genera. Sharing food from

Fig. 3. Phylogenetic inference of isolate LBT 7063 detected in *Carollia perspicillata* bat from Rio Branco municipality, Acre state, Brazil, in the *T. cruzi* clade. Tree construction from SSU rRNA followed the maximum likelihood (ML) method under Kimura's two-parameter model and gamma distributed with invariant sites (K2 + G + I). Numbers at nodes indicate support from 1000 bootstrap replicates. The isolate clustered into the *T. cruzi* clade associated with trypanosomes described in neotropical bats (Lima *et al.* 2015). *Trypanosoma* sp H25, G8 and probably *T.* sp 15 and 17 have now been named *T. noyesi* (Botero *et al.* 2016)

adult animals to their offspring is a common pattern of behaviour in the animal world and may favour parasite transmission. This may especially be the case for mammalian trypanosomatids if the bat meal consists of blood. Of note, blood-feeding bats share food between adults, i.e. adult individuals regurgitate the blood they obtained for both their puppies and adult bats that could not feed themselves (Wilkinson *et al.* 2016). The care of offspring includes feeding other female pups and creating maintenance clusters of young as a crèche (Wilkinson *et al.* 2016). Bats are excellent reservoirs of *Trypanosoma* sp. and probably the ancestral host of *T. cruzi* (Molyneux, 1991; Hamilton *et al.* 2012). Nevertheless, *Trypanosoma* sp. of bats is a little explored universe. In fact, the increase in the analytical power of the molecular tools has resulted in a growing number of new species of *Trypanosoma* sp. described in Chiropterans over the last several years. Chagas disease is currently a health threat in the Amazon region and in Acre State, where cases and outbreaks are becoming frequent; moreover, studies about *Trypanosoma* sp. infection of the mammalian fauna including bat species of Acre State are scarce.

Bats fidelity to their refuges is a factor that favours the establishment and maintenance of triatomine colonies. The association of *Cavernicola pilosa* with caves and *T. cruzi* and *T. c. marinkellei* transmission with bats is a nice example. Knowledge of how animal, especially bat, behaviour can alter and modulate the transmission of their parasites is still in the early stages.

Table 4. *Trypanosoma* sp. diversity in bats from Acre State, Amazon Biome

Genus	*T. cruzi* TcI	*T. cruzi* TcIV	*T. c. marinkellei*	*T. dionisii*	*Trypanosoma* sp. + *T. cruzi* DTU I	*T. cruzi* TcIV + *T. c. marinkellei*	*T. rangeli*	*Trypanosoma* sp.
Carollia	24			3	1		1	4
Artibeus	8	2	2	1			1	5
Phyllostomus	2		8			1		1
Anoura	1							
Dermanura	1							
Glossophaga	1	1						
Lasiurus	1							
Hsunycteris	1							
Lophostoma			2					
Platyrhinus	1							
Sturnira				1				1
Tonatia	1							
Trachops	2			1				
Uroderma	1							
Vampyressa	1							
Total	45	3	12	6	1	1	2	11

Phyllostomidae constitutes a diverse group that includes generalist feeders such as *Phyllostomus* sp. as well as specialist feeders such as the hematophagous bat species *Desmodus rotundus*, essentially frugivores bat species *Carollia perspicillata* and *Artibeus jamaicensis*, and nectarivorous *Glossophaga soricina*. Moreover, insectivory is part of the diet to a greater or lesser extent for most bats (Carrillo-Araujo *et al.* 2015). *Phyllostomus* sp. displayed a high infection rate (75%) by both *T. cruzi* and *T. c. marinkellei*, which were found in single and mixed infections, but *Trypanosoma* infection in this bat genera was only noted in SC. Although *C. pilosa* is considered the vector species of these two *Trypanosoma* species, also *Rhodnius* species may act as a vector based on it was successfully used in bat xenodiagnosis (Marinkelle, 1976; Garcia *et al.* 2012).

Carollia sp., which also displayed high *Trypanosoma* sp. infection rates, are considered resilient bats in terms of their diet (they feed on fruits and insects) and roosting sites, although they have also been described as understory specialists (Bernard, 2001). They live in colonies that may include hundreds of individuals that can be set in hollow trees, dense foliage, caves and tunnels, and they mainly forage at night. It is likely these bats acquire the *Trypanosoma* sp. infection in their roosting sites by ingesting triatomines or by the contaminative route because several of their roosting places are classical habitats of triatomines. This food and habit eclecticism in *Carollia* sp. probably explains the observation that this taxon not only had the highest rates of *T. cruzi* infection, but it also demonstrated a great diversity of *Trypanosoma* species that included *T. rangeli*, *T. dionisii* and at least one still-undescribed *Trypanosoma* species. Of note, we do not know if all trypanosomatids that could not be diagnosed at the species level constitute only a single species or include more than one species.

The diversity of *Trypanosoma* species that were found to infect bats from Acre state was astonishing, and further studies may reveal new aspects of *Trypanosoma* ecology and phylogeny. Additionally, *Carollia*, *Artibeus* and *Phyllostomus* were bat genres that also showed high rates of infection by a great diversity of *Trypanosoma* species. Concerning *T. cruzi* DTUs, bats were infected by TcI and TcIV, and both genotypes are associated with Chagas disease in the Amazon biome (Tables 3–4). In the infected bats, *T. cruzi* DTU TcI was found to infect all but two bat genera. This was an expected result because DTU TcI is ubiquitous in the transmission cycle of *T. cruzi* in the wild. *Lophostoma* sp. and *Sturnira* sp., two bat genera that were not infected by *T. cruzi*, harboured *T. c. marinkellei* and *T. dionisii*, respectively. The number of individuals of these two genera was very low, at only 3 and 2, respectively, and they were only collected in SC – the pristine vegetation area. Both Phyllostomidae and *Sturnira* are considered very abundant bat genera. Additionally, *Lupinus silvicola* is considered an abundant bat species. This species has a unique habitat because it is able to prepare and roost in cavities of active termite nests (Dechmann *et al.* 2004).

In addition to Marinkelle's seminal description of *T. rangeli* infection in bats (Marinkelle, 1976), there are only a few reports of infection by this taxon in chiropterans. *Trypanosoma rangeli* infecting *Artibeus planirostris* and *Platyrrhinus lineatus* bats was described by Maia da Silva *et al.* (2009) in central Brazil. Moreover, the authors observed a new lineage of *T. rangeli* in *P. lineatus* that was named lineage E. Here, we expand the occurrence area of lineage A of *T. rangeli* in bats to the northern region of Brazil, i.e. the Amazon biome. This lineage is widely distributed and infects many mammalian host species; therefore, it would be expected to be found in bats from other biomes. It is interesting to note that the two isolates of *T. rangeli* were obtained from two bats (*C. perspicillata* and *A. planirostris*) of the best-preserved area (SC), where the diversity of bats and trypanosomes was higher. The sequence analysis grouped one isolate from a *C. perspicillata* (LBT 7063) between *T. wauwau* and *Trypanosoma* sp RMNO, described in *Pteronotus* and *Tracops cirrhosus* bats in northern Brazil (Lima *et al.* 2015; da Costa *et al.* 2016). This result demonstrates that another trypanosome species is circulating in neotropical bats from Acre, reinforcing how little is known about the trypanosome diversity that circulate among bats. Even if it is an apparently well-preserved urban forest, the PZ area already shows a loss of bat and *Trypanosoma* sp. diversity, as demonstrated by the lower number of bat species captured and lower diversity of *Trypanosoma* species (Table 2).

The Trypanosomes that did not grow in culture media or presented with non-sustainable growth give a clue about how much remains to be known about the realm of bat trypanosomes without considering the trypanosomes that are not cultivable. In fact, there is still much to uncover about the assemblage of extant *Trypanosoma* species, their biology and ecology.

One phenomenon that is ripe for a thorough re-evaluation is host specificity in Trypanosomatids. It has been increasingly observed that numerous genotypes and species of *Trypanosoma* (*Schizotrypanum*) are far more eclectic with respect to their hosts. This is the case for Tcbat, a *T. cruzi* genotype that was exclusively associated with bats but has already been observed to infect humans (Ramírez *et al.* 2014), and for *T. dionisii*, a species of the subgenus *T.* (*Schizotrypanum*) that is usually associated with bats and has been detected in the cardiac tissue of a human individual who died from Chagas disease (Dario *et al.* 2016). Beyond parasites of medical or veterinary interest, trypanosomes are a fascinating model of the parasite–host interaction that constantly challenges and surprises us.

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