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Doutorado em Biologia Parasitária

# Diversidade, especificidade e distribuição geográfica de *Trypanosoma* spp. em mamíferos

MARINA SILVA RODRIGUES

Rio de Janeiro
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Ministério da Saúde

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# INSTITUTO OSWALDO CRUZ

## Programa de Pós-Graduação em Biologia Parasitária

*MARINA SILVA RODRIGUES*

Diversidade, especificidade e distribuição geográfica de *Trypanosoma* spp. em mamíferos

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

**Orientadora:** Profa. Dra. Ana Maria Jansen

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**Aprovada em: 30/05/2019**

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Rio de Janeiro, 30 de maio de 2019

Ministério da Saúde

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Ata da defesa de tese de doutorado em Biologia Parasitária de **Marina Silva Rodrigues**, sob orientação da Drª. Ana Maria Jansen-Franken. Ao trigésimo dia do mês de maio de dois mil e dezenove, realizou-se às nove horas e trinta minutos, na Sala 14B - Pavilhão Hélio & Peggy Pereira, o exame da tese de doutorado intitulada: **"Diversidade, especificidade e distribuição geográfica de *Trypanosoma spp.* em mamíferos"**, 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: Epidemiologia de Doenças Infecciosas e Parasitárias e Hospedeiros. A banca examinadora foi constituída pelos Professores: Drª. Constança Felicia de Paoli de Carvalho Britto - IOC/FIOCRUZ (Presidente), Drª. Marta Maria Geraldes Teixeira - USP/SP, Dr. Reginaldo Peçanha Brazil - IOC/FIOCRUZ e como suplentes: Dr. Cícero Brasileiro de Mello Neto – UFF/RJ e Dr. Otacilio da Cruz Moreira – IOC/FIOCRUZ. Após arguir a candidata e considerando que a mesma demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela APROVAÇÃO da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutora em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 30 de maio de 2019.

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

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Aos meus pais, Edima e Carlos, e meu esposo Leonardo

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“O que sabemos é uma gota; o que ignoramos é um oceano.”

Isaac Newton

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# INSTITUTO OSWALDO CRUZ

**Diversidade, especificidade e distribuição geográfica de *Trypanosoma* spp. em mamíferos**

**RESUMO**

**TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA**

**Marina Silva Rodrigues**

O gênero *Trypanosoma* é um táxon heterogêneo que inclui hematozoários flagelados que são parasitas obrigatórios. Esses tripanosomas infectam invertebrados e todas as classes de vertebrados. Contudo, a extensão da diversidade de espécies e genótipos, espectro de hospedeiros e distribuição geográfica do gênero *Trypanosoma* permanecem ainda parcialmente desconhecidos. Neste estudo tivemos dois objetivos principais: 1) testar o gene mitocondrial COI como código de barras de DNA para identificação e discriminação de espécies e genótipos do gênero *Trypanosoma*; 2) utilizar PCR de DNA obtido de coágulos sanguíneos como método mais sensível e menos seletivo para identificação da diversidade de *Trypanosoma* spp. que infectam mamíferos silvestres de vida livre. O gene COI demonstrou ser um alvo eficiente para a identificação de espécies do gênero *Trypanosoma*, distinguindo *T. cruzi* de *T. cruzi marinkellei*, *T. rangeli* e *T. dionisii.* Além disso, foi possível discriminar cinco das sete DTUs de *T. cruzi.* Com o uso de COI foi possível observar diversidade em *T. c. marinkellei*, *T. rangeli* e intra-DTUs de *T. cruzi*. Paralelamente, o caso de uma criança de 2 anos que veio à óbito em decorrência de doença de Chagas aguda adquirida por via oral nos fez conduzir um estudo sobre as DTUs que infectaram a criança e o cenário ecoepidemiológico deste caso. No tecido cardíaco da criança foram identificadas as DTUs TcI, TcII, TcIII e TcIV de *T. cruzi*, além de *T. dionisii*. O encontro de *T. dionisii*, até aquele momento descrito como restrito a quirópteros, mostrou que o espectro de hospedeiros desta espécie estava sendo subestimado. Além disso, essa infecção mista que incluiu vários genótipos de *T. cruzi*, além de *T. dionisii,* levanta a questão sobre a importância de infecções mistas na patogenicidade da doença de Chagas. A PCR de DNA de coágulos sanguíneos demonstrou ser uma técnica sensível porque foi possível detectar infecção por *T. cruzi*, mesmo em animais com sorologia e hemocultura negativas, além de identificar *Trypanosoma* spp. que são de difícil crescimento em cultura. Infecção por *Trypanosoma* spp. foi detectada em 95/120 (79,2%) amostras. *T. cascavelli*, *T. dionisii* e *T. lainsoni* apresentaram um maior espectro de espécies de hospedeiros e distribuição geográfica. Entretanto, não é possível atribuir qual o papel desempenhado por esses hospedeiros na manutenção e no ciclo de transmissão dessas espécies de tripanosoma. Nossos resultados ampliaram o conhecimento da distribuição geográfica de *T. cruzi* TcII, *T. rangeli* A, *T.* sp. Neobats 2 e 3, *T. janseni* e *T. gennarii*, além de identificarmos duas novas Unidades Taxonômicas Operacionais Moleculares (MOTUs). Nosso estudo confirmou que a diversidade, distribuição geográfica e espectro de hospedeiros de *Trypanosoma* spp. ainda são subestimados.

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**Diversity, specificity and geographic distribution of *Trypanosoma* spp. in mammals**

**ABSTRACT**

**PHD THESIS IN PARASITE BIOLOGY**

**Marina Silva Rodrigues**

The genus *Trypanosoma* is a heterogeneous taxon that includes flagellated hematozoa that are obligatory parasites. These trypanosomes infect invertebrates and all vertebrate classes. However, the extent of species and genotype diversity, host spectrum, and geographic distribution of the *Trypanosoma* genus remain largely unknown. In this study we had two main objectives: 1) to test the mitochondrial gene COI as a DNA barcode for identification and discrimination of species and genotypes of the genus *Trypanosoma*; 2) to use PCR of DNA obtained from blood clots as a more sensitive and less selective method to identify the diversity of *Trypanosoma* spp. which infect free-ranging wild mammals. The COI gene has been shown to be an efficient target for the identification of species of the genus *Trypanosoma*, distinguishing *T. cruzi* from *T. cruzi marinkellei*, *T. rangeli* and *T. dionisii*. In addition, it was possible to discriminate five of the seven *T. cruzi* DTUs. With the use of COI it was possible to observe diversity in *T. c. marinkellei*, *T. rangeli* and intra-DTUs of *T. cruzi*. At the same time, the case of a 2-year-old child who died due to acute Chagas' disease acquired orally led us to conduct a study on the DTUs that infected the child and the ecoepidemiological scenario of this case. In the cardiac tissue of the child, *T. cruzi* DTUs TcI, TcII, TcIII and TcIV, as well as *T. dionisii*, were identified. The encounter of *T. dionisii*, until that moment described as restricted to chiropterans, showed that the host spectrum of this species was being underestimated. In addition, this mixed infection that included several genotypes of *T. cruzi*, in addition to *T. dionisii*, raises the question about the importance of mixed infections in the pathogenicity of Chagas disease. PCR of DNA from blood clots proved to be a sensitive technique because it was possible to detect *T. cruzi* infection, even in animals with negative serology and hemoculture, in addition to identifying *Trypanosoma* spp. which are difficult to grow in culture. *Trypanosoma* spp. was detected in 95/120 (79.2%) samples. *T. cascavelli*, *T. dionisii* and *T. lainsoni* presented a larger spectrum of host species and geographical distribution. However, it is not possible to determine the role played by these hosts in the maintenance and transmission cycle of these trypanosome species. Our results increased the knowledge of the geographical distribution of *T. cruzi* TcII, *T. rangeli* A, *T*. sp. Neobats 2 and 3, *T. janseni* and *T. gennarii*, besides identifying two new Molecular Operational Taxonomic Units (MOTUs). Our study confirmed that the diversity, geographic distribution and host spectrum of *Trypanosoma* spp. are still underestimated.

**ÍNDICE**

# ÍNDICE DE FIGURAS

## LISTA DE SIGLAS E ABREVIATURAS

| | |
|---|---|
| kDNA | DNA do cinetoplasto |
| DTU | Unidade Discreta de Tipagem |
| DNA | Ácido desoxirribonucleico |
| COI | Citocromo c oxidase subunidade 1 |
| COII | Citocromo c oxidase subunidade 2 |
| Cytb | Citocromo b |
| gGAPDH | Gliceraldeído-3-fosfato desidrogenase glicossomal |
| GPI | Glicose 6-fosfato isomerase |
| iBOL | consórcio internacional de código de barras da vida |
| µL | microlitro |
| MOTUs | Unidades Taxonômicas Operacionais Moleculares |
| ND1 | NADH desidrogenase subunidade 1 |
| rDNA | Ácido desoxirribonucleico ribossomal |
| RIFI | reação de imunofluorescência indireta |
| SSU | Subunidade menor |
| PCR | Reação em Cadeia da Polimerase |

# 1 INTRODUÇÃO

## 1.1 O gênero *Trypanosoma*

O gênero *Trypanosoma* Gruby, 1843 (Euglenozoa Cavalier-Smith, 1981; Kinetoplastea Honigberg, 1963; Trypanosomatida Hollande, 1952; Trypanosomatidae Doflein, 1951) inclui hematozoários flagelados que são parasitas obrigatórios. *Trypanosoma* spp. infectam invertebrados e todas as classes de vertebrados: anfíbios, aves, mamíferos, peixes e répteis (Hoare, 1972; Viola et al., 2009; Lemos et al., 2015; Cooper et al., 2017; Spodareva et al., 2018).

As espécies do gênero *Trypanosoma* diferem consideravelmente em relação ao espectro de seus hospedeiros, vetores e a capacidade de colonizar tecidos. O gênero *Trypanosoma* inclui parasitas descritos como especialistas, como é o caso de *Trypanosoma caninum* até o momento considerado restrito a cães (Barros et al., 2015; Oliveira et al., 2015), e espécies consideradas generalistas, como *Trypanosoma cruzi* que infecta quase todos os tecidos de centenas de espécies de mamíferos (Hoare, 1972).

As estratégias de dispersão de *Trypanosoma* spp. através da eliminação das formas infectivas pelas fezes ou pela saliva do vetor fez com que Hoare (1972) propusesse a divisão das espécies em dois grandes grupos: seção Stercoraria e seção Salivaria (Figura 1). A observação de formas infectivas nas fezes e na saliva e a morfologia semelhante a *T. lewisi* fez com que *T. rangeli* fosse primeiramente classificado no subgênero *Herpetosoma*, seção Stercoraria, como uma espécie atípica (Hoare, 1972). Añez em 1982 propôs a mudança de *T. rangeli* para a seção Salivaria e a criação do subgênero *Tejeraia*. Recentemente, Molinari & Moreno (2018) recomendaram a substituição do homônimo júnior *Tejeraia* por *Aneza* como nome válido. A mudança de seção e criação de um novo subgênero aconteceu porque *T. rangeli* apresenta no tubo digestivo do vetor formas tripomastigotas que, no entanto, não são infectivas. De fato *T. rangeli* multiplica-se no intestino de triatomíneos (gênero *Rhodnius*), completam seu desenvolvimento nas glândulas salivares e a transmissão acontece exclusivamente pela picada do inseto (Maia da Silva et al., 2009). Atualmente se sabe que *T. rangeli*, incluído no clado *T. cruzi*, apresenta maior proximidade filogenética com os tripanosomas do velho mundo do que com *Schizotrypanum* (Espinosa-Álvarez et al., 2018). Até o momento não há

evidências inequívocas de multiplicação de *T. rangeli* no hospedeiro vertebrado (Guhl & Vallejo, 2003; de Sousa et al., 2008; Ferreira et al., 2015) e formas intracelulares ainda não foram confirmadas (Espinosa-Álvarez et al., 2018).

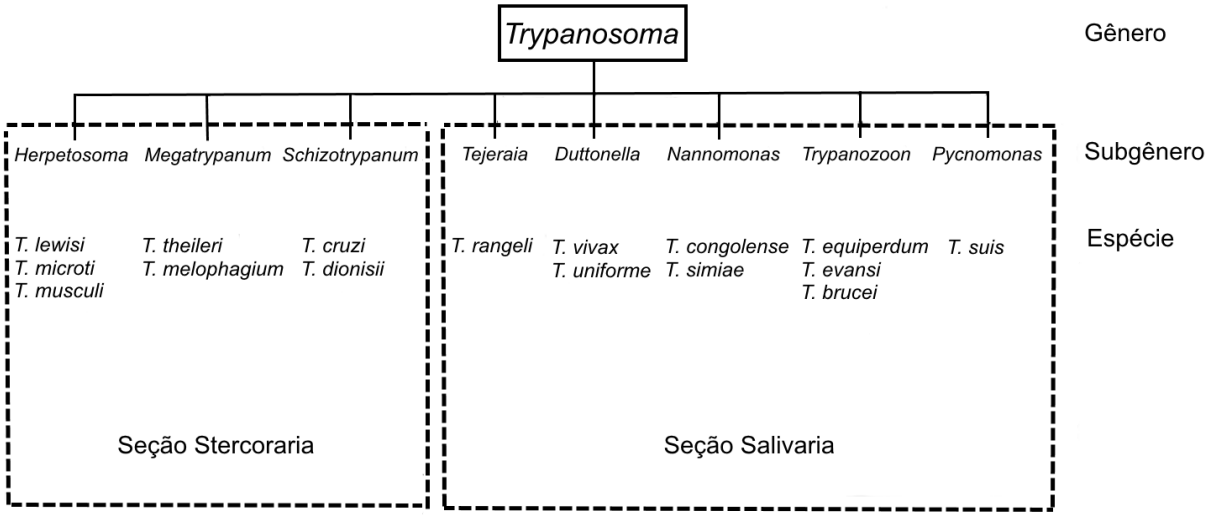


**Figura 1:** Espécies do gênero *Trypanosoma*: seções Stercoraria e Salivaria.
Fonte: adaptado de World Health Organization (WHO) *technical report*, 1986.

A seção Stercoraria se caracteriza por incluir espécies cujo desenvolvimento é finalizado no trato digestivo posterior do inseto e a transmissão é contaminativa, ou seja, as formas metacíclicas, que são as infectivas, são diferenciadas na ampola retal e eliminadas nas fezes do inseto vetor.

A seção Salivaria engloba espécies cujo desenvolvimento é finalizado no trato anterior do tubo digestivo do inseto vetor e a transmissão é inoculativa, ou seja, as formas metacíclicas são passadas ao hospedeiro mamífero durante o repasto sanguíneo do inseto vetor. O desenvolvimento no tubo digestivo e nas glândulas salivares do inseto varia de acordo com as espécies de tripanosoma (Hoare, 1972; Claes et al., 2005; Desquesnes et al., 2013; Ooi et al., 2016; Ponte-Sucre, 2016).

## 1.2 Filogenia do gênero *Trypanosoma*

O gênero *Trypanosoma* é monofilético, ou seja, deriva de um ancestral comum a todas as espécies (Leonard et al., 2011). Atualmente, a separação deste gênero tem sido feita em pequenos grupos denominados clados, que reúnem espécies ou unidades taxonômicas que compartilham o mesmo ancestral (Hamilton & Stevens, 2017). Na proposta de filogenia mais recente obtida com os genes 18S

(SSU rDNA) e gGAPDH, o gênero *Trypanosoma* pode ser dividido em 11 clados principais (Acosta et al., 2013; Botero et al., 2013; Ferreira et al., 2017; Hamilton & Stevens, 2017; Ortiz et al., 2018) (Figura 2).

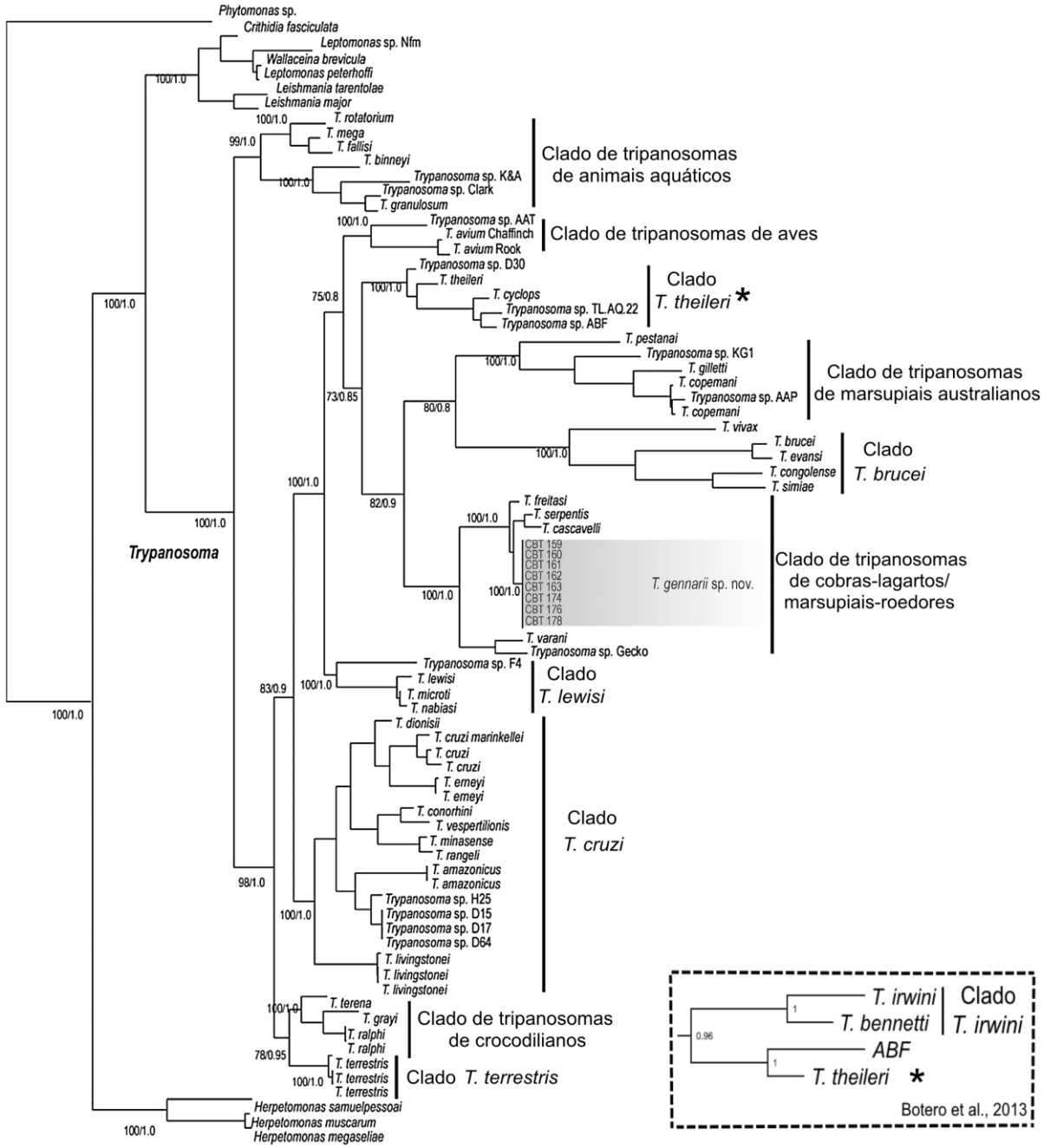


**Figura 2:** Clados de espécies do gênero *Trypanosoma*.
Fonte: adaptado de Botero et al., 2013 e Ferreira et al., 2017.

CLADO *T. CRUZI*: reúne tripanosomas de mamíferos do velho e do novo mundo. Triatomíneos e cimicídios são reconhecidos como vetores de algumas

espécies deste clado. Entretanto, informações do ciclo de transmissão e vetores da maior parte das espécies deste clado permanecem desconhecidas.

CLADO DE TRIPANOSOMAS DE COBRAS-LAGARTOS/MARSUPIAIS-ROEDORES: classicamente considerado como restrito a répteis. Recentemente foram posicionados neste clado tripanosomas identificados em mamíferos, a saber, *Trypanosoma freitasi* e *Trypanosoma lainsoni* de marsupiais e roedores, respectivamente (Ortiz et al., 2018). Pouco se sabe sobre o ciclo de transmissão desses tripanosomas. Os achados de flebotomíneos infectados por tripanosoma de lagarto (*T. varani*) e por um tripanosoma filogeneticamente próximo aos tripanosomas identificados em cobras (*Trypanosoma* sp. 910) sugerem que flebotomíneos sejam possíveis vetores de tripanosomas desse clado (Minter-Goedbloed et al., 1993; Viola et al., 2008).

CLADO *T. BRUCEI*: inclui espécies que causam doenças severas em humanos e animais e que são transmitidos pela via inoculativa. Os tripanosomas deste clado são transmitidos por moscas dos gêneros *Glossina*, *Tabanus* ou *Stomoxys*. Alguns tripanosomas deste clado causam a doença conhecida como Nagana, amplamente distribuída pelo cinturão tsé-tsé. Essa doença acomete principalmente o gado bovino causando perdas econômicas na produção de carne, leite e na agricultura, onde esses animais são utilizados na tração rural. O gado bovino da África Ocidental e animais silvestres são aparentemente resistentes devido a mecanismos ainda não conhecidos (Connor, 1994; Abebe et al., 2017).

CLADO DE TRIPANOSOMAS DE ANIMAIS AQUÁTICOS: inclui tripanosomas descritos em animais aquáticos e semiaquáticos: peixes, anfíbios, répteis e no mamífero ornitorrinco. Contudo, este clado também inclui um tripanosoma identificado em camaleão (*T. thereziene*), um exemplo de mudança evolutiva de hospedeiro aquático para terrestre (Stevens et al., 2001). Sanguessugas são vetores de tripanosomas deste clado.

CLADO *T. THEILERI*: compreende tripanosomas que infectam gado (bovino e bubalino) e marsupiais. Recentemente *T. theileri* foi descrito no morcego hematófago *Desmodus rotundus* (Ramírez et al., 2014). Bois e búfalos que vivem em simpatria são infectados por diferentes genótipos de *T. theileri*, sendo um exemplo de especificidade parasitária (Garcia et al., 2011). Tabanídeos e carrapatos são considerados vetores desses tripanosomas.

CLADO *T. LEWISI*: compreende tripanosomas identificados em diversas espécies de roedores, em lagomorfos e primatas (Maia da Silva et al, 2010; Ortiz et

al., 2018). *T. lewisi*, a espécie mais conhecida deste clado, está amplamente dispersa no velho e no novo mundo (Ortiz et al., 2018). Além de infectar mais de 100 espécies de roedores, *T. lewisi* foi identificado em humanos (Desquesnes et al., 2016; Ortiz et al., 2018). Pulgas são vetores confirmados de alguns tripanosomas deste clado, o que poderia justificar a transmissão de *T. lewisi* de roedores para humanos.

CLADO DE TRIPANOSOMAS DE AVES: inclui tripanosomas descritos em diversas espécies de aves. Contudo, *T. avium*, *Trypanosoma* sp. AAT e outros genótipos deste clado foram identificados em marsupiais na Austrália (Averis et al., 2009; Cooper et al., 2018), sugerindo que tripanosomas desse clado podem apresentar um caráter mais generalista com relação aos seus hospedeiros. Mosquitos e insetos da família Simulidae são considerados vetores desses tripanosomas.

CLADO DE TRIPANOSOMAS DE MARSUPIAIS AUSTRALIANOS: leva este nome por, inicialmente, incluir apenas tripanosomas isolados de marsupiais da Austrália. Atualmente engloba tripanosomas identificados em texugo (*Trypanosoma pestanai*, no Reino Unido) e cão (*Trypanosoma caninum*, no Brasil) (Lizundia et al., 2011; Barros et al., 2015). O genótipo G1 de *T. copemani* é encontrado somente no sangue enquanto o genótipo G2 é capaz de invadir células de marsupiais e é proposto como responsável pelo declínio de uma espécie de marsupial ameaçada (Botero et al., 2016; Cooper et al., 2018). Carrapatos e pulgas são possíveis vetores desses tripanosomas (Lizundia et al., 2011; Thompson & Thompson, 2015).

CLADO *T. IRWINI*: composto por tripanosomas identificados em mamíferos e aves, indicando que esses tripanosomas infectam hospedeiros filogeneticamente distantes. Carrapatos são possíveis vetores (Thompson & Thompson, 2015).

CLADO DE TRIPANOSOMAS DE CROCODILIANOS: inclui tripanosomas de crocodilianos da África e América do Sul. Moscas tsé-tsé são vetores de alguns tripanosomas deste clado Hamilton & Stevens, 2017.

CLADO *T. TERRESTRIS*: até o momento composto somente pela espécie *Trypanosoma terrestris* descrita em antas (*Tapirus terrestris*). Em infecção experimental *T. terrestris* não infectou camundongos BALB/c, o que para os autores é uma sugestão de especificidade em relação ao hospedeiro (Acosta et al., 2013). O vetor deste tripanosoma permanece desconhecido.

Inicialmente a separação de tripanosomas em clados correspondia também a um determinado grupo de hospedeiros (espécie, ordem). Contudo, com o avanço no

conhecimento da diversidade de *Trypanosoma* spp. e de seus hospedeiros observamos que poucos clados permanecem restritos.

### 1.3 *Trypanosoma cruzi*

*T. cruzi*, agente etiológico da doença de Chagas ou Tripanosomíase Americana (Carlos Chagas,1909), é uma das espécies mais estudadas do gênero *Trypanosoma*. Os ciclos de vida e de transmissão de *T. cruzi* são dos mais conhecidos (Figura 3). Contudo, algumas questões permanecem não respondidas mesmo após cem anos de sua descoberta.

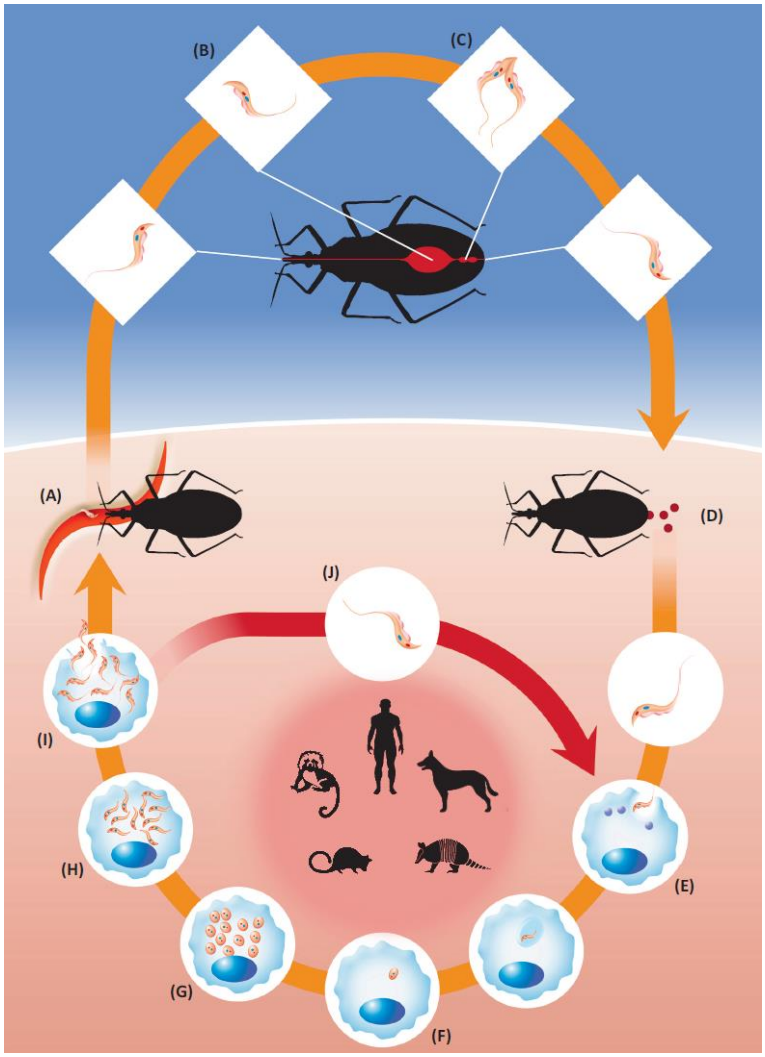


**Figura 3:** Ciclo de vida de *T. cruzi*. A partir de um hospedeiro infectado, (A) o triatomíneo (Hemiptera: Reduviidae) realiza o repasto sanguíneo ingerindo tripomastigotas no sangue. (B) Os parasitas se diferenciam em epimastigotas e entram no intestino médio do triatomíneo, (C) onde se replicam por fissão binária. (D) No intestino posterior, os parasitas se diferenciam nas formas infectantes

tripomastigotas metacíclicas, que são depositadas nas fezes do triatomíneo no local da picada. (E) Os tripomastigotas metacíclicos entram na corrente sanguínea do hospedeiro através do local da picada do inseto ou por mucosas ou abrasão na pele infectando as células hospedeiras e (F) diferenciam-se em amastigotas, (G) replicam-se por fissão binária, (H) diferenciam-se novamente em tripomastigotas. (I) Quando as células rompem, tripomastigotas são eliminados na corrente sanguínea e (J) infectam novas células hospedeiras.
Fonte: adaptado de Perez et al., 2014.

*T. cruzi* é um organismo diploide com genoma distribuído em pares de cromossomos homólogos (Zingales, 2018). Esse parasita apresenta linhagens genéticas bem definidas com uma estrutura populacional predominantemente clonal (Tibayrenc & Ayala, 2015), com raros eventos de trocas genéticas e hibridização (Tomasini & Diosque, 2015; Zingales, 2018). Contudo, heterogeneidade em *T. cruzi* é relatada desde Carlos Chagas (1909) que observou diferentes formas do parasita.

Técnicas biológicas, bioquímicas e moleculares têm sido utilizadas na identificação e discriminação de *T. cruzi*, suas formas e subpopulações o que levou a diferentes designações (biodemas, zimodemas, linhagens, DTUs, clados mitocondriais (mtTcI, mtTcII e mtTcIII) (Andrade, 1974 apud Andrade & Magalhães, 1997; Miles et al., 1978 apud Miles et al., 1981; Souto et al., 1996; Tibayrenc, 1998; Barnabé et al., 2016). Atualmente permanece a designação DTUs (do inglês *discrete typing units*) ou unidades discretas de tipagem. No táxon são reconhecidas seis DTUs e um genótipo nomeado Tcbat que, provavelmente, será classificado como sétima DTU (Marcili et al., 2009; Zingales et al., 2012). Contudo, ainda não há um consenso sobre qual é a metodologia mais sensível e adequada para identificação dessas DTUs (Brenière et al., 2016).

Correlações da forma ou linhagem de *T. cruzi* com a manifestação clínica da doença de Chagas, hospedeiros e distribuição geográfica são propostas desde a descoberta do parasita (Zingales et al., 2012). A DTU TcI, por exemplo, foi durante muitos anos correlacionada com didelfídeos (O'Connor et al., 2007) e TcIII associada com tatus (Llewellyn et al., 2009; Acosta et al., 2017). Até bem pouco tempo pensava-se que TcII estava restrita às regiões abaixo da bacia Amazônica (Zingales et al., 2012). Contudo, esta DTU foi descrita em diferentes estados brasileiros da região Amazônica, Colômbia, Suriname, México e Estados Unidos

(Ibáñez-Cervantes et al., 2013; Lima et al., 2014; Ramírez et al., 2014; Herrera et al., 2015; Lima et al., 2015a). Associação de *T. cruzi* com manifestação clínica, espécie hospedeira ou bioma nunca foi inequivocamente comprovada (del Puerto et al., 2010; Jansen et al., 2018; Roman et al., 2018).

*T. cruzi* é transmitido através das fezes de dezenas de espécies de triatomíneos (Hemiptera: Reduviidae) que entram em contato com mucosas ou abrasões na pele (via contaminativa), por via oral, transfusão de sangue, transplante de órgãos e por via congênita. Atualmente a via oral é a via com maior número de casos, principalmente na região Amazônica (Coura, 2015). *T. cruzi* infecta quase todos os tipos de células de mais de 100 espécies de mamíferos. O parasita está distribuído do sul dos Estados Unidos ao sul da América Latina (Brenière et al., 2016). No Brasil, é encontrado em todos os biomas (Jansen et al., 2018).

A Tripanosomíase Americana é, primariamente, uma enzootia silvestre, portanto a via oral é, provavelmente, a mais comum e importante no ciclo silvestre por predação de mamíferos e triatomíneos infectados (Coura, 2015). Marsupiais e morcegos são reconhecidos como antigos hospedeiros de *T. cruzi* e outros tripanosomatídeos (Stevens et al., 1998; Briones et al., 1999; Hamilton et al., 2012) e descritos como “bioacumuladores” de parasitas através da predação (Cleaveland et al., 2006; Jansen et al., 2018).

## 1.4 Ordem Didelphimorphia e infecções por *Trypanosoma* spp.

A origem dos marsupiais é controversa, uma das hipóteses indica que a divergência entre mamíferos metatérios (marsupiais) e eutérios (placentários) ocorreu durante o Cretáceo no “supercontinente norte” (Laurasia) que englobava América do Norte, Europa e Ásia, com achados fósseis de marsupiais na América do Norte e na Ásia que datam entre 110 e 125 milhões de anos (Luo et al., 2003; Krause & Krause, 2006; Svartman, 2009; Williamson et al., 2014; Bennett et al., 2018). Outros autores sugerem a origem no “supercontinente sul” (Gondwana) na atual América do Sul (Reig, 1961 apud Jansen, 2002; Nilsson et al., 2010). Atualmente, marsupiais são encontrados somente nas Américas e na Austrália. Contudo, indivíduos da ordem Didelphimorphia são encontrados somente no continente americano.

Dentre os marsupiais, a ordem Didelphimorphia é a mais rica em número de espécies e está presente nas três Américas (Svartman, 2009). Esta ordem é

composta por 19 gêneros e 100 espécies (Rossi & Bianconi, 2011). No Brasil foram descritos 16 gêneros e 55 espécies (Rossi & Bianconi, 2011; Loyola et al., 2012).

Marsupiais do gênero *Didelphis* spp. (conhecido popularmente como gambá, saruê, cassaco) são considerados um sucesso evolutivo por terem sofrido poucas mudanças anatômicas ao longo de sua história (Austad, 1988; Krause & Krause, 2006). A ampla distribuição geográfica do gênero *Didelphis* é devido a sua adaptabilidade ao uso de abrigos utilizados previamente por outros animais, alimentação generalista, dieta com característica sazonal que modifica de acordo com a disponibilidade no ambiente e uso de diferentes estratos (arbóreo e terrestre).

*Didelphis* spp. e triatomíneos ocupam, frequentemente, o mesmo habitat, como palmeiras, ocos de árvores e tocas abandonadas de outros animais (Rossi & Bianconi, 2011). Por utilizarem todos os estratos florestais, serem nômades e onívoros; didelfídeos estão expostos a todos os ciclos de transmissão de *T. cruzi* e outros tripanosomas no ambiente natural e provavelmente se infectam com frequência por via oral. De fato, esses marsupiais são conhecidos reservatórios de *T. cruzi* (Deane et al., 1984; Jansen et al., 2018). Anteriormente, a origem do clado *T. cruzi* foi associada à fauna marsupial com a hipótese de que tripanosomas que infectavam os ancestrais dos marsupiais provavelmente se dispersaram com seus hospedeiros pelo “supercontinente sul” (Stevens et al., 1998; Stevens et al., 1999; Lopes et al., 2018).

Didelfídeos são também hospedeiros de numerosas outras espécies de *Trypanosoma*: *T. rangeli, T. freitasi, T. gennarii*, *T. janseni, T. dionisii* e *T. cascavelli* (Rêgo et al., 1957 apud Rocha e Silva et al., 1976; Maia da Silva et al., 2007; Dario et al., 2017a; Ferreira et al., 2017; Lopes et al., 2018). Destas espécies, *T. freitasi, T. gennarii* e *T. janseni* foram descritas somente em Didelphimorphia. *T. dionisii* e *T. cascavelli* eram associados, respectivamente, a morcegos e répteis, mostrando que o gênero *Trypanosoma* é mais generalista em relação aos seus hospedeiros do que suposto até o presente.

## 1.5 Ordem Chiroptera e infecções por *Trypanosoma* spp.

A origem dos morcegos permanece desconhecida, com achados fósseis na América do Norte, Europa, África e Austrália (Gunnell & Simmons, 2005). A datação mais antiga tem aproximadamente 50 milhões de anos (início do Eoceno) (Gunnell &

Simmons, 2005; Simmons et al., 2008). Os quirópteros são encontrados em todos os ambientes, exceto Antártida (Gunnell & Simmons, 2005).

Dentre os mamíferos, é na ordem Chiroptera que se encontra a segunda maior riqueza de espécies incluindo mais de 175 gêneros e 1300 espécies (Moratelli & Calisher, 2015). No Brasil foram identificados 68 gêneros e 178 espécies (Nogueira et al., 2014).

Morcegos são os únicos mamíferos capazes de voar, o que lhes permite longos deslocamentos. Morcegos são longevos e, comparativamente, a maioria vive mais do que qualquer outro mamífero placentário do mesmo tamanho (Wilkinson & South, 2002; Podlutsky et al., 2005; Austad, 2010; Healy et al., 2014). Os morcegos podem utilizar abrigos naturais (cavernas, folhas de grande porte, oco de árvore) e artificiais (prédios abandonados, porões, sótãos, pontes). Algumas espécies formam colônias compostas por dezenas, centenas e, em alguns casos, milhares de indivíduos (Moratelli & Calisher, 2015).

Chiroptera inclui representantes insetívoros, frugívoros, nectarívoros, piscívoros, carnívoros (alimentam-se de pequenos roedores, sapos, aves), hematófagos e também as espécies onívoras (Moratelli & Calisher, 2015). Contudo, a insetivoria é conhecida como a dieta ancestral dos morcegos (Carrilo-Araujo et al., 2015) e, com exceção dos hematófagos, os quirópteros mantem esse hábito alimentar até o presente, o que significa a possibilidade de adquirir infecções por via oral na ingestão de triatomíneos (Thomas et al., 2007) ou outros artrópodes infectados.

Na última década foram descritas novas espécies e unidades taxonômicas de tripanosomas infectando exclusivamente morcegos. Esses parasitas são, em sua maioria, morfologicamente semelhantes e filogeneticamente próximos e passaram a constituir o clado *T. cruzi* (Lima et al., 2012; Lima et al., 2013; Cottontail et al., 2014; Lima et al., 2015b; Barbosa et al., 2016). A presença de tripanosomas do clado *T. cruzi* na África e América do Sul, associado ao fato da maioria dessas espécies serem aparentemente restritas a quirópteros apoiam a hipótese “bat-seeding” que sugere os morcegos como hospedeiros ancestrais de tripanosomas do clado *T. cruzi* e que esses parasitas se dispersaram juntamente com os morcegos entre os continentes (Hamilton et al., 2012). O compartilhamento de abrigos entre morcegos, insetos hematófagos e mamíferos não-voadores possivelmente possibilitou a adaptação de espécies como *T. cruzi* e *T. rangeli*, que passaram a infectar outros mamíferos (Lima et al., 2013).

As hipóteses que indicam marsupiais ou morcegos como hospedeiros ancestrais de tripanosomas do clado *T. cruzi* não são mutuamente excludentes. A proposta mais recente sugere que um ancestral comum dos tripanosomas passou por distintos processos de diversificação nessas duas ordens de mamíferos dando origem a atual diversidade do clado *T. cruzi* (Lopes et al., 2018).

## 1.6 Identificação de *Trypanosoma* spp. por "código de barras de DNA"

Nas últimas duas décadas o método de identificação micro-genômica de espécies "código de barras de DNA" ou *DNA barcoding* começou a ser difundido por Paul Hebert e colaboradores (2003a). A proposta do código de barras de DNA é utilizar o mesmo marcador para identificar todos os taxa e torná-los comparáveis. O método utiliza sequências curtas de DNA de regiões específicas do genoma do espécimen em estudo. A sequência adquirida é comparada às sequências de indivíduos cuja identidade já está bem estabelecida (Hajibabaei et al., 2007). A região de escolha como código de barras deve ser variável o suficiente para captar a diversidade e variabilidade interespecífica e, de preferência, intraespecífica (Hutchinson & Stevens, 2018).

O gene de escolha para essa iniciativa foi o gene mitocondrial citocromo c oxidase subunidade 1 (COI) para identificação de espécies animais (Hebert et al., 2003a; Hutchinson & Stevens, 2018). O COI é um gene que codifica uma proteína homônima que desempenha papel chave na cadeia de transporte de elétrons (Strüder-Kypke & Lynn, 2010). O COI foi proposto pela ausência de íntrons, a rara frequência de *indels*, a ausência de amplificação de pseudogenes em estudos com *primers* degenerados e o relato de alta incidência de substituição nucleotídica, quando comparado a outros alvos mitocondriais, possibilitando a discriminação de espécies próximas (Hebert et al., 2003a; Hebert et al., 2003b).

COI tem sido utilizado como padrão de identificação da maior parte dos animais e mostrou ser aplicável em estudos com vários taxa de organismos multicelulares (Frézal & Leblos, 2008). O uso de COI para identificar protozoários e outros organismos unicelulares ainda está em seu estágio inicial, mas tem sido demonstrado como promissor para dinoflagelados, amebas, *Paramecium* sp. (Stern et al., 2010; Zhao et al., 2013; Chakraborty et al., 2014). Alguns autores defendem que a evolução do gene COI é rápida o suficiente para permitir a discriminação entre espécies próximas e para averiguar a diversidade intraespecífica (Hebert et al.,

2003a; Hebert et al., 2003b). Até o momento, COI apresenta um número limitado de sequências-referência para o gênero *Trypanosoma* depositadas em bancos de dados públicos como o GenBank. Além disso, análises conjuntas de marcadores nucleares e mitocondriais podem ser necessárias para distinguir todas as DTUs de *T. cruzi* e outras espécies (Blaxter, 2016). Adicionalmente, ainda não existem estudos utilizando COI como marcador de discriminação de *Trypanosoma* spp.

Outra região que tem sido proposta como código de barras de DNA é a subunidade menor do DNA ribossomal (SSU rDNA), 16S em procariotos e 18S em eucariotos (Yilmaz et al., 2014; Hutchinson & Stevens, 2018; Tomé et al., 2018; Santacruz et al., 2019). O gene 18S (SSU rDNA) está entre as regiões mais utilizadas para identificação de eucariotos, incluindo tripanosomas, o que implica em maior número de sequências de diferentes espécies depositadas em banco de dados públicos (Xie et al., 2011; D'Avila-Levy et al., 2015; Hutchinson & Stevens, 2018). Quase toda a filogenia atual dos tripanosomas foi construída com sequências de 18S (SSU rDNA) (Hutchinson & Stevens, 2018). Os *primers* mais utilizados atualmente para essa região são aplicáveis à detecção de espécies da família Trypanosomatidae (Noyes et al., 1999; Smith et al., 2008).

## 1.7 Amostras utilizadas na identificação de *Trypanosoma* spp.

Tradicionalmente, a identificação morfológica e molecular de *Trypanosoma* spp. é realizada após amplificação em meios de cultura, que é uma metodologia tida como altamente seletiva e pouco sensível. DNA de *T. cruzi* tem sido extraído a partir de amostras de sangue total, soro, coágulos sanguíneos e outros tecidos de pacientes com doença de Chagas (Fitzwater et al., 2008; Melo et al., 2015; Schijman, 2018).

Dentre esses materiais, o coágulo sanguíneo é considerado uma fonte negligenciada de DNA de parasitas, geralmente descartada após a separação do soro. Fitzwater et al. (2008) hipotetizaram que com a centrifugação para separação do soro, as formas tripomastigotas de *T. cruzi* ficariam presas na rede de fribrina e, portanto, seriam uma fonte rica de DNA. Coágulos foram descritos como adequados para serem armazenados por longos períodos, além de requererem baixos volumes (100 - 1000 µL) para extração de DNA (Fitzwater et al., 2008; Lundblom et al., 2011; Bank et al., 2013; de Abreu et al., 2018). Além disso, coágulos mostraram ser mais eficientes quando comparados ao sangue total (Fitzwater et al., 2008) e já foram

utilizados com sucesso no diagnóstico de infecções por *T. cruzi*, *Leishmania* spp., *Plasmodium* spp. e *Aspergillus* spp. (Fitzwater et al., 2008; McCulloch, et al., 2009; Lundblom et al., 2011; Costa et al., 2015; Curtis-Robles et al., 2017; de Abreu et al., 2018).

# 2 JUSTIFICATIVA

A iniciativa código de barras de DNA tem como objetivo estabelecer um marcador molecular de identificação taxonômica universal. Neste sentido, o gene mitocondrial COI vem sendo adotado, com sucesso, na identificação de espécies pluricelulares quanto em unicelulares, incluindo mamíferos, insetos e protozoários. Em relação ao gênero *Trypanosoma* ainda não havia nenhum estudo com este alvo molecular sendo o nosso, o primeiro. Como apresentado neste trabalho, utilizando o COI foi possível não apenas distinguir espécies do clado *T. cruzi*, como também alguns genótipos.

Um aspecto que ainda merece estudo em relação ao gênero *Trypanosoma* é a extensão da diversidade de espécies e genótipos deste grupo. Um dos obstáculos é representado pela pressão seletiva exercida pelos meios de cultivo, além de parasitemias muito baixas e dificuldade/impossibilidade de cultivo. A caracterização de DNA extraído diretamente de coágulos sanguíneos de mamíferos silvestres de vida livre mostrou ser uma estratégia menos seletiva, complementar ao hemocultivo, que se caracteriza por sua baixa sensibilidade.

# 3 OBJETIVOS

## 3.1 Objetivo Geral

Ampliar o conhecimento da diversidade de *Trypanosoma* spp. de mamíferos silvestres de vida livre utilizando código de barras de DNA e coágulos sanguíneos

## 3.2 Objetivos Específicos

- Testar o gene COI como alvo para identificação e diferenciação de *T. cruzi* e espécies próximas;
- Verificar a eficiência do gene COI na discriminação das principais DTUs de *T. cruzi* (TcI e TcII);
- Identificar, em tecido cardíaco, o(s) genótipo(s) de *T. cruzi* envolvido(s) no caso fatal de doença de Chagas no Espírito Santo;
- Padronizar a obtenção de DNA a partir de coágulo sanguíneo para diagnóstico e caracterização de *Trypanosoma* spp.;
- Identificar a diversidade de *Trypanosoma* spp. em mamíferos silvestres de vida livre a partir de DNA obtido de coágulo sanguíneo utilizando PCR *nested*;
- Verificar a presença de infecções mistas por tripanosomas em animais silvestres de vida livre através da técnica de clonagem gênica.

# 4 RESULTADOS

Os resultados obtidos serão apresentados no formato de artigos:

**Artigo 1**. Cytochrome c oxidase subunit 1 gene as a DNA barcode for discriminating *Trypanosoma cruzi* DTUs and closely related species. Parasites & Vectors 10 (2017) 488: doi: 10.1186/s13071-017-2457-1.

**Artigo 2.** 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 3.** Uncovering *Trypanosoma* spp. diversity of wild mammals by the use of DNA from blood clots. International Journal for Parasitology: Parasites and Wildlife 8 (2019) 171-181: doi: 10.1016/j.ijppaw.2019.02.004.

**Artigo 1. Cytochrome c oxidase subunit 1 gene as a DNA barcode for discriminating *Trypanosoma cruzi* DTUs and closely related species**

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Identificar os organismos vivos e compará-los sempre foi uma preocupação humana. Especificamente em relação ao *T. cruzi*, devido a sua extrema heterogeneidade, diferentes métodos de identificação de suas subpopulações têm sido utilizados desde sua descoberta. Mais recentemente, diferentes ferramentas moleculares têm sido empregadas sem chegar a uma conclusão sobre o agrupamento dos genótipos de *T. cruzi*. Nossa hipótese é que o gene mitocondrial citocromo c oxidase subunidade 1 (COI) pode ser utilizado como uma ferramenta molecular única para identificação de *T. cruzi* e suas principais DTUs. Esse gene é largamente utilizado como código de barras para identificação de diversos organismos eucariotos, inclusive protozoários. Em relação à *Trypanosoma* spp., poucas sequências estão disponíveis para comparação em bancos de dados públicos. Assim sendo, nosso objetivo foi testar o gene COI na identificação de *T. cruzi*, discriminação de suas subpopulações (DTUs) e de espécies filogeneticamente próximas. Utilizamos DNA extraído de cultura proveniente de sangue de mamíferos silvestres e conteúdo intestinal de triatomíneos. A PCR e sequenciamento de fragmento do gene COI demonstrou ser uma ferramenta sensível para a identificação de espécies do gênero *Trypanosoma*, distinguindo *T. cruzi* de *T. cruzi marinkellei*, *T. rangeli* e *T. dionisii.* Além disso, a PCR/sequenciamento desse fragmento permitiu discriminar as DTUs TcI, TcII, TcIII, TcIV e Tcbat de *T. cruzi*, além de observar diversidade intra-DTU (TcI, TcII e TcIII). Foi possível também utilizando esse marcador verificar heterogeneidade em *T. c. marinkellei*, bem como reconhecer as linhagens C e D de *T. rangeli*.

Rodrigues *et al. Parasites & Vectors* (2017) 10:488
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Parasites & Vectors

RESEARCH Open Access

# Cytochrome *c* oxidase subunit 1 gene as a DNA barcode for discriminating *Trypanosoma cruzi* DTUs and closely related species

Marina Silva Rodrigues[1*], Karina Alessandra Morelli[2] and Ana Maria Jansen[1]

## Abstract

**Background:** The DNA barcoding system using the cytochrome *c* oxidase subunit 1 mitochondrial gene (*cox*1 or *COI*) is highly efficient for discriminating vertebrate and invertebrate species. In the present study, we examined the suitability of *cox*1 as a marker for *Trypanosoma cruzi* identification from other closely related species. Additionally, we combined the sequences of *cox*1 and the nuclear gene glucose-6-phosphate isomerase (*GPI*) to evaluate the occurrence of mitochondrial introgression and the presence of hybrid genotypes.

**Methods:** Sixty-two isolates of *Trypanosoma* spp. obtained from five of the six Brazilian biomes (Amazon Forest, Atlantic Forest, Caatinga, Cerrado and Pantanal) were sequenced for *cox*1 and *GPI* gene fragments. Phylogenetic trees were reconstructed using neighbor-joining, maximum likelihood, parsimony and Bayesian inference methods. Molecular species delimitation was evaluated through pairwise intraspecific and interspecific distances, Automatic Barcode Gap Discovery, single-rate Poisson Tree Processes and multi-rate Poisson Tree Processes.

**Results:** Both *cox*1 and *GPI* genes recognized and differentiated *T. cruzi*, *Trypanosoma cruzi marinkellei*, *Trypanosoma dionisii* and *Trypanosoma rangeli*. *Cox*1 discriminated Tcbat, TcI, TcII, TcIII and TcIV. Additionally, TcV and TcVI were identified as a single group. *Cox*1 also demonstrated diversity in the discrete typing units (DTUs) TcI, TcII and TcIII and in *T. c. marinkellei* and *T. rangeli*. *Cox*1 and *GPI* demonstrated TcI and TcII as the most genetically distant branches, and the position of the other *T. cruzi* DTUs differed according to the molecular marker. The tree reconstructed with concatenated *cox*1 and *GPI* sequences confirmed the separation of the subgenus *Trypanosoma* (*Schizotrypanum*) sp. and the *T. cruzi* DTUs TcI, TcII, TcIII and TcIV. The evaluation of single nucleotide polymorphisms (SNPs) was informative for DTU differentiation using both genes. In the *cox*1 analysis, one SNP differentiated heterozygous hybrids from TcIV sequences. In the *GPI* analysis one SNP discriminated Tcbat from TcI, while another SNP distinguished TcI from TcIII.

**Conclusions:** DNA barcoding using the *cox*1 gene is a reliable tool to distinguish *T. cruzi* from *T. c. marinkellei*, *T. dionisii* and *T. rangeli* and identify the main *T. cruzi* genotypes.

**Keywords:** Cytochrome *c* oxidase subunit 1, *Trypanosoma cruzi*, Discrete typing units, Glucose-6-phosphate isomerase, Barcoding, Subgenus *Trypanosoma* (*Schizotrypanum*)

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## Background

How many species are there on Earth? Estimations suggest that approximately 90% of species remain undescribed [1]. The identification and classification of biodiversity is a practice that has always fascinated humankind. The Greek philosopher Aristotle (4th century BC) developed the first classification system, which was used for nearly 2000 years [2]. In the 1700s, Carl Linnaeus [3] developed the concept of binomial nomenclature using Latin, which was the language of educated men at his time. Binomial nomenclature is a standard method for scientists that speak different languages to classify living things to clearly communicate their discoveries. Recently, DNA sequences have been applied in the classification of life forms. However, different methods and DNA regions have been used to compare the same taxonomic groups, frequently leading to conflicting results.

In the search for a simple method to identify and compare species, Hebert et al. [4] proposed DNA barcoding, a new system of species identification using the cytochrome *c* oxidase subunit 1 mitochondrial gene (*cox*1 or *COI*) as a standardized single molecular marker for the classification of animal species. One of the requirements of the DNA barcoding approach is that species identification is associated with a voucher belonging to a curated biological collection, enabling follow up and a strategy for corroborating species identification [5]. Until recently, national barcode networks have been established in 11 countries, including Brazil, which uses the Brazilian Barcode of Life (BrBOL) [6].

In addition to the identification of known and new species, barcoding with the *cox*1 gene is suggested as a standard for cryptic taxa discovery, association of different life stages of the same species and wildlife conservation genetics [5]. *Cox*1 appears to have a better phylogenetic signal than the other mitochondrial genes [7]. Some authors argue that the evolution of the *cox*1 gene is sufficiently rapid to discriminate between closely related species and investigate intraspecific diversity [4, 8]. The *cox*1 region is highly efficient for discriminating vertebrate and invertebrate species [5, 9, 10], but is not suited for plants and some fungal species [4, 11, 12]. The use of *cox*1 for identifying protozoa and other unicellular organisms is still in its initial stage but has been demonstrated as a promising barcode marker for dinoflagellates, *Paramecium* sp., Nemertea [13–15]. Moreover, joint analyses of nuclear and mitochondrial markers may be necessary to distinguish species [1].

Therefore, the small subunit ribosomal DNA (SSU rDNA) has been proposed as a first step for a protist barcode, followed by the use of a second marker specific for each group [6]. To confirm a gene as a barcode marker, it is essential to calculate the "barcoding gap", that is, the gap between the maximum intraspecific and minimum interspecific genetic distances that establish the limits between species [16]. Several molecular targets for trypanosomatid identification have previously been used: internal transcribed spacer (ITS) [17], mini-exon [18], glucose-6-phosphate isomerase (*GPI*) [19–21], cytochrome *b* (*cytb*) [22, 23], multilocus sequencing typing (MLST) [24], the variable regions V7 V8 of SSU rDNA, and glyceraldehyde-3-phosphate dehydrogenase (*gGAPDH*) genes [22, 25, 26]. However, there is no consistency concerning which DNA region to use as a trypanosomatid barcode, and none of these markers were evaluated as a universal marker for biodiversity analysis.

With the emergence of molecular techniques with higher analytical power, an increasing number of new species of *Trypanosoma* have been reported [26, 27] and it is becoming increasingly clear that there is a high diversity of *Trypanosoma* spp. within the subgenus *Schizotrypanum* of *Trypanosoma*. The extreme morphological similarity and phylogenetic proximity of these species, and the use of tools with lower analytical power, resulted in the isolation of numerous species of *T.* (*Schizotrypanum*) from the wild transmission cycle remaining only at the generic level or wrongly diagnosed as *Trypanosoma cruzi*, highlighting the importance of a universal method to characterize *T. cruzi*, its subpopulations and representatives of the subgenus *Schizotrypanum*.

*Trypanosoma cruzi* (Trypanosomatida: Trypanosomatidae) is a successful parasite that is capable of establishing an extracellular cycle in dozens of triatomine species (Hemiptera: Reduvidae) and infecting almost every cellular type from more than 100 mammal species distributed from the south of the USA to the south of Latin America [28, 29]. Although described as one taxon, *T. cruzi* has a remarkable genetic heterogeneity that was already recognized by the very first authors who researched it [30–32]. The *T. cruzi* population structure has been classified as clonal or, at least, primarily clonal [32, 33] and hybridization events have also been observed, suggesting that genetic recombination occurs in this group [33, 34]. Moreover, the extent to which these hybridization events are responsible for the enormous heterogeneity of this parasite remains a controversial issue [19, 35–37]. Several techniques, (biological, biochemical and molecular) applied to define *T. cruzi* subpopulations have led to different designations (Fig. 1) [25, 30, 35, 38–51]. Presently, *T. cruzi* subpopulations are assembled in six discrete typing units (DTUs) - named TcI, TcII, TcIII, TcIV, TcV, TcVI [29] - and a genotype called Tcbat, initially described as being associated with Chiroptera [25], although it was previously isolated from a child [52] and from mummified human tissue [53]. However, Barnabé et al. [51] recently proposed the subdivision of *T. cruzi* into three mitochondrial clades (mtTcI, mtTcII and mtTcIII) based on the analysis of the nucleotide sequences available in the GenBank database, showing that

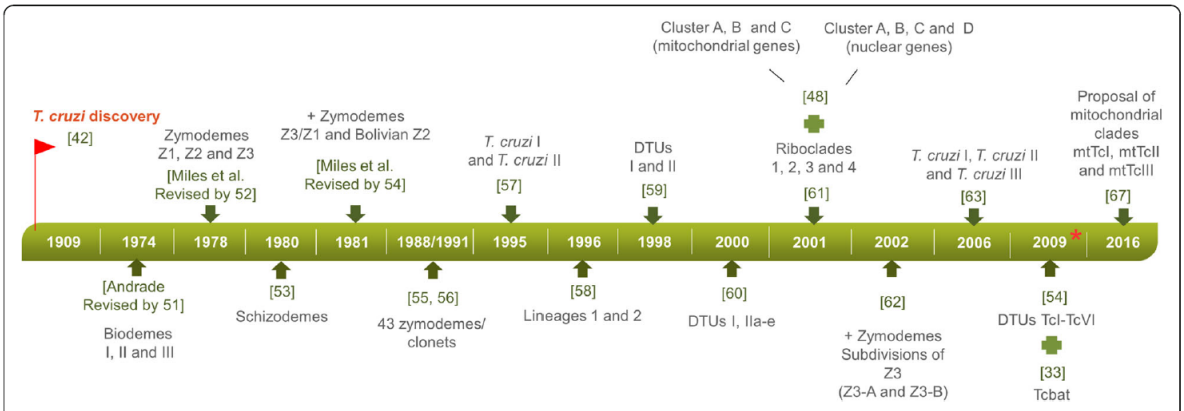


**Fig. 1** Designations of *T. cruzi* subpopulations through time and according to the method employed. The numbers represent dates of publication. The red star indicates current consensus of *T. cruzi* nomenclature and subdivisions

the classification of *T. cruzi* subpopulations remains a debatable issue.

In the present study, we tested *cox*1 as a DNA barcode to identify *T. cruzi* from other closely related species belonging to the subgenus *Schizotrypanum* and to examine the genetic diversity within *T. cruzi* and its DTUs to further understand the ecology of the species of *T.* (*Schizotrypanum*). Additionally, we evaluated *cox*1 as a target to also identify *Trypanosoma* (*Tejeraia*) *rangeli*, as this trypanosome shares the geographical distribution, vectors and mammal hosts of *T. cruzi* and is also included in the *T. cruzi* clade [54]. To evaluate the occurrence of mitochondrial introgression events and the presence of hybrid genotypes we tested a combination of *cox*1 (uniparental inheritance) and the nuclear gene glucose-6-phosphate isomerase (*GPI*) (biparental inheritance). *GPI* is one of the genes sequenced for an extensive number of *T. cruzi* isolates distributed over several geographic regions [51], enabling a comparison of the sequences generated in the present study. Thus, the precise identification of these *Trypanosoma* species is of the utmost importance. In addition, the present study will enhance the amount of nucleotide sequences available for comparison, since the GenBank database still lacks a broader deposit on trypanosomatid sequences for the *cox*1 gene.

## Methods

### Samples

The present study included 62 isolates of *Trypanosoma* spp. obtained from 16 different genera of free-ranging wild mammals and from triatomines of the genera *Rhodnius* and *Triatoma*, distributed in five of the six Brazilian biomes (Amazon Forest, Atlantic Forest, Caatinga, Cerrado and Pantanal) (Table 1 Fig. 2b). Isolates were cryopreserved and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, Fiocruz - COLTRYP (Oswaldo Cruz Foundation, Rio de Janeiro - RJ/Brazil) in previous studies. Details on animal capture and parasite culture methods are described elsewhere [55–57]. After thawing, the cells were sown in axenic culture media (NNN and LIT). When the cultures reached the exponential growth phase, DNA was extracted using the phenol-chloroform method, as described elsewhere [58]. The *T. dionisii* samples used in the present study were molecularly identified using 18S (SSU) and gGAPDH sequences and subsequently deposited in COLTRYP.

Nine *T. cruzi* references were also genotyped and used as DTU standards. Colombiana (COLPROT 004), Dm28c (COLPROT 010), Y (COLPROT 106), 3663 (COLPROT 608), 4167 (COLPROT 607) DNA samples obtained from the Protozoa Collection - COLPROT (FIOCRUZ, Rio de Janeiro, Brazil); Sc43cl1, Bug2148cl1, CLBrener and Tulacl2 DNA samples were a kind gift from Dr. Michael Miles from the London School of Hygiene & Tropical Medicine (London, UK). The *T. rangeli* lineage R1625 DNA sample (COLPROT 002) was obtained from COLPROT (Table 1). In addition, *cox*1 and *GPI* sequences were retrieved from the GenBank database and used as references (Table 1).

### PCR and sequencing

A fragment of the mitochondrial gene *cox*1 was amplified using a set of forward (5′-CCA WAC AAC AAA CAT ATG ATG CTG C-3′) and reverse (5′-TCC HGA TAT GGT WTT KCC YCG-3′) primers. Polymerase chain reaction (PCR) was conducted in a 50 μl final reaction volume containing 2.5 mM $MgCl_2$ (Invitrogen, Carlsbad, California, USA), 0.25 mM of each nucleotide (Thermo Scientific, Waltham, Massachusetts, USA), 0.25 mM of each primer (IDT, Coralville, Iowa, USA), 1.0 U of Platinum Taq DNA polymerase (Invitrogen), and 50 ng of DNA template. The amplification was performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, California, USA) with the following cycle conditions: denaturation at

**Table 1** Molecular identification, geographical and host origin of the COLTRYP isolates and reference stocks under study

| Strain code | Host or Vector | State/Biome | Lineage | Hap (*cox*1)[a] | GenBank accession number | |
|---|---|---|---|---|---|---|
| | | | | | *cox*1 | *GPI* |
| *T. cruzi* | | | | | | |
| TCC 1994 | *Myotis levis* | SP/Brazil | Tcbat | hap9 | KT327226[b] | KT327312[b] |
| COLTRYP 038 | *Rattus rattus* | CE/Caatinga | TcI | hap3 | KU145414 | KT390200 |
| COLTRYP 039 | *Rattus rattus* | CE/Caatinga | TcI | hap3 | KU145415 | KT390201 |
| COLTRYP 048 | *Didelphis albiventris* | CE/Caatinga | TcI | hap3 | KU256221 | KU256227 |
| COLTRYP 087 | *Didelphis albiventris* | CE/Caatinga | TcI | hap3 | KU145426 | KT390212 |
| COLTRYP 128 | *Didelphis albiventris* | CE/Caatinga | TcI | hap3 | KU145433 | KT390219 |
| COLTRYP 018 | *Thylamys macrurus* | MS/Pantanal | TcI | hap1 | KU256219 | KU256225 |
| COLTRYP 084 | *Oecomys* sp. | MS/Pantanal | TcI | hap1 | KU145425 | KT390211 |
| COLTRYP 103 | *Monodelphis domestica* | MS/Pantanal | TcI | hap3 | KU145428 | KT390214 |
| COLTRYP 115 | *Thylamys macrurus* | MS/Pantanal | TcI | hap1 | KU145430 | KT390216 |
| COLTRYP 368 | *Nasua nasua* | MS/Pantanal | TcI | hap3 | KU145441 | KT390227 |
| COLTRYP 468 | *Oecomys mamorae* | MS/Pantanal | TcI | hap1 | KU145443 | KT390229 |
| COLTRYP 053 | *Rhodnius pictipes* | PA/Amazon | TcI | hap2 | KU145418 | KT390204 |
| COLTRYP 055 | *Didelphis marsupialis* | PA/Amazon | TcI | hap2 | KU145419 | KT390205 |
| COLTRYP 126 | *Didelphis marsupialis* | PA/Amazon | TcI | hap2 | KU145432 | KT390218 |
| COLTRYP 220 | *Didelphis marsupialis* | PA/Amazon | TcI | hap5 | KU145435 | KT390221 |
| COLTRYP 339 | *Rhodnius pictipes* | PA/Amazon | TcI | hap2 | KU145438 | KT390224 |
| Epinet 88,115 | *Rhodnius robustus* | PA/Amazon | TcI | hap3 | KU145452 | KT390238 |
| COLTRYP 356 | *Akodon cursor* | RJ/Atlantic Forest | TcI | hap3 | KU145439 | KT390225 |
| Epinet 88,127 | *Rhodnius* sp. | RJ/Atlantic Forest | TcI | hap4 | KU145454 | KT390240 |
| Epinet 88,132 | *Philander frenatus* | RJ/Atlantic Forest | TcI | hap4 | KU145456 | KT390242 |
| Epinet 88,135 | *Philander frenatus* | RJ/Atlantic Forest | TcI | hap4 | KU145457 | KT390243 |
| COLTRYP 003 | *Didelphis aurita* | SC/Atlantic Forest | TcI | hap1 | KU145410 | KT390196 |
| COLTRYP 036 | *Didelphis albiventris* | GO/Cerrado | TcI | hap8 | KU145413 | KT390199 |
| COLTRYP 042 | *Desmodus rotundus* | TO/Cerrado | TcI | hap4 | KU145416 | KT390202 |
| COLTRYP 136 | *Phyllostomus albicola* | TO/Cerrado | TcI | hap4 | KU145434 | KT390220 |
| COLTRYP 224 | *Carollia perspicillata* | TO/Cerrado | TcI | hap4 | KU145436 | KT390222 |
| COLTRYP 305 | *Philander opossum* | TO/Cerrado | TcI | hap6 | KU145437 | KT390223 |
| COLTRYP 362 | *Gracilinanus* sp. | TO/Cerrado | TcI | hap6 | KU145440 | KT390226 |
| Colombiana | *Homo sapiens* | Colombia | TcI | hap7 | KU168553 | KU168558 |
| Dm28c | *Didelphis marsupialis* | Venezuela | TcI | hap4 | KU168554 | KU168559 |
| Silvio | *Homo sapiens* | PA/Amazon | TcI | hap2 | FJ203996[b] | |
| OPS21cl11 | *Homo sapiens* | Venezuela | TcI | | | AY484472[b] |
| COLTRYP 061 | *Leontopithecus chrysomelas* | BA/Atlantic Forest | TcII | hap11 | KU145420 | KT390206 |
| COLTRYP 062 | *Leontopithecus chrysomelas* | BA/Atlantic Forest | TcII | hap11 | KU145421 | KT390207 |
| COLTRYP 063 | *Leontopithecus chrysomelas* | BA/Atlantic Forest | TcII | hap11 | KU145422 | KT390208 |
| COLTRYP 072 | *Leontopithecus chrysomelas* | BA/Atlantic Forest | TcII | hap11 | KU145423 | KT390209 |
| COLTRYP 081 | *Leontopithecus chrysomelas* | BA/Atlantic Forest | TcII | hap11 | KU145424 | KT390210 |
| COLTRYP 099 | *Leontopithecus chrysomelas* | BA/Atlantic Forest | TcII | hap11 | KU145427 | KT390213 |
| COLTRYP 006 | *Thrichomys apereoides* | PI/Caatinga | TcII | hap10 | KU256218 | KU256224 |
| COLTRYP 021 | *Leontopithecus rosalia* | RJ/Atlantic Forest | TcII | hap12 | KU145411 | KT390197 |

**Table 1** Molecular identification, geographical and host origin of the COLTRYP isolates and reference stocks under study *(Continued)*

| Strain code | Host or Vector | State/Biome | Lineage | Hap (*cox*1)[a] | GenBank accession number | |
|---|---|---|---|---|---|---|
| | | | | | *cox*1 | *GPI* |
| COLTRYP 121 | *Leontopithecus rosalia* | RJ/Atlantic Forest | TcII | hap10 | KU145431 | KT390217 |
| Epinet 88,130 | *Philander frenatus* | RJ/Atlantic Forest | TcII | hap11 | KU145455 | KT390241 |
| COLTRYP 043 | *Triatoma tibiamaculata* | SC/Atlantic Forest | TcII | hap10 | KU145417 | KT390203 |
| Epinet 88,121 | *Triatoma tibiamaculata* | SC/Atlantic Forest | TcII | hap10 | KU145453 | KT390239 |
| Y | *Homo sapiens* | SP/Brazil | TcII | hap11 | KU168555 | KU168560 |
| Esmeraldo | *Homo sapiens* | BA/Brazil | TcII | hap11 | DQ343646[b] | |
| Tu18cl2 | *Triatoma infestans* | Bolivia | TcII | | | AY484477[b] |
| COLTRYP 113 | *Monodelphis domestica* | GO/Cerrado | TcIII | hap14 | KU145429 | KT390215 |
| COLTRYP 370 | *Rhodnius pictipes* | PA/Amazon | TcIII | hap15 | KU145442 | KT390228 |
| COLTRYP 029 | *Galictis vittata* | RJ/Atlantic Forest | TcIII | hap13 | KU145412 | KT390198 |
| 3663 | *Panstrongylus geniculatus* | AM/Brazil | TcIII | hap13 | KU168556 | KU168561 |
| M6241 cl6 | *Homo sapiens* | PA/Brazil | TcIII | | | AY484478[b] |
| COLTRYP 041 | *Thrichomys pachyurus* | MS/Pantanal | TcIV | hap16 | KU256220 | KU256226 |
| COLTRYP 471 | *Oecomys mamorae* | MS/Pantanal | TcIV | hap16 | KU145444 | KT390230 |
| COLTRYP 524 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145445 | KT390231 |
| COLTRYP 526 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145446 | KT390232 |
| COLTRYP 527 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145447 | KT390233 |
| COLTRYP 528 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145448 | KT390234 |
| COLTRYP 529 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145449 | KT390235 |
| COLTRYP 531 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145450 | KT390236 |
| COLTRYP 532 | *Triatoma* sp. | MS/Pantanal | TcIV | hap16 | KU145451 | KT390237 |
| 4167 | *Rhodnius brethesi* | AM/Brazil | TcIV | hap16 | KU168557 | KU168562 |
| CANIIIcl1 | *Homo sapiens* | PA/Brazil | TcIV | | | AY484474[b] |
| Sc43cl1 | *Triatoma infestans* | Bolivia | TcV | hap17 | KU686477 | KU686481 |
| Bug2148 | *Triatoma infestans* | RS/Brazil | TcV | hap17 | KU686478 | KU686482 |
| CLBrener | *Triatoma infestans* | SP/Brazil | TcVI | hap17 | KU686479 | KU686483 |
| Tulacl2 | *Homo sapiens* | Chile | TcVI | hap17 | KU686480 | KU686484 |
| CLBrener | *Triatoma infestans* | SP/Brazil | TcVI | hap17 | DQ343645[b] | |
| Bug2148_1 | *Triatoma infestans* | RS/Brazil | TcV | | | HQ452737[b] |
| Bug2148_2 | *Triatoma infestans* | RS/Brazil | TcV | | | HQ452738[b] |
| CLBrener_1 | *Triatoma infestans* | SP/Brazil | TcVI | | | HQ452739[b] |
| CLBrener_2 | *Triatoma infestans* | SP/Brazil | TcVI | | | HQ452740[b] |
| *T. c. marinkellei* | | | | | | |
| COLTRYP 107 | *Phyllostomus discolor* | GO/Cerrado | | hap18 | KU145458 | KT390244 |
| COLTRYP 117 | *Phyllostomus discolor* | GO/Cerrado | | hap18 | KU256222 | KU256228 |
| COLTRYP 143 | *Phyllostomus discolor* | GO/Cerrado | | hap18 | KU256223 | KU256229 |
| COLTRYP 576 | *Phyllostomus hastatus* | AC/Amazon | | hap19 | KX620471 | KX620476 |
| COLTRYP 577 | *Phyllostomus hastatus* | AC/Amazon | | hap19 | KX620472 | KX620477 |
| B7 | *Phyllostomus discolor* | BA/Brazil | | hap18 | KC427240[b] | AY484485[b] |
| TCC 344 | *Carollia perspicillata* | RO/Brazil | | hap19 | KT327227[b] | KT327313[b] |
| *T. dionisii* | | | | | | |
| COLTRYP 596 | *Anoura geoffroyi* | ES/Atlantic Forest | | hap21 | KX274234 | KX274236 |

**Table 1** Molecular identification, geographical and host origin of the COLTRYP isolates and reference stocks under study *(Continued)*

| Strain code | Host or Vector | State/Biome | Lineage | Hap (*cox*1)[a] | GenBank accession number | |
|---|---|---|---|---|---|---|
| | | | | | *cox*1 | *GPI* |
| COLTRYP 598 | *Carollia* sp. | ES/Atlantic Forest | | hap21 | KX274235 | KX274237 |
| COLTRYP 621 | *Anoura geoffroyi* | ES/Atlantic Forest | | hap21 | KX620468 | KX620473 |
| COLTRYP 622 | *Carollia* sp. | ES/Atlantic Forest | | hap21 | KX620469 | KX620474 |
| COLTRYP 623 | *Carollia* sp. | ES/Atlantic Forest | | hap21 | KX620470 | KX620475 |
| *T. rangeli* | | | | | | |
| R1625 | *Homo sapiens* | El Salvador | | hap23 | KU176138 | KU176137 |
| RGB | *Canis familiaris* | Colombia | | | | AY484486[b] |
| SC58 | *Echimys dasythrix* | SC/Brazil | | hap24 | KJ803830[b] | |

[a]Hap (*cox*1): haplotype inferred for *cox*1 in DnaSP v5.10.01
[b]Sequences retrieved from GenBank
*Abbreviations*: Brazilian states: *AC* Acre, *AM* Amazonas, *BA* Bahia, *CE* Ceará, *ES* Espírito Santo, *GO* Goiás, *MS* Mato Grosso do Sul, *PA* Pará, *PI* Piauí, *RJ* Rio de Janeiro, *RO* Rondônia, *RS* Rio Grande do Sul, *SC* Santa Catarina, *SP* São Paulo, *TO* Tocantins

95 °C for 3 min; followed by 35 cycles at 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. Nucleotide sequences were also determined using a fragment of the nuclear gene *GPI*. The primers and cycling conditions are described elsewhere [20]. The PCR products were separated on 1.5% agarose gels and stained with GelRed (Biotium Inc., Fremont, California, USA). The fragments were purified using the Wizard Genomic DNA Purification Kit, according to manufacturer's instructions (Promega, Madison, Wisconsin, USA), and direct sequencing of both strands of DNA was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using an ABI 3730 DNA sequencer available at the RPT01A/FIOCRUZ sequencing facilities.

### Data analysis

The sequences were manually edited using Geneious software version 8.1.6. (Biomatters, Auckland, New Zealand) and aligned using the CLUSTAL X version 2.1 multiple alignment program [59]. All sequences were translated to confirm the absence of premature stop codons. All sequences generated were deposited in the GenBank database (Table 1).

The heterozygous hybrid lineages (TcV and TcVI) amplified for *GPI* were subjected to haplotype reconstruction using the PHASE algorithm implemented in DnaSP v5.10.01 [60].

The neighbor-joining (NJ) method and Kimura 2-parameters (K2P) model were applied for both *cox*1 and *GPI* genes according to the barcode approach [4]. NJ analyses were performed with MEGA version 6 [61]. For each node, bootstrap percentages (BP) were computed after 1000 resamplings.

The maximum likelihood (ML) method was also applied to each topology. The model of nucleotide substitution that best fitted the *cox*1 data was the Hasegawa-Kishino-Yano's model (HKY), with a gamma-distributed rate (Γ). For *GPI*, the best-fit model was the Tamura-Nei model, with a gamma-distributed rate. These models were selected using the Akaike Information Criterion corrected for small samples (AICc) approach implemented in the program jModelTest [62]. ML analyses were performed using PhyML 3.0 [63]. For each node, BP were computed after 1000 resamplings.

Maximum Parsimony (MP) analyses were performed using PAUP* 4.0b10 [64]. For the tree search and bootstrap we used a heuristic search with 100 random sequence addition replicates through tree bisection and reconnection (TBR) branch-swapping algorithm. Bayesian inference (BI) was run in MrBayes v3.2.6 [65] with a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR + Γ + I). The runs converged after 1,000,000 generations, by sampling every 100th generation and discarding the first 25% of the trees as 'burn-in'. *Cox*1 and *GPI* sequences were concatenated in SequenceMatrix 1.8 [66] and submitted to NJ, ML, MP and BI analysis as described above.

The number of haplotypes, nucleotide diversity (π) and haplotype diversity (Hd) were calculated for both genes, except for Tcbat, which had a single sequence available in GenBank. The analyses were run in DnaSP v5.10.01 [60].

Molecular species delimitation was evaluated using distance-based methods and coalescent-based models. Distance-based analyses included the pairwise intraspecific and interspecific distances calculated using MEGA version 6 [61] and the Automatic Barcode Gap Discovery (ABGD) method, which detects a gap in the distribution of pairwise distances and uses this information to partition the sequences into groups of hypothetical species [67]. ABGD analysis was conducted in the web version: Jukes Cantor,

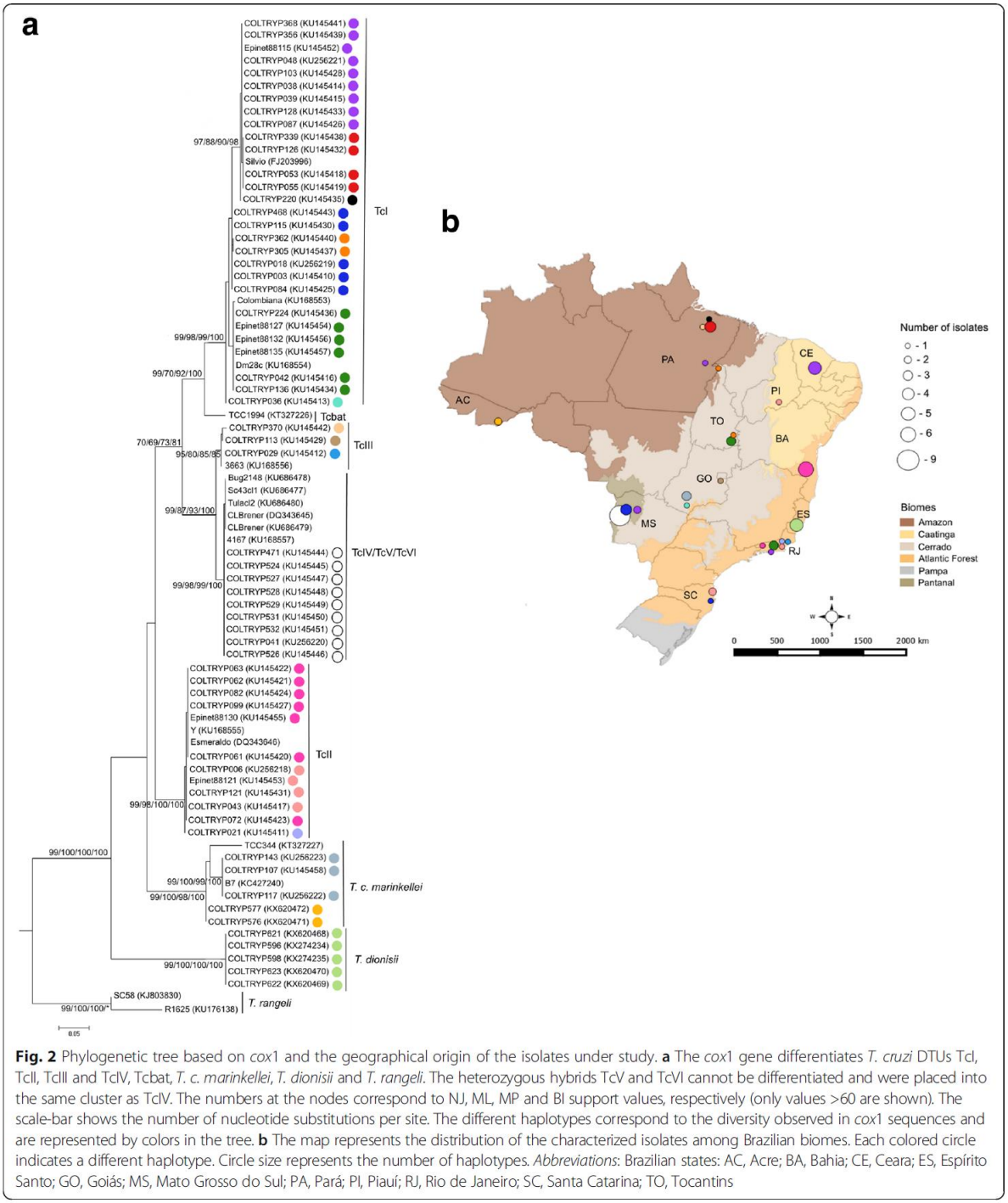


**Fig. 2** Phylogenetic tree based on *cox*1 and the geographical origin of the isolates under study. **a** The *cox*1 gene differentiates *T. cruzi* DTUs TcI, TcII, TcIII and TcIV, Tcbat, *T. c. marinkellei*, *T. dionisii* and *T. rangeli*. The heterozygous hybrids TcV and TcVI cannot be differentiated and were placed into the same cluster as TcIV. The numbers at the nodes correspond to NJ, ML, MP and BI support values, respectively (only values >60 are shown). The scale-bar shows the number of nucleotide substitutions per site. The different haplotypes correspond to the diversity observed in *cox*1 sequences and are represented by colors in the tree. **b** The map represents the distribution of the characterized isolates among Brazilian biomes. Each colored circle indicates a different haplotype. Circle size represents the number of haplotypes. *Abbreviations*: Brazilian states: AC, Acre; BA, Bahia; CE, Ceara; ES, Espírito Santo; GO, Goiás; MS, Mato Grosso do Sul; PA, Pará; PI, Piauí; RJ, Rio de Janeiro; SC, Santa Catarina; TO, Tocantins

K2P and p distances were calculated, and the remaining parameters were used as default [67]. Coalescent-based analysis included the single-rate Poisson Tree Processes (PTP) model [68], which considers that every species evolved at the same rate, and the multi-rate Poisson Tree Processes (mPTP) [69], which assumes a different evolution rate for each species. PTP and mPTP analyses were conducted using the web version of this software [69].

## Results

*Cox*1 and *GPI* gene fragments were successfully amplified for a panel of 62 *Trypanosoma* spp. isolates and ten reference strains (Table 1). All sequences were translated to amino acids and compared to *cox*1 and *GPI* proteins. No indels (insertions/deletions) or stop codons were detected. No pseudo genes or contaminants were observed.

### Phylogenetic tree reconstruction using *cox*1 as barcode

The clusters observed in the *cox*1 trees were the same for all methods tested, indicating that these groups are robust and do not depend on the evolutionary methods selected (Fig. 2a). *Cox*1 discriminated species belonging to the subgenus *Schizotrypanum* and *T. cruzi* DTUs. TcI and Tcbat were closely related but clearly constitute two different DTUs with a statistical support of 99, 70, 92 and 100 in NJ, ML, MP and BI analysis, respectively (Fig. 2a). TcIII and TcIV sequences were separated into two different clusters with bootstrap values of 99, 87, 93 and 100 in NJ, ML, MP and BI analysis, respectively. For the heterozygous hybrid lineages, TcV and TcVI formed an indistinguishable group in the same cluster as TcIV.

### Phylogenetic tree reconstruction using *GPI*

Both *GPI* and *cox*1 helped recognize *Schizotrypanum* species, but not *T. cruzi* DTUs. Depending on the method used for the phylogenetic tree reconstruction there was a slightly different topology. Tcbat and TcI clustered together and could not be differentiated in the tree (Fig. 3). In NJ analysis TcIII constituted a separate DTU close to TcI (Fig. 3a). However, with ML, MP and BI methods TcI, Tcbat and TcIII clustered together (bootstrap of 80, 77 and 82, respectively) (Fig. 3b). *GPI* sequences generated for TcV and TcVI presented electropherograms with double peaks (i.e. with two bases at the same position) and were submitted to haplotype reconstruction prior to use in the final alignments and tree reconstructions. This analysis resulted in two sequences for each hybrid sample corresponding to alleles. One allele was closer to TcII, and the other allele was closer to TcIII (Fig. 3).

The geographical distribution of the trypanosomatid isolates under study is represented in Fig. 2b. Both *cox*1 and *GPI* sequences demonstrated the differences between *T. cruzi*, *T. c. marinkellei*, *T. dionisii* and *T. rangeli* and also, to some degree, *T. cruzi* lineages. *Cox*1 and *GPI* phylogenies equally demonstrated that TcI and TcII are the most genetically distant branches, but showed differences concerning the positions of the DTUs TcIII, TcIV, TcV, TcVI and Tcbat in the phylogenetic trees. The mitochondrial gene *cox*1 may be a better discriminator of *T. cruzi* lineages, identifying five DTUs and TcV/TcVI as a single group (Fig. 2a). Additionally, these differences between mitochondrial and nuclear tree topologies, no incongruence was observed in DTU assignment (Table 1), and mitochondrial introgression events were absent in the present sample set.

### Identification of *T. cruzi* DTUs through single nucleotide polymorphisms (SNPs)

Some *T. cruzi* sequences were not clearly assigned to a DTU based solely on information from the trees. In the *cox*1 tree, TcIV and TcV/TcVI sequences were arranged in the same cluster (Fig. 2a), whereas in *GPI* analyses, TcI, TcIII and Tcbat separation was blurred (Fig. 3). Therefore, the multiple sequence alignment of *cox*1 and *GPI* data was considered for the evaluation of single nucleotide polymorphisms (SNPs) using *T. cruzi* sequences only. These polymorphisms were informative to DTU differentiation for both genes.

In the *cox*1 gene fragment analysis, we identified 84 polymorphic sites. We observed a single nucleotide polymorphism (SNP) that differentiates the heterozygous hybrids from TcIV sequences. A T (thymine) was present at position 1264 of the *cox*1 gene in all ten TcIV sequences analyzed, whereas TcV and TcVI sequences display a C (cytosine) at the same site. No polymorphism differentiating TcV from TcVI was observed (Additional file 1: Figure S1).

In the *GPI* analysis we identified 20 polymorphic sites concerning all *T. cruzi* sequences. A thymine at position 315 separates Tcbat from TcI (cytosine) and one guanine to adenine change separates TcI from TcIII at position 396 of the gene (Additional file 2: Figure S2). No polymorphism discriminating TcV from TcVI sequences was observed.

### Phylogenetic tree reconstruction using concatenated data

The concatenation of *cox*1 and *GPI* gene fragments confirmed, with robust statistical support values, the separation of species belonging to the subgenus *Schizotrypanum* and the *T. cruzi* DTUs.

TcI, TcII, TcIII and TcIV sequences constituted clearly separated clades. In addition, concatenated data supported Tcbat as a sister clade to TcI. The heterozygous hybrids TcV and TcVI could not be differentiated and formed a cluster separate from TcIV. The topologies observed in the trees were the same for the four methods tested (NJ, ML, MP and BI) and were supported by values above 80 in the main branches (Fig. 4).

### Genetic diversity evaluated with *cox*1 and *GPI* genes

Although the *cox*1 gene did not distinguish TcV from TcVI, this gene demonstrated diversity in *T. cruzi* DTUs TcI, TcII and TcIII and *T. c. marinkellei* and *T. rangeli* (Additional file 3: Table S1, Fig. 2b). *GPI* sequences also

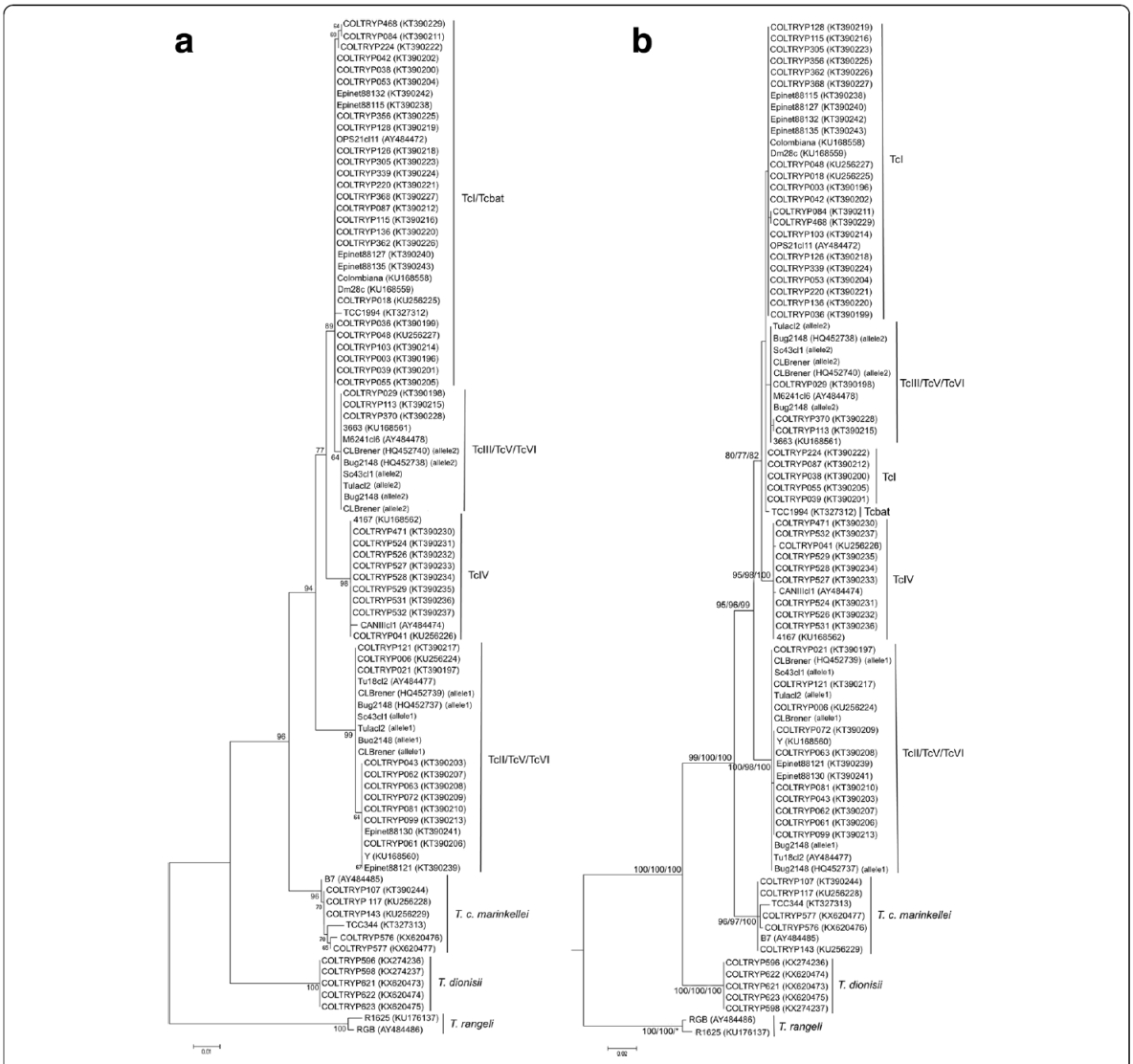


**Fig. 3** Phylogenetic tree based on nuclear gene *GPI*. **a** Tree inferred with neighbor-joining method and Kimura-2-parameter model. *GPI* recognizes and differentiates *T. cruzi* DTUs TcI, TcII, TcIII and TcIV, *T. c. marinkellei*, *T. dionisii* and *T. rangeli*. One allele of TcV and TcVI sequences cluster with TcII, and the other allele clusters with TcIII. Tcbat was placed in the same cluster as TcI. **b** The tree inferred from maximum likelihood, parsimony and Bayesian inference. *T. cruzi*, *T. c. marinkellei*, *T. dionisii* and *T. rangeli* are clearly separated from each other. DTUs TcI and TcII are the most genetically divergent. Tcbat, TcI and TcIII fall in the same cluster. One allele of TcV and TcVI clustered with TcII, and the other allele clustered with the group comprising TcI, TcIII and Tcbat. The numbers at the nodes correspond to ML, MP and BI support values, respectively (only values >60 are shown). The scale-bar shows the number of nucleotide substitutions per site

displayed distinct haplotypes in *T. c. marinkellei* and *T. rangeli*, but lower intra-DTU diversity. The correlation between haplotype and geographical area or host species was not evident.

In the *cox*1 analysis, TcI was demonstrated as the most diverse DTU with the highest nucleotide diversity and haplotype diversity of all DTUs, followed by TcIII and TcII. The TcI isolates and reference strains in the present study were distributed in eight haplotypes throughout five Brazilian biomes (Fig. 2b) and six different host orders (Carnivora, Chiroptera, Didelphimorphia, Hemiptera, Primates and Rodentia) (Table 1). In the Amazon, we observed the highest number of different TcI haplotypes in the Para state (hap 2, 3 and 5). Haplotypes 2 and 5 were observed only in the Para State, while haplotype 3 was widely distributed and detected in four

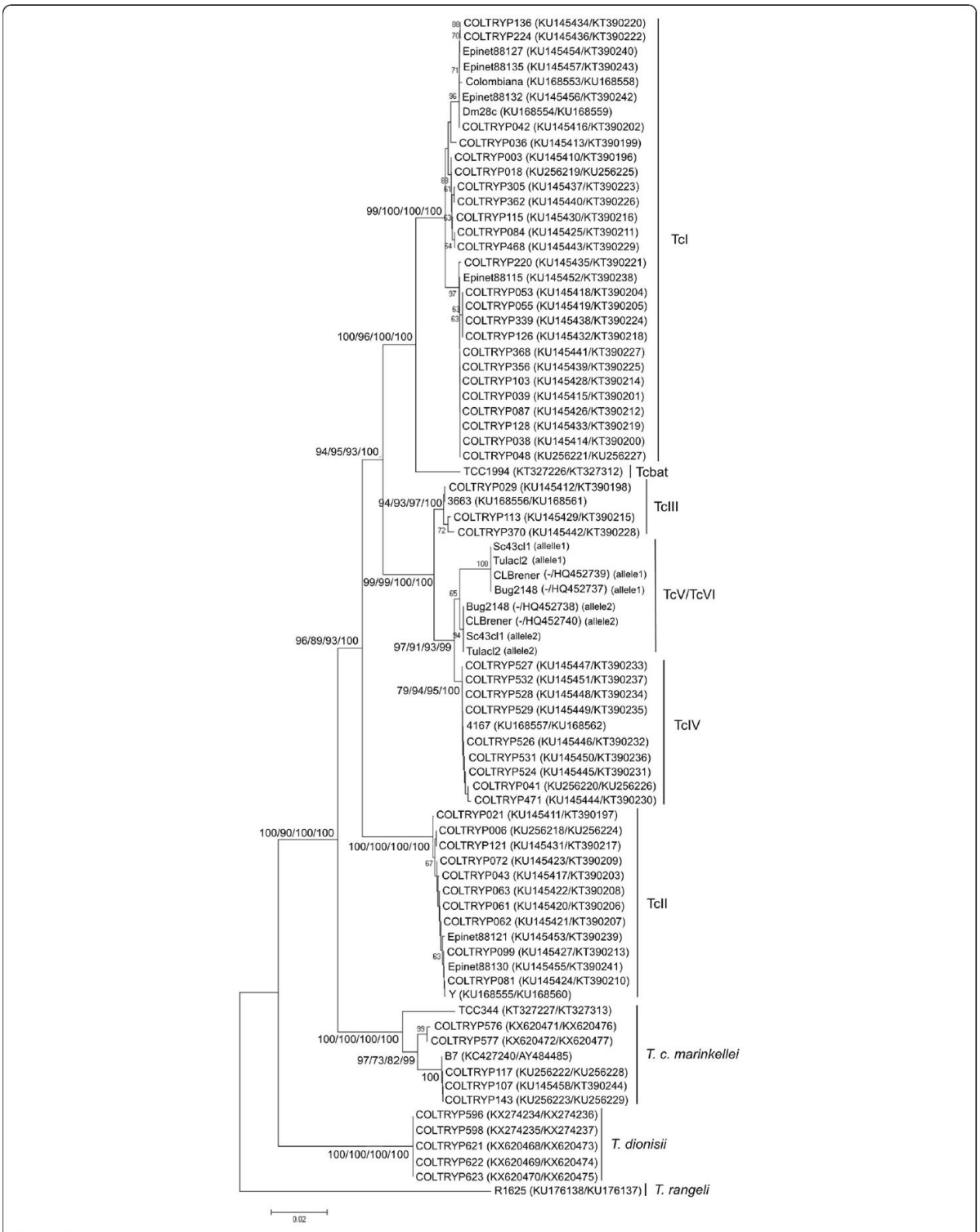


**Fig. 4** (See legend on next page.)

(See figure on previous page.)
**Fig. 4** Phylogenetic tree based on the concatenation of *cox*1 and *GPI* sequences. The concatenated data show a clear separation between *T. cruzi* DTUs TcI, TcII, TcIII and TcIV, Tcbat, *T. c. marinkellei*, *T. dionisii* and *T. rangeli*. The heterozygous hybrids TcV and TcVI were not differentiated from each other. The numbers at the nodes correspond to NJ, ML, MP and BI support values, respectively (only values >60 are shown). The scale-bar shows the number of nucleotide substitutions per site

different biomes (Amazon, Atlantic Forest, Caatinga and Pantanal). Additionally, in Para, we observed two different haplotypes in three TcI isolates derived from *Didelphis marsupialis* (Table 1). Thus, establishing a correlation between the TcI haplotype and location or host species would be premature. Substantial genetic diversity was also observed in TcIII sequences. In four TcIII isolates derived from *Monodelphis domestica* (Cerrado biome), *Galictis vittata* (Atlantic Forest), *Rhodnius pictipes* (Amazon biome) and a reference strain from *Panstrongylus geniculatus* (Amazon biome), we identified three different haplotypes (Table 1, Fig. 2b). These TcII isolates were divided into three haplotypes and originated from primates, a marsupial and triatomines captured in the Atlantic Forest and from a rodent captured in the Caatinga biome. The reference strains were isolated from humans in the Atlantic Forest. The three TcII haplotypes were distributed in the state of Rio de Janeiro, isolated from a *Philander frenatus* and two *Leontopithecus rosalia* (Table 1). Genetic diversity was not detected among the TcIV, TcV and TcVI isolates. Diversity could not be evaluated for Tcbat, reflecting the unique sequence available for this DTU.

The seven *T. c. marinkellei* isolates displayed three haplotypes (Additional file 3: Table S1, Fig. 2b). One haplotype was formed by isolates originated from *P. discolor* from the Goiás State (Cerrado) and the Bahia state (Atlantic Forest); another haplotype comprised isolates originated from *P. hastatus* from the Acre State (Amazon biome); and a third group was formed by the reference strain TCC 344, isolated from *C. perspicillata* (Amazon) (Table 1, Fig. 2a). Despite the low number of isolates, an apparent correlation between haplotype and host species was observed.

We compared two *T. rangeli* isolates previously identified as lineages C and D [54, 70, 71]. Sequences generated with *cox*1 exhibited two different haplotypes (Table 1, Additional file 3: Table S1). *Trypanosoma dionisii* sequences showed no diversity. Isolates derived from two different species of phyllostomid bats from the same geographical area and collected during the same field expedition (Table 1, Additional file 3: Table S1).

In the *GPI* analysis, intra-DTU diversity was lower than observed with *cox*1. TcI, TcII, TcIII and TcIV sequences displayed two different haplotypes each. In TcIV, one haplotype was formed by the nine isolates identified herein and the reference strain 4167, while the other haplotype only comprised the reference strain CANIII, which was not available for *cox*1 analysis. No diversity within TcV/TcVI sequences was detected.

The highest diversity in *GPI* sequences was observed in *T. c. marinkellei* with five haplotypes. One haplotype comprised three isolates from the Goiás state (Cerrado) and the other haplotypes corresponded to the other four isolates (Additional file 3: Table S1, Fig. 3). The two *T. rangeli* sequences generated with *GPI* were identified elsewhere as lineage C [71]. However, these sequences displayed distinct haplotypes (Additional file 3: Table S1, Fig. 3). No diversity within *T. dionisii* sequences was observed. No correlation between haplotype, host or geographical location could be established.

### Barcoding gap and species delimitation with *cox*1 and *GPI* sequences

Based on the analysis of the barcode gaps we assessed and compared the efficiency of *cox*1 and *GPI* for the identification of trypanosomatids. In *cox*1 the mean genetic distance between *T. cruzi* sequences and *T. c. marinkellei* was 13%. The mean intraspecific divergence for *T. cruzi* was 7.6%, a value lower than the mean interspecific value. However, comparing the minimum and maximum values, we observed an overlap of the genetic distances between *T. cruzi* and *T. c. marinkellei* (10.1–15.3%) and the intraspecific divergence for *T. cruzi* (0–12.7%), indicating the absence of a limit that separates these two subspecies (i.e. absence of a barcoding gap). The divergence between *T. cruzi* and *T. dionisii* limits was 13.0–16.0%, while the divergence between *T. cruzi* and *T. rangeli* was 14.7–21.3%, indicating that the genetic distance separating *T. cruzi*, *T. dionisii* and *T. rangeli* as different species is 0.3 and 2.0%, respectively (Additional file 4: Table S2).

*GPI* was not as discriminative as *cox*1. The mean interspecific divergence between *T. cruzi* sequences and *T. c. marinkellei*, *T. dionisii* and *T. rangeli* was 3.6, 7.8 and 13.7%, respectively.

We observed differences between *T. c. marinkellei* sequences in the trees (Fig. 3) confirmed by an intraspecific distance ranging from 0 to 1.0% (Additional file 5: Table S3). Distance within *T. rangeli* sequences was 0.7% and there was no genetic difference between *T. dionisii* sequences. For *T. cruzi*, the intraspecific genetic distance ranged from 0 to 3.0%, i.e. lower than the mean interspecies values (Table 2). Similar to *cox*1, we observed an overlap of the genetic distances between *T. cruzi* and *T. c.*

*marinkellei* with *GPI*, indicating the absence of a "barcoding gap".

*Trypanosoma cruzi* and *T. c. marinkellei* were separated into distinct groups according to ABGD, PTP and mPTP analysis using *cox*1 and *GPI* data. Here, we report the results for the three substitution models and both initial and recursive partitions in the output of ABGD. The results varied from 6 to 21 for *cox*1 depending on the substitution model used, confirming diversity in *T. cruzi* sequences and the separation of *T. cruzi*, *T. c. marinkellei*, *T. rangeli* and *T. dionisii* (Table 2). *Trypanosoma cruzi* sequences were divided into a minimum of three groups in the three models tested. One group corresponded to Tcbat and TcI; another group corresponded only to TcII sequences only; and a third group comprised TcIII, TcIV, TcV and TcVI sequences. A maximum of 13 different groups were observed, with TcI sequences divided into eight different groups, showing the higher intra-DTU diversity of TcI compared to the other DTUs (Additional file 6: Table S4). In all models and partitions, *T. dionisii* sequences were arranged in one group.

The number of ABGD groups for *GPI* data varied from 2 to 14 depending on the model applied (Table 2). *Trypanosoma cruzi* sequences were separated into different groups. TcI sequences were grouped together, showing less variability with *GPI*. TcII sequences were divided into two groups: one group with only TcII sequences and another group with TcII, TcV (allele 1) and TcVI (allele 1). TcIII sequences were grouped together with sequences representing the other TcV and TcVI alleles. TcIV sequences were combined in one group, except for the reference strain CANIII, which was placed in a separated group. *Trypanosoma cruzi marinkellei* and *T. rangeli* sequences were divided into groups, reaffirming their diversity (Additional file 7: Table S5), while *T. dionisii* sequences formed one group in all tests (Table 2, Additional file 7: Table S5).

The number of groups recovered by ABGD was higher than the number of species studied. However, this finding confirms the genetic diversity within *T. cruzi* DTUs, *T. c. marinkellei* and *T. rangeli* observed in the phylogenetic trees (Figs. 2a and 3).

The PTP and mPTP models identified, respectively, a total of 10 and 7 putative species in the *cox*1 dataset (Table 3). Four of these putative species were subdivisions of *T. cruzi*, indicating the heterogeneity of this taxon. The PTP model also recognized diversity within *T. c. marinkellei* and *T. rangeli* sequences.

PTP and mPTP provided a similar number of putative species for *GPI* sequences (Table 3). *Trypanosoma cruzi* was divided into three groups, and *T. c. marinkellei* sequences were allocated into one group. The difference between models was observed in the *T. rangeli* sequences, separated into two groups or placed into one group.

In *cox*1 and *GPI* analysis using both models, *T. cruzi*, *T. c. marinkellei*, *T. rangeli* and *T. dionisii* were recognized as different species. The diversity of *T. cruzi* was confirmed, and no diversity was observed in *T. dionisii* sequences.

## Discussion

In the present study, the DNA barcoding approach using the *cox*1 gene has been demonstrated to be efficient at

**Table 2** *cox*1 and *GPI* sequences division in groups based on ABGD analysis

| Substitution model | X[a] | Partition | Prior intraspecific divergence (P) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.059948 | 0.035938 | 0.021544 | 0.012915 | 0.007743 | 0.004642 | 0.002783 | 0.001668 | 0.001000 |
| *cox*1 | | | | | | | | | | | |
| Jukes Cantor | 1.5 | Initial | 6 | 6 | 6 | 10 | 10 | 10 | 10 | 10 | 10 |
| | | Recursive | | | 8 | 11 | 14 | 14 | 14 | 21 | 21 |
| K2P[b] | 1.5 | Initial | 6 | 6 | 6 | 11 | 11 | 11 | 11 | 11 | 11 |
| | | Recursive | | | 8 | | 14 | 14 | 14 | 19 | 19 |
| p-distance | 1.5 | Initial | | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| | | Recursive | | 0 | 8 | 9 | 9 | 9 | 11 | 11 | 11 |
| *GPI* | | | | | | | | | | | |
| Jukes Cantor | 1.5 | Initial | 2 | 2 | 3 | 3 | 5 | 7 | 3 | 14 | 14 |
| | | Recursive | | | | | | | 7 | | |
| K2P[b] | 1.5 | Initial | 2 | 2 | 3 | 3 | 5 | 7 | 3 | 14 | 14 |
| | | Recursive | | | | | | | 7 | | |
| p-distance | 1.5 | Initial | 2 | 2 | 3 | 3 | 5 | 5 | 5 | 7 | 7 |
| | | Recursive | | | | | | | | | |

[a]X, relative gap width
[b]*K2P* Kimura 2-parameter

**Table 3** Number of species according to PTP and mPTP delimitation methods

| Gene and taxon | PTP | mPTP |
|---|---|---|
| | Number of putative species | |
| *cox*1 | | |
| *T. cruzi* | 4 | 4 |
| *T. c. marinkellei* | 3 | 1 |
| *T. dionisii* | 1 | 1 |
| *T. rangeli* | 2 | 1 |
| Total | 10 | 7 |
| *GPI* | | |
| *T. cruzi* | 3 | 3 |
| *T. c.marinkellei* | 1 | 1 |
| *T. dionisii* | 1 | 1 |
| *T. rangeli* | 2 | 1 |
| Total | 7 | 6 |

recognizing *Trypanosoma* species and their major subpopulations. With *cox*1, we distinguished *T. cruzi* from *T. c. marinkellei*, *T. donisii* and *T.* (*Tejeraia*) *rangeli*, fulfilling the main DNA barcode demands of a short gene fragment that can be sequenced in diverse sample sets and generating comparable sequences that enable the distinction of species from each other [4]. We also generated a library of trypanosome sequences for *cox*1 and *GPI* genes. Each specimen analyzed is linked to an identification number, collection date, country, region and host of origin, geographical coordinates and other information that enable the tracking of the origin of the specimen and ensure the reproducibility of subsequent experiments.

*Trypanosoma cruzi* is currently divided into seven DTUs [29]. Using *cox*1, we identified five *T. cruzi* groups (TcI, TcII, TcIII, TcIV and Tcbat). The DTUs TcI and TcII are consistently shown as the most genetically distant groups, well separated by *cox*1 in all four methods tested (Fig. 2a). This structure has been observed by other authors in trees with high bootstrap support values, sustaining TcI and TcII as the two discernible DTUs, independently of gene or method used [25, 51, 72, 73]. Furthermore, this system showed the potential for separating genetically closer DTUs. We observed Tcbat as a separated cluster within *T. cruzi* and its proximity to TcI, consistent with Marcili et al. [25]. According to other studies using *cytb*, *V7 V8* rRNA and *gGAPDH* genes this relationship is unanimous [26, 51, 74, 75]. However, the data on Tcbat are still limited, as only one sequence was generated with *cox*1 available in GenBank. Our *cox*1 sequences also showed the homozygous hybrids TcIII and TcIV forming distinct groups in all methods tested (Fig. 2a). These DTUs are proposed to have been originated from the genetic exchange between TcI and TcII and evolved separately giving origin to TcIII and TcIV [76, 77]. The genetic proximity between TcIII and TcIV is undeniable, and their separation and position in the phylogenetic trees is altered by the gene and method of inference selected. Based on *cytb* (inferred by MP), *V7 V8*, *gGAPDH*, *GPI* genes and MLST approaches, TcIII and TcIV were identified as two separate DTUs [24–26, 28, 51]. However, in other studies using the *cytb* gene (inferred by neighbor-joining and maximum likelihood), TcIII and TcIV were identified as a single group [34, 51, 77]. TcV and TcVI sequences were indistinguishable and clustered with TcIV (Fig. 2a), consistent with previous *cytb* mitochondrial gene results [25]. In some studies, independently of the molecular marker and phylogenetic method applied, TcV and TcVI were also indistinguishable from each other. However, these hybrids clustered together with TcII or TcIII when analyzed using nuclear markers [25, 26, 34, 78]. In contrast, in studies using 4 to 10 gene fragments and neighbor-joining trees, TcV and TcVI appeared as two distinct DTUs [24, 79]. In the *cox*1 analysis, we observed one SNP that differentiates TcIV sequences from TcV/TcVI (Additional file 1: Figure S1). This nucleotide polymorphism, combined with the phylogenetic tree, was demonstrated as relevant to DTU assignment. However, TcV and TcVI are the less conspicuous lineages, and their separation remains an issue.

*Cox*1 was also suitable to determine diversity within DTUs TcI, TcII and TcIII (Additional file 3: Table S1). The number of sequences classified as TcI and the number of different haplotypes in these DTU sequences were the highest, compared to the other DTUs. The diversity within TcI is consistent with previous studies and may be explained by TcI being a multi-host lineage widely distributed throughout Brazilian biomes, representing the DTU with the largest set of samples analyzed, and consequently, the DTU with the most published studies compared to the other DTUs [20, 51, 52, 80, 81]. The nucleotide and haplotype diversity of the TcII sequences generated using *cox*1 were lower. However, this effect may not reflect the reality, but rather may show subsampling. In the Rio de Janeiro State, we observed one TcII haplotype circulating in a specimen of *L. rosalia* and a different haplotype in another specimen of *L. rosalia*. This observed diversity may reflect primates captured in different years and the changes in TcII haplotype circulation in that area. Nevertheless, the same host can harbor different haplotypes from the same parasite, and one haplotype can prevail over another in different moments of isolation. Diversity within TcII has previously been demonstrated through the sequencing of the glycoprotein 72 gene (*gp72*) and showed that this DTU has a higher distribution range than

previously considered [56]. The high haplotype diversity observed in TcIII could result from overestimation, since we identified three different haplotypes in the four sequences analyzed, belonging to isolates from different Brazilian regions. However, this scenario shows TcIII distributed in a wide geographical range, infecting marsupials, carnivores and triatomines (Table 1). This finding clearly indicates that the richness within TcIII, and its dispersion is yet to be explored. Diversity in TcIII has previously been observed based on *V7 V8*, *cytb*, *GPI*, MLST approaches, but no correlation with geographical area or host species was evident [24, 25, 28]. In the present study, TcIV samples were isolated from triatomines and rodents from the same geographical area (Table 1). This aspect might explain the observation of only one haplotype in TcIV sequences. However, isolates from rodents were collected 11 years before the parasites isolated from the intestinal content of triatomines. Thus, we propose that TcIV haplotype circulation in the Pantanal area was at least equally predominant throughout more than a decade. However, the TcIV reference was isolated from a triatomine in the Amazon region (Table 1) and had the same haplotype as the Pantanal isolates. This finding could reflect the conservation of the *cox*1 region in TcIV. A correlation between TcIV diversity and the geographical region has been suggested by other studies based on mitochondrial genes *cytb* and cytochrome *c* oxidase subunit 2-NADH dehydrogenase subunit 1 (*cox*2-*nad*1) [28, 34, 77].

The *cox*1 tree topologies, independently of the method applied, showed *T. c. marinkellei* as a sister clade to the monophyletic clade formed by all *T. cruzi* DTUs (Fig. 2a); we also observed genetic diversity within *T. c. marinkellei* (Additional file 3: Table S1). Even with the characterization of a low number of isolates, the samples were separated into two groups, and a sequence retrieved from GenBank was positioned in a third group (Fig. 2a). Heterogeneity within *T. c. marinkellei* has previously been reported [75, 82, 83]. Subdivision into two major groups (T.c.m.I and II) and a potential third group (lineage Z) was proposed using multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD). No association with a host or geographical distribution was confirmed [82]. This lack of evidence for an association and the different markers used prevented the comparison of these data.

We also observed genetic differences between *T. rangeli* reference sequences R1625 and SC58 classified, respectively, as lineage C and lineage D [54, 70]. Previous studies have proposed the subdivision of *T. rangeli* in five lineages (A-E), based on spliced leader and SSU rDNA [54, 70, 71]. Even with only two sequences, we suggest that *cox*1 can distinguish different *T. rangeli* lineages and is a promising tool for use in species identification.

In the present study, we showed the first *T. dionisii* sequences for the *cox*1 gene. The nucleotide sequences were deposited in GenBank, contributing to the enhancement of the barcode public library for *Trypanosoma* species. We did not observe diversity among these sequences (Fig. 2a, Additional file 3: Table S1), likely because the samples were collected from bats of the same area. No subdivisions in groups or subpopulations have been proposed for *T. dionisii* until recently. Although potential diversity within this species can be observed in *cytb* and 18S (SSU) phylogenetic trees, these data were not reported [84].

The concomitant analysis of the mitochondrial gene *cox*1 and the nuclear gene *GPI* enable the confirmation of the absence of mitochondrial introgression events in the sample set. The frequency of this genetic phenomenon is unknown and has primarily been observed in heterozygous hybrid DTUs TcV and TcVI, where uniparental inheritance of maxicircle kDNA is the rule [28, 36, 56, 85]. *Cox*1 has limitations and does not work as a single barcode in all situations [1], and since *T. cruzi* possesses heterozygous hybrid lineages and mitochondrial introgression events have previously been reported, we proposed this *cox*1-*GPI* barcoding system. Additionally, the concatenated analysis of *cox*1 and *GPI* confirmed *T. cruzi* DTU separation (Fig. 4).

Consistent with the *cox*1 results, *GPI* distinguished *T. cruzi* from *T. c. marinkellei*, *T. donisii* and *T.* (*Tejeraia*) *rangeli.* Additionally, with *GPI*, TcI and TcII were separated into two conspicuous groups. This nuclear gene recognizes a lower number of *T. cruzi* groups (Fig. 3) and considerably lower intra-DTU diversity compared to *cox*1 (Additional files 4 and 5: Tables S2 and S3). In contrast to the *cox*1 results, Tcbat and TcI were clustered together. The lower power of resolution to discriminate DTUs and intra-DTU diversity might reflect the fact that *GPI* is a housekeeping nuclear gene, which shows a lower evolution rate than mitochondrial genes [28, 85]. Furthermore, we compared the *GPI* sequences with the single Tcbat sequence available in GenBank and the results may change depending on the number of sequences available. It is likely that a larger set of Tcbat sequences would resolve the incongruence between the mitochondrial and nuclear gene trees. In addition, we also observed differences in DTU placement in the trees according to the method of inference in the *GPI* analyses. TcIII sequences formed a cluster separate from TcI when we applied the neighbor-joining method and clustered together with TcI when maximum likelihood, parsimony and Bayesian inference were used. This effect may reflect the fact that the neighbor-joining method was based on the genetic distance matrix, where a pairwise distance matrix is produced, and the tree is inferred from this matrix; maximum likelihood, parsimony and

Bayesian inference were character-based methods of inference where each position of the alignment is analyzed [86]. However, in the *GPI* analysis, we detected SNPs that enable the differentiation of TcI, TcIII and Tcbat (Additional file 2: Figure S2). We observed diversity within *T. c. marinkellei* and generated the first five *T. dionisii* sequences for *GPI*. We could not compare *T. rangeli* sequences generated with *GPI*, as both sequences analyzed belonged to lineage C [54, 70].

Barcoding gap in trypanosomatids is still an unresolved issue. Therefore, there are no parameters or cut-off values available to compare with these results. As expected, we did not observe a barcoding gap between *T. cruzi* and *T. c. marinkellei* because *T. c. marinkellei* is considered a subspecies of *T. cruzi* [87] (Additional files 4 and 5: Tables S2 and S3).

The debate concerning the definition of species will always exist since species are not discrete units, but rather continuous entities.

## Conclusions

The use of partial sequences of *cox*1 and *GPI* genes can clearly identify and separate *T. cruzi* samples from *T. c. marinkellei*, *T. dionisii* and *T. rangeli*. The two-locus barcoding system using *cox*1 and the nuclear gene *GPI* revealed that mitochondrial introgression was absent from the sample set. Additionally, the resolution of *cox*1 at the intraspecific level shows great potential for DTU characterization, separating five DTUs and recognizing the heterozygous hybrids TcV and TcVI as one group different from all the other DTUs; the resolution of *cox*1 at the intraspecific level also demonstrates intra-DTU genetic diversity. Moreover, with *cox*1, we evaluated the diversity within *T. c. marinkellei* sequences and identified two *T. rangeli* lineages. Therefore, the *cox*1 gene is a promising DNA barcode for studying the genus *Trypanosoma* and represents a simple, fast and reliable marker.

## Additional files

**Additional file 1: Figure S1.** Comparison between TcIV and TcV/TcVI nucleotide sequences generated with the *cox*1 barcode. **a** Alignment of TcIV sequences with TcV and TcVI shows one single nucleotide polymorphism differentiating TcIV samples from the hybrids. **b** Electropherogram confirms the presence of a T (thymine) in TcIV in the same position, showing a C (cytosine) in TcV and TcVI sequences. (TIFF 724 kb)

**Additional file 2: Figure S2.** Comparison between TcI, Tcbat and TcIII nucleotide sequences generated with *GPI*. **a** Sequence alignment shows one single nucleotide polymorphism differentiating TcI from Tcbat and one polymorphism separating TcI from TcIII sequences. **b** Electropherogram confirms the presence of A (adenine) in TcIII sequences in the same position, showing a G (guanine) in TcI sequences. The Tcbat sequence was retrieved from GenBank and the electropherogram is not publicly available. (TIFF 527 kb)

**Additional file 3: Table S1.** Number of haplotypes, nucleotide diversity and haplotype diversity of sequences generated with *cox*1 and *GPI*. (DOCX 12 kb)

**Additional file 4: Table S2.** Inter- and intraspecific genetic distance based on *cox*1 sequences. (DOCX 14 kb)

**Additional file 5: Table S3.** Inter- and intraspecific genetic distance based on *GPI* sequences. (DOCX 15 kb)

**Additional file 6: Table S4.** *Cox*1 sequences partition into groups inferred with ABGD, based on Kimura 2-parameters. (DOCX 14 kb)

**Additional file 7: Table S5.** *GPI* sequences partition into groups inferred with ABGD, based on Kimura 2-parameters. (DOCX 14 kb)

### Abbreviations

ABGD: Automatic Barcode Gap Discovery; BI: Bayesian inference; BrBOL: Brazilian Barcode of Life; COLPROT: Protozoa Collection; COLTRYP: Trypanosomatid collection from wild and domestic mammals and vectors; *cox*1: cytochrome *c* oxidase subunit 1 gene; *cox*2-*nad*1: cytochrome *c* oxidase subunit 2-NADH dehydrogenase subunit 1 gene region; *cytb*: cytochrome b; DTU: Discrete typing unit; gGAPDH: glyceraldehyde-3-phosphate dehydrogenase; *GPI*: Glucose-6-phosphate isomerase; ITS: Internal transcribed spacer; K2P: Kimura 2-parameter; LIT: Liver infusion tryptose; ML: Maximum likelihood; MLST: Multilocus sequencing typing; MP: Maximum parsimony; mPTP: multi-rate Poisson Tree Processes; NJ: Neighbor-joining; NNN: Novy-McNeal-Nicole medium; PTP: single-rate Poisson Tree Processes; SSU rDNA: Small subunit ribosomal DNA; V7 V8: Variable regions 7 and 8

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### Availability of data and materials

All sequences analyzed are available in GenBank under the accession numbers within the article.

### Authors' contributions

MSR, KAM and AMJ conceived and designed the experiments. MSR performed and analyzed the molecular characterization. KAM provided additional suggestions on content. MSR and AMJ drafted the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable. No special permission was required for the present study. We used DNA extracted from the cultures obtained from animals collected during previous field expeditions conducted by our group. The field expeditions were endorsed by the Ethics Committee of FIOCRUZ (Oswaldo Cruz Foundation, Brazil) (CEUA L-015/04; CEUA P-292-06).

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

## Publisher's Note

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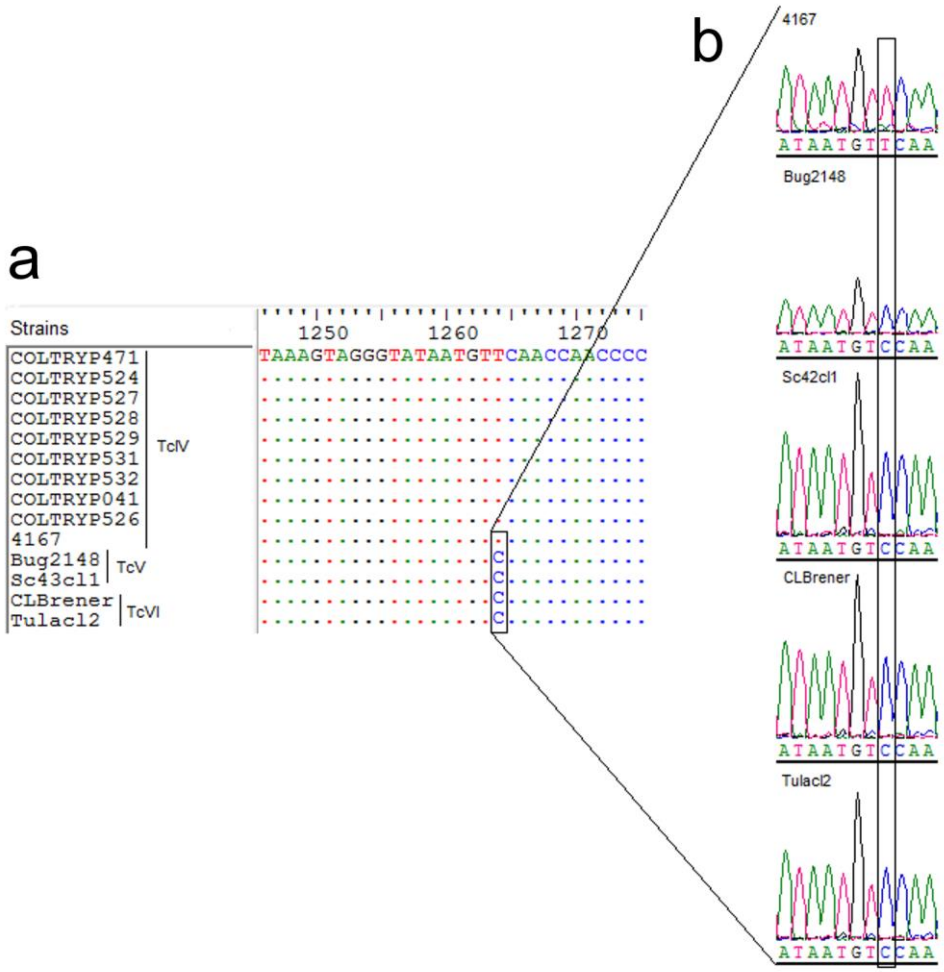
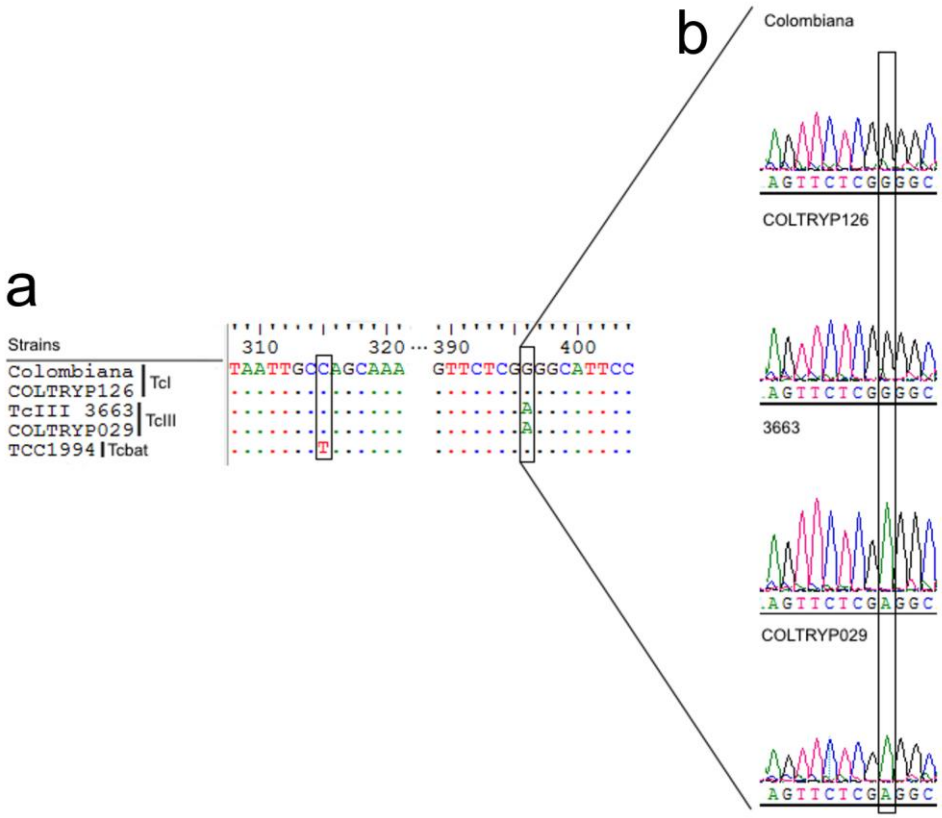



Additional file 1: Figure S1. Comparison between TcIV and TcV/TcVI nucleotide sequences generated with the *cox*1 barcode. **a** Alignment of TcIV sequences with TcV and TcVI shows one single nucleotide polymorphism differentiating TcIV samples from the hybrids. **b** Electropherogram confirms the presence of a T (thymine) in TcIV in the same position, showing a C (cytosine) in TcV and TcVI sequences.




**Additional file 2: Figure S2.** Comparison between TcI, Tcbat and TcIII nucleotide sequences generated with *GPI*. **a** Sequence alignment shows one single nucleotide polymorphism differentiating TcI from Tcbat and one polymorphism separating TcI from TcIII sequences. **B** Electropherogram confirms the presence of A (adenine) in TcIII sequences in the same position, showing a G (guanine) in TcI sequences. The Tcbat sequence was retrieved from GenBank and the electropherogram is not publicly available

**Additional file 3: Table S1** Number of haplotypes, nucleotide diversity and haplotype diversity of sequences generated with COI and GPI.

| Group | N | Haplotype | π ± SD | Hd ± SD |
|---|---|---|---|---|
| COI | | | | |
| TcI | 31 | 8 | 0.01276 ± 0.00074 | 0.832 ± 0.035 |
| TcII | 14 | 3 | 0.00143 ± 0.00038 | 0.538 ± 0.115 |
| TcIII | 4 | 3 | 0.00485 ± 0.00192 | 0.833 ± 0.222 |
| TcIV | 10 | 1 | 0.00000 ± 0.00000 | 0.000 ± 0.000 |
| TcV/TcVI | 4 | 1 | 0.00000 ± 0.00000 | 0.000 ± 0.000 |
| *T. c. marinkellei* | 7 | 3 | 0.02493 ± 0.00872 | 0.667 ± 0.160 |
| *T. dionisii* | 5 | 1 | 0.00000 ± 0.00000 | 0.000 ± 0.000 |
| *T. rangeli* | 2 | 2 | 0.05593 ± 0.02796 | 1.000 ± 0.500 |
| Group | N | Haplotype | π ± SD | Hd ± SD |
| GPI | | | | |
| TcI | 31 | 2 | 0.00026 ± 0.00014 | 0.094 ± 0.049 |
| TcII | 14 | 2 | 0.00089 ± 0.00016 | 0.423 ± 0.076 |
| TcIII | 5 | 2 | 0.00096 ± 0.00017 | 0.533 ± 0.095 |
| TcIV | 11 | 2 | 0.00040 ± 0.00023 | 0.173 ± 0.101 |
| TcV/TcVI | 4 | 1 | 0.00000 ± 0.00000 | 0.000 ± 0.000 |
| *T. c. marinkellei* | 7 | 5 | 0.00338 ± 0.00080 | 0.736 ± 0.107 |
| *T. dionisii* | 5 | 1 | 0.00000 ± 0.00000 | 0.000 ± 0.000 |
| *T. rangeli* | 2 | 2 | 0.00585 ± 0.00179 | 0.667 ± 0.204 |

N = total number of sequences used in the present study and from GenBank; π = nucleotide diversity; Hd = haplotype diversity, SD = standard deviation.

*GPI haplotypes were inferred after haplotype reconstruction with the algorithm PHASE

[a] TcV/TcVI sequences have one haplotype for each allele.

**Additional file 4: Table S2** Inter- and intraspecific genetic distance based on COI sequences.

| Groups | Min | Max | Mean |
|---|---|---|---|
| **Inter-DTU** | | | |
| Tcbat X TcI | 0.054 | 0.064 | 0.060 |
| Tcbat X TcII | 0.099 | 0.105 | 0.103 |
| Tcbat X TcIII | 0.100 | 0.105 | 0.102 |
| Tcbat X TcIV | 0.105 | 0.105 | 0.105 |
| Tcbat X TcV/TcVI | 0.108 | 0.108 | 0.108 |
| TcI X TcII | 0.094 | 0.118 | 0.109 |
| TcI X TcIII | 0.100 | 0.113 | 0.107 |
| TcI X TcIV | 0.105 | 0.113 | 0.110 |
| TcI X TcV/TcVI | 0.105 | 0.116 | 0.112 |
| TcII X TcIII | 0.105 | 0.114 | 0.111 |
| TcII X TcIV | 0.119 | 0.124 | 0.123 |
| TcII X TcV/TcVI | 0.122 | 0.127 | 0.126 |
| TcIII X TcIV | 0.020 | 0.025 | 0.022 |
| TcIII X TcV/TcVI | 0.023 | 0.027 | 0.025 |
| TcIV X TcV/TcVI | 0.002 | 0.002 | 0.002 |
| | | | |
| **Interspecific** | | | |
| *T. cruzi* X *T.c.marinkellei* | 0.101 | 0.153 | 0.130 |
| *T.cruzi* X *T. dionisii* | 0.130 | 0.160 | 0.150 |
| *T. cruzi* X *T. rangeli* | 0.147 | 0.213 | 0.182 |
| *T.c.marinkellei* X *T. dionisii* | 0.135 | 0.163 | 0.156 |
| *T.c.marinkellei* X *T. rangeli* | 0.157 | 0.198 | 0.178 |
| *T. dionisii* X *T. rangeli* | 0.151 | 0.188 | 0.169 |
| **Intra-DTU** | | | |
| Tcbat | NC | NC | NC |
| TcI | 0.000 | 0.028 | 0.013 |
| TcII | 0.000 | 0.004 | 0.001 |

| | | | |
|---|---|---|---|
| TcIII | 0.000 | 0.009 | 0.005 |
| TcIV | 0.000 | 0.000 | 0.000 |
| TcV/TcVI | 0.000 | 0.000 | 0.000 |
| | | | |
| **Intraspecific** | | | |
| *T. cruzi* | 0.000 | 0.127 | 0.076 |
| *T.c.marinkellei* | 0.000 | 0.062 | 0.026 |
| *T. dionisii* | 0.000 | 0.000 | 0.000 |
| *T. rangeli* | NC | NC | 0.059 |

NC = not calculated. Low number of sequences available for calculation.

Top: genetic distance between *Trypanosoma* species *T. cruzi*, *T. c. marinkellei*, *T. dionisi*i and *T. rangeli*

Bottom: genetic distance within *T. cruzi* subpopulations, *T. c. marinkellei*, *T. dionisii* and *T. rangeli*

**Additional file 5: Table S3** Inter- and intraspecific genetic distance based on GPI sequences.

| Groups | Min | Max | Mean |
|---|---|---|---|
| **Inter-DTU** | | | |
| Tcbat X TcI | 0.002 | 0.005 | 0.003 |
| Tcbat X TcII | 0.025 | 0.028 | 0.027 |
| Tcbat X TcIII | 0.005 | 0.005 | 0.005 |
| Tcbat X TcIV | 0.015 | 0.017 | 0.015 |
| Tcbat X TcV/TcVI | 0.005 | 0.025 | 0.015 |
| TcI X TcII | 0.022 | 0.028 | 0.024 |
| TcI X TcIII | 0.002 | 0.005 | 0.003 |
| TcI X TcIV | 0.012 | 0.015 | 0.013 |
| TcI X TcV/TcVI | 0.002 | 0.022 | 0.013 |
| TcII X TcIII | 0.025 | 0.028 | 0.027 |
| TcII X TcIV | 0.027 | 0.033 | 0.029 |
| TcII X TcV/TcVI | 0.000 | 0.028 | 0.014 |
| TcIII X TcIV | 0.015 | 0.017 | 0.015 |
| TcIII X TcV/TcVI | 0.000 | 0.025 | 0.012 |
| TcIV X TcV/TcVI | 0.015 | 0.030 | 0.021 |
| **Interspecific** | | | |
| *T. cruzi* X *T.c.marinkellei* | 0.028 | 0.046 | 0.036 |
| *T.cruzi* X *T. dionisii* | 0.071 | 0.087 | 0.078 |
| *T. cruzi* X *T. rangeli* | 0.129 | 0.145 | 0.137 |
| *T.c.marinkellei* X *T. dionisii* | 0.056 | 0.064 | 0.059 |
| *T.c.marinkellei* X *T.rangeli* | 0.132 | 0.142 | 0.138 |
| *T. dionisii* X *T. rangeli* | 0.127 | 0.130 | 0.128 |
| **Intra-DTU** | | | |
| Tcbat | NC | NC | NC |
| TcI | 0.000 | 0.002 | 0.000 |
| TcII | 0.000 | 0.002 | 0.001 |

| | | | |
|---|---|---|---|
| TcIII | 0.000 | 0.000 | 0.000 |
| TcIV | 0.000 | 0.002 | 0.000 |
| TcV/TcVI | 0.000 | 0.025 | 0.010 |
| | | | |
| **Intraspecific** | | | |
| *T. cruzi* | 0.000 | 0.030 | 0.013 |
| *T.c.marinkellei* | 0.000 | 0.010 | 0.000 |
| *T. dionisii* | 0.000 | 0.000 | 0.000 |
| *T. rangeli* | NC | NC | 0.007 |

NC = not calculated. Low number of sequences available for calculation

Top: genetic distance between *Trypanosoma* species *T. cruzi*, *T. c. marinkellei*, *T. dionisi*i and *T. rangeli*

Bottom: genetic distance within *T. cruzi* subpopulations, *T. c. marinkellei*, *T. dionisii* and *T. rangeli*.

**Additional file 6: Table S4** COI sequences partition into groups inferred with ABGD, based on Kimura 2-parameters.

| Groups | Nº of sequences | Sequence code | DTU |
|---|---|---|---|
| *T. cruzi* | | | |
| Group 1 | 1 | TCC1994 | Tcbat |
| Group 2 | 5 | COLTRYP115,COLTRYP468,COLTRYP018, COLTRYP003, COLTRYP084 | TcI |
| Group 3 | 5 | COLTRYP126, COLTRYP339, Silvio, COLTRYP053, COLTRYP055 | TcI |
| Group 4 | 1 | COLTRYP036 | TcI |
| Group 5 | 9 | COLTRYP128,COLTRYP356,COLTRYP36, Ep88115, COLTRYP048, COLTRYP103, COLTRYP038,COLTRYP039,COLTRYP087 | TcI |
| Group 6 | 7 | COLTRYP136, COLTRYP224, Ep88127, Ep88132, Ep88135, Dm28c, COLTRYP042 | TcI |
| Group 7 | 1 | COLTRYP220 | TcI |
| Group 8 | 2 | COLTRYP305, COLTRYP362 | TcI |
| Group 9 | 1 | Colombiana | TcI |
| Group 10 | 14 | COLTRYP043,COLTRYP062,COLTRYP063, COLTRYP072,COLTRYP081,COLTRYP099, Ep88121, Ep88130, Y, Esmeraldo, COLTRYP006,COLTRYP021,COLTRYP061, COLTRYP121 | TcII |

| | | | |
|---|---|---|---|
| Group 11 | 4 | COLTRYP029,COLTRYP113,COLTRYP370, 3663 | TcIII |
| Group 12 | 10 | 4167, COLTRYP471, COLTRYP524, COLTRYP527,COLTRYP528,COLTRYP529, COLTRYP531,COLTRYP532, COLTRYP041, COLTRYP526 | TcIV |
| Group 13 | 5 | Bug2148, CLBrener, Sc43, Tulacl2, CLBrener (GenBank) | TcV/TcVI |
| *T.c.marinkellei* | | | |
| Group 1 | 4 | COLTRYP107,B7, COLTRYP117,COLTRYP143 | |
| Group 2 | 2 | COLTRYP576, COLTRYP577 | |
| Group 3 | 1 | TCC344 | |
| *T. dionisii* | | | |
| Group 1 | 5 | COLTRYP596,COLTRYP598, COLTRYP621, COLTRYP622,COLTRYP623 | |
| *T. rangeli* | | | |
| Group 1 | 1 | R1625 | |
| Group 2 | 1 | SC58 | |

**Additional file 7: Table S5** GPI sequences partition into groups inferred with ABGD, based on Kimura 2-parameters.

| Groups | Nº of sequences | Sequence code | DTU |
|---|---|---|---|
| *T. cruzi* | | | |
| Group 1 | 1 | TCC1994 | Tcbat |
| Group 2 | 31 | OPS21cl11, COLTRYP115, COLTRYP126, COLTRYP128, COLTRYP136, COLTRYP220, COLTRYP224, COLTRYP305, COLTRYP339, COLTRYP356, COLTRYP362, COLTRYP368, COLTRYP468, Ep88115, Ep88127, Ep88132, Ep88135, Colombiana, Dm28c, COLTRYP048, COLTRYP018, COLTRYP103, COLTRYP053, COLTRYP003, COLTRYP036, COLTRYP038, COLTRYP039, COLTRYP042, COLTRYP055, COLTRYP084, COLTRYP087 | TcI |
| Group 3 | 10 | COLTRYP043, COLTRYP062, COLTRYP063, COLTRYP072, COLTRYP081, COLTRYP099, Ep88130, Y, COLTRYP061, Ep88121 | TcII |
| Group 4 | 10 | COLTRYP121, COLTRYP006, COLTRYP021, Tu18cl2, CLBrener allele1(GenBank), Bug2148 allele1(GenBank), Sc43 allele1, Tulacl2 allele1, Bug2148 allele1, CLBrener allele1 | TcII/TcV/TcVI |

| | | | |
|---|---|---|---|
| Group 5 | 11 | COLTRYP029, COLTRYP113, COLTRYP370, 3663, M6241cl6, CLBrener allele2(GenBank), Bug2148 allele2(GenBank), Sc43 allele2, Tulacl2 allele2, Bug2148 allele2, CLBrener allele2 | TcIII/TcV/TcVI |
| Group 6 | 10 | 4167, COLTRYP471, COLTRYP524, COLTRYP527, COLTRYP528, COLTRYP529, COLTRYP531, COLTRYP532, COLTRYP041, COLTRYP526 | TcIV |
| Group 7 | 1 | CANIIIcl1 | TcIV |
| *T. c. marinkellei* | | | |
| Group 1 | 4 | COLTRYP107, B7, COLTRYP117, COLTRYP143 | |
| Group 2 | 2 | COLTRYP576 | |
| Group 3 | | COLTRYP577 | |
| Group 4 | 1 | TCC344 | |
| *T. dionisii* | | | |
| Group 1 | 5 | COLTRYP596,COLTRYP598, COLTRYP621, COLTRYP622, COLTRYP623 | |
| *T. rangeli* | | | |
| Group 1 | 1 | R1625 | |
| Group 2 | 1 | RGB | |

**Artigo 2. Ecological scenario and *Trypanosoma cruzi* DTU characterization of a fatal acute Chagas disease case transmitted orally (Espírito Santo state, Brazil)**

Maria Augusta Dario, **Marina Silva Rodrigues**, Juliana Helena da Silva Barros, Samanta Cristina das Chagas Xavier, Paulo Sérgio D'Andrea, André Luiz Rodrigues Roque, Ana Maria Jansen

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Analisamos o DNA de tecido cardíaco de uma criança de 2 anos que veio à óbito em decorrência de doença de Chagas (DC) aguda adquirida por via oral, no estado do Espírito Santo. O estudo teve por objetivo determinar quais DTUs de *T. cruzi* estavam relacionadas com este caso fatal de DC. A criança havia manipulado e levado à boca um triatomíneo da espécie *Triatoma vitticeps*, considerado um vetor secundário na transmissão de *T. cruzi*. A extrema gravidade e rapidez com que evoluiu este quadro de DC nos fez conduzir um estudo sobre o cenário ecoepidemiológico deste caso. Assim sendo, DNA foi extraído a partir de blocos de parafina em que estava incluído o tecido cardíaco da criança, gentilmente cedido pela Secretaria de Saúde. Análise molecular por PCR, sequenciamento e clonagem gênica da região V7V8 do gene 18S (SSU) possibilitaram a identificação de infecção mista pelas DTUs TcI, TcII, TcIII e TcIV, além da presença de *T. dionisii*, que até o momento havia sido identificado somente em morcegos. Foram testados também os animais do peridomicílio, que tiveram resultados negativos no hemocultivo. O estudo demonstra a importância de se considerar infecções mistas por espécies e genótipos do gênero *Trypanosoma* no estabelecimento da patogenia da DC.

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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)

Maria Augusta Dario[1], Marina Silva Rodrigues[1], Juliana Helena da Silva Barros[1], Samanta Cristina das Chagas Xavier[1], Paulo Sérgio D'Andrea[2], André Luiz Rodrigues Roque[1] and Ana Maria Jansen[1*]

## 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)

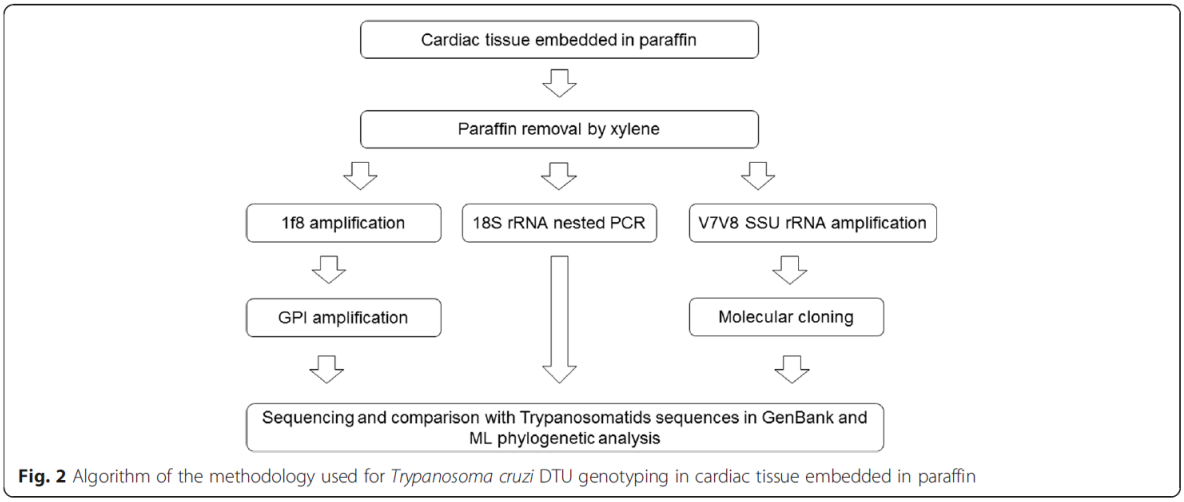


**Fig. 2** Algorithm of the methodology used for *Trypanosoma cruzi* DTU genotyping in cardiac tissue embedded in paraffin

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

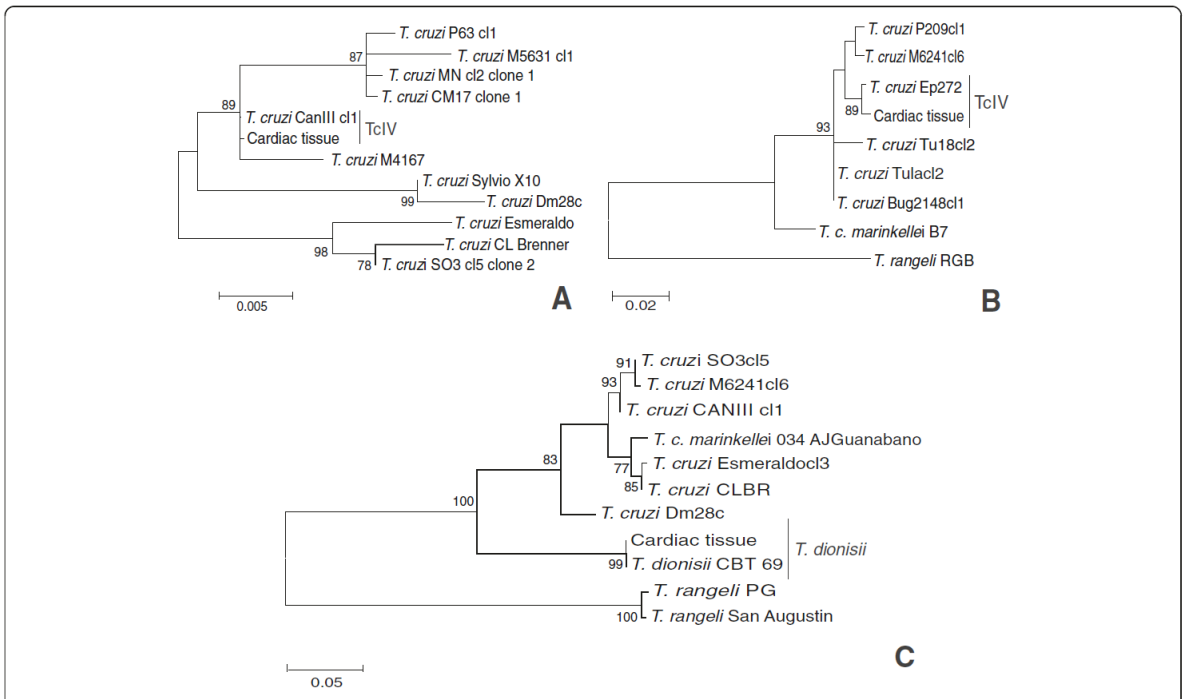


**Fig. 3** Molecular phylogenetic analysis by maximum likelihood methods. **a** for 1f8 gene with HKY parameter and bootstrap value of 1000 replicates; **b** for GPI gene with T92 + G parameter and bootstrap value of 1000 replicates; and **c** for 18S rRNA K2 parameter and bootstrap value of 1000 replicates. 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

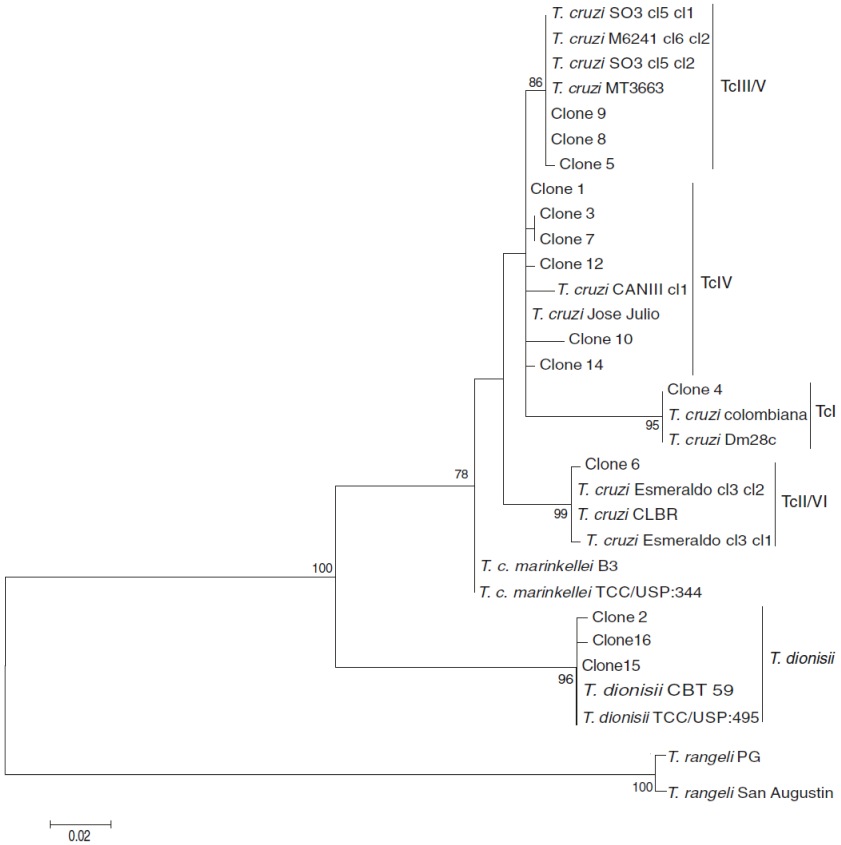


**Fig. 4** Molecular phylogenetic analysis by maximum likelihood of the V7V8 SSU rRNA gene. K2 + G parameter and bootstrap value of 1000 replicates. The clones from cardiac tissue clustered with Jose Julio and CANIII strains (TcIV); SO3cl5 clone1 and clone2, M6241cl6 clone2 and MT3663 strains (TcIII/TcV); 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

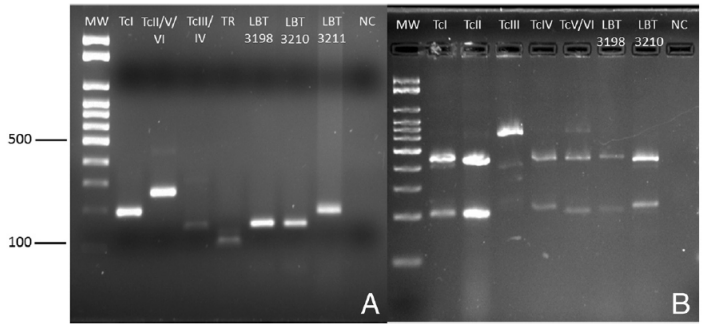


**Fig. 5** *Trypanosoma cruzi* DTU molecular characterization of *T. vitticeps* isolates LBT 3198, LBT 3210 and LBT 3211. **a** Products amplified by the mini-exon gene and analyzed by electrophoresis on 2 % agarose gel stained with ethidium bromide. The sample LBT 3198 and LBT 3210 presented an amplicon 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 Tcl 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*)

### Acknowledgment

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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 3. Uncovering *Trypanosoma* spp. diversity of wild mammals by the use of DNA from blood clots**

**Marina Silva Rodrigues**, Luciana Lima, Samanta Cristina das Chagas Xavier, Heitor Miraglia Herrera, Fabiana Lopes Rocha, André Luiz Rodrigues Roque, Marta Maria Geraldes Teixeira, Ana Maria Jansen

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Estudos anteriores do nosso grupo trouxeram à tona questionamentos sobre especificidade parasitária e a pressão seletiva exercida pelos métodos de cultivo de *Trypanosoma* spp. Infecções por tripanosomas são comumente detectadas através de testes parasitológicos indiretos e de baixa sensibilidade (hemocultivo, xenodiagnóstico). Este trabalho teve por objetivos padronizar e analisar o uso de coágulos sanguíneos como um método menos seletivo e de baixo custo para o diagnóstico da infecção e identificação da diversidade de *Trypanosoma* spp. em mamíferos silvestres de vida livre. A extração de DNA de tripanosomas diretamente de coágulo, seguida de PCR e sequenciamento, proporcionou a identificação e caracterização de *Trypanosoma* spp. em 95/120 (79,2%) amostras, na sua maioria proveniente de indivíduos que apresentaram hemocultura e testes sorológicos negativos. Nos coágulos identificamos duas novas Unidades Taxonômicas Operacionais Moleculares (MOTUs) e infecções mistas. Além disso, foi possível observar um espectro mais amplo de hospedeiros e distribuição geográfica para as espécies *T. dionisii*, *T. lainsoni* e *T. cascavelli*, além de expandir o conhecimento sobre as áreas de distribuição de *T. cruzi* TcII, *T. rangeli* A, *T*. sp. Neobats 2 e 3, *T. janseni* e *T. gennarii*.

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# Uncovering *Trypanosoma* spp. diversity of wild mammals by the use of DNA from blood clots

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ABSTRACT

*Trypanosoma* spp. infection in wild mammals is detected mainly through parasitological tests that usually display low sensitivity. We propose the use of DNA extracted directly from blood clots (BC), which are neglected sources of DNA for diagnosis and identification of *Trypanosoma* spp. This approach followed by nested PCR targeting the 18S SSU rDNA demonstrated to be sensitive and suitable to evaluate the diversity of trypanosomes infecting sylvatic mammals, including subpatent and mixed infections. Infection was detected in 95/120 (79.2%) samples from bats, carnivores and marsupials that included negative serological and hemoculture testing mammals. Thirteen *Trypanosoma* spp. or Molecular Operational Taxonomic Units (MOTUs) were identified, including two new MOTUs. The high diversity of trypanosomes species and MOTUs infecting bats and marsupials showed that these hosts can be considered as bio-accumulators of *Trypanosoma* spp., with specimens of *Didelphis* spp. displaying the highest trypanosome diversity. The use of blood clots allowed direct access to non-culturable parasites, mixed infections, besides bypassing the selective pressure on the parasites inherent to cultivation procedures. *Trypanosoma cruzi* was the species found infecting the highest number of individuals, followed by *T. lainsoni*. Positive PCR for *T. cruzi* was observed in 16 seronegative individuals and 30 individuals with negative hemocultures. Also, *T. lainsoni*, previously found only in rodents, showed to be capable of infecting bats and marsupials. This finding makes it clear that some species of *Trypanosoma* are more generalist than previously thought. Molecular diagnosis using nested PCR from DNA extracted from BC allowed the increase of the knowledge about host-spectrum and distribution of *Trypanosoma* spp. and allowed the identification of new MOTUs.

## 1. Introduction

The genus *Trypanosoma* Gruby, 1843 (Kinetoplastea; Trypanosomatida; Trypanosomatidae) is a monophyletic taxon (Stevens et al., 2001; Leonard et al., 2011). This genus is characterized by wide dispersion, as regards to geographic distribution and host range (Hoare, 1972; Spodareva et al., 2018; Jansen et al., 2018). All its representatives are parasites although they present quite different life strategies (Hoare, 1972). The diversity of *Trypanosoma* spp. species remains underestimated. This is largely due to the existence of numerous non-culturable taxa, non-sensitive parasitological diagnostic methods, and the low accessibility due to the high cost of next-generation sequencing methods (NGS).

*Trypanosoma* spp. includes species that have been described as highly specialists as it is the case of *T. minasense*, to date associated only to non-human primates (Martínez et al., 2016); other trypanosomes are generalists, and capable of infecting hosts from different orders, as observed in *T. cruzi* and *T. rangeli* (Jansen et al., 2018; Espinosa-Álvarez et al., 2018).

In addition to including representatives related to severe human and

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animals' diseases, the genus *Trypanosoma* presents numerous and important still unanswered questions regarding diversity, host specificity, distribution, occurrence and consequence of mixed infections and phylogenetic relatedness between clades, species and molecular operational taxonomic units (MOTUs).

Molecular tools with higher analytical power have arisen in the last decade and have increased the recognition and description of several new *Trypanosoma* species as well as new MOTUs infecting vertebrates of all taxa and all habitats worldwide (Viola et al., 2009; Cottontail et al., 2014; Lemos et al., 2015; Cooper et al., 2017; Spodareva et al., 2018). The current awareness of the deep interdependence of human, animal and environmental health has contributed to the increasing recognition of the importance of including parasites in biodiversity studies (Robertson et al., 2014). Also, it has stimulated the search for knowledge of parasites not necessarily related to human or animal diseases. Obviously, the analysis of the phylogenetic relations between these parasites has been and will be constantly altered as new host species and trypanosome taxa are described. Additionally, only recently a broader spectrum of orders and species of wild animals became the subject of integrated studies among parasitologists and other health professionals.

*T. cruzi*, the etiological agent of Chagas disease (Chagas, 1909), is genetically heterogeneous and is presently grouped in seven genotypes (TcI to TcVI and Tcbat) or discrete typing units (DTUs) (Zingales et al., 2012). *T. cruzi* is primarily an enzooty of wild mammals, infects hundreds of mammalian species and is transmitted by dozens of triatomine species throughout all of the biomes of America between the southern United States and South Argentina (Brenière et al., 2016; Jansen et al., 2018). Marsupials and bats are recognized as very ancient hosts of *T. cruzi* clade (Stevens et al., 1998; Hamilton et al., 2012; Lopes et al., 2018). However, the origin of this clade still remains inconclusive. Marsupials and bats have also been described as bio-accumulators of *Trypanosoma* species, due to their ability to host an expressive diversity of trypanosome species and taxonomic units (MOTUs) (Jansen et al., 2018).

Carnivores, a poorly studied mammalian taxon due to the difficulty in trapping and handling them, are important in the maintenance of *T. cruzi* in nature as well (Rocha et al., 2013b). As top chain predators, they are exposed to *T. cruzi* infection through the oral route and have also been proposed as *Trypanosoma* spp. bio-accumulators of parasites (Rocha et al., 2013a, b).

Serological tests display high sensitivity but low specificity and are restricted to availability of species specific conjugate as well as species-specific parasite antigens (Jansen et al., 2015)., consequently they are only rarely performed. Diagnosis of trypanosome infection in sylvatic mammals is made mainly through hemoculture and fresh blood smears examination. Positive fresh blood smears and hemocultures display low sensitivity, but are irreplaceable tools that indicate the competence of the animal to be a source of infection for the vector (Gomes et al., 1999; Siriano et al., 2011; Teston et al., 2016; Jansen et al., 2018). The isolation and maintenance methods allow further morphological and biological studies, but exert selective pressure on the subpopulations of the parasite, favoring some and excluding others. As a result, what grows in the culture media does not necessarily reflect the original composition of the parasitic populations in the host (Lopes et al., 2018; Jansen et al., 2018).

The analysis of trypanosome DNA using PCR is a sensitive and specific approach that allows the detection of infection in initial stages, low parasitemias, and identification of known and unknown species (Hutchinson and Stevens, 2018). A neglected source of DNA is the blood clot (BC), which is usually discarded after serum separation (Fitzwater et al., 2008; Lundblom et al., 2011; Bank et al., 2013). Fitzwater et al. (2008) hypothesized that *T. cruzi* trypomastigotes would be trapped in the cellular portion of blood clots and, thus would be a rich source of trypanosome DNA. BC was described as being suitable to be stored for long periods besides requiring low volumes (100–1000 μL) for DNA extraction (Fitzwater et al., 2008; Lundblom et al., 2011; Bank et al., 2013; de Abreu et al., 2018). This material has already been used to diagnose infection by *T. cruzi* and *Plasmodium falciparum* in humans (Fitzwater et al., 2008; Lundblom et al., 2011), *T. cruzi* in dogs (Curtis-Robles et al., 2017), *Plasmodium* spp. in humans and non-human primates (de Abreu et al., 2018), *Aspergillus* spp. in experimental rodent model (McCulloch et al., 2009) and *Leishmania* spp. in dogs (Costa et al., 2015).

Considering these pros and cons, it becomes clear that trypanosome identification should be performed with different and complementary methodologies. Here we used fresh blood smears examination, hemoculture, serology, and molecular characterization. We propose the use of DNA extracted directly from blood clots followed by PCR, based on the very probably underestimation of *Trypanosoma* spp. diversity. We are confident that this will be a cost effective, less selective methodology that can be applied in trypanosome research in any mammal host. In this study, we evaluated the diversity of trypanosomes using DNA from blood clots of sylvatic free-ranging mammals. We focused primarily on marsupials, bats and carnivores, all of them already proposed as *Trypanosoma* bio-accumulators (Rocha et al., 2013a; Jansen et al., 2015; Roman et al., 2018).

## 2. Materials and methods

### 2.1. Fresh blood smears examination, hemocultures and serological tests

Blood samples (approximately 5 μL) were examined by light microscope for the presence of flagellates; 300 μL were cultured in two tubes containing Novy, McNeal and Nicolle plus Liver Infusion Tryptose (NNN/LIT) medium. The hemocultures were examined fortnightly for five months. Positive hemocultures, which demonstrated parasite growth, were amplified, cryopreserved, and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, COLTRYP/Fiocruz.

Serological diagnoses were obtained using an adapted version of the IFAT described by Camargo (1966). Reference strains I00/BR/00F (TcI) and MHOM/BR/1957/Y (TcII) from axenic cultures were mixed in equal proportions (1:1) and used as antigens. Carnivore sera were tested with anti-dog IgG coupled to fluorescein isothiocyanate (Sigma, St. Louis, Missouri, USA). Didelphimorphia were tested with the specific intermediary antibody anti-*Didelphis* spp. IgG raised in rabbits, and the reaction was revealed by an anti-rabbit IgG conjugate. The cut-off values adopted by LABTRIP were 1:20 and 1:40, respectively, for Carnivore and Didelphimorphia (Rocha et al., 2013a; Xavier et al., 2014). Chiroptera has not been serologically tested due to the absence of specific antibodies for this group.

### 2.2. Blood clot samples

Table 1 and Supplementary Table S1 display the data of the wild mammal species, their geographical origin, hemoculture and serology results, as well as molecular identification of *Trypanosoma* spp. in blood clots. In short, a total of 120 samples were obtained from free-ranging mammals of the orders Carnivora (n = 15), Chiroptera (n = 30) and Didelphimorphia (n = 75). These samples were derived from 24 species included in 17 genera, of five Brazilian biomes (Amazon Forest, Atlantic Forest, Cerrado, Pampa and Pantanal) (Table 1). Mammals were captured as part of prior studies (Rocha et al., 2013a; Dario et al., 2017b). Our selection criteria were: i) mammals with negative fresh blood smears, hemoculture and serology (excepting bats) (Supplementary Table S1); ii) mammals with positive hemocultures (n = 3) and/or serology. The serological diagnosis of *T. cruzi* infection and hemoculture were performed before the molecular characterization in the Laboratório de Biologia de Tripanosomatídeos (LABTRIP – Instituto Oswaldo Cruz, Fiocruz, Brazil) (details in section 2.1).

**Table 1**
Host species, geographical origin, positive IFAT and molecular identification of *Trypanosoma* spp. in blood clots from Carnivora, Chiroptera, and Didelphimorphia.

| Host species | State/Biome | Number of specimens | Positive IFAT (*T. cruzi*) | Molecular identification (18S SSU) |
|---|---|---|---|---|
| **Carnivora** | | | | |
| *Cerdocyon thous* | MS/Pantanal | 10 | 3 | TcI (4) |
| | RS/Pampa | 2 | 1 | TcI (2) |
| *Lycalopex gymnocercus* | RS/Pampa | 3 | 3 | TcI (2); TcI/*T. dionisii* (1) |
| **Chiroptera**[a] | | | | |
| *Artibeus. fimbriatus* | RJ/Atlantic Forest | 1 | | TcII (1) |
| *Artibeus lituratus* | PB/Atlantic Forest | 6 | | TcII (1); *T.* sp. Neobat 2 (1); *T.* sp. Neobat 3 (3) |
| | RJ/Atlantic Forest | 5 | | TcII (1); *T.* sp. Neobat 3 (2) |
| *Artibeus planirostris* | PB/Atlantic Forest | 5 | | *T. lainsoni* (1); *T.* sp. Neobat 2 (2) |
| *Carollia perspicillata* | PB/Atlantic Forest | 2 | | TcII (1) |
| | RJ/Atlantic Forest | 1 | | *T.* sp. Neobat 1 (1) |
| *Desmodus rotundus* | PB/Atlantic Forest | 1 | | TcI (1) |
| | RJ/Atlantic Forest | 1 | | |
| *Glossophaga soricina* | PB/Atlantic Forest | 1 | | TcI (1) |
| *Phyllostomus hastatus* | RJ/Atlantic Forest | 2 | | TcII (1) |
| *Platyrrhinus lineatus* | PB/Atlantic Forest | 3 | | TcI (1); *T. lainsoni* (1) |
| *Sturnira lilium* | RJ/Atlantic Forest | 2 | | *T. dionisii* (1) |
| **Didelphimorphia** | | | | |
| *Didelphis albiventris* | GO/Cerrado | 2 | 0 | *T. dionisii* (1); *T.* sp. DID (1) |
| | PB/Atlantic Forest | 17 | 5 | TcI (2); *T. cascavelli* (6); *T. janseni* (4) |
| *Didelphis aurita* | RJ/Atlantic Forest | 5 | 0 | *T. janseni* (2); *T.* sp. DID (3) |
| *Didelphis marsupialis* | AC/Amazon | 3 | 1 | TcI (1); TcII (2) |
| *Gracilinanus agilis* | GO/Cerrado | 33 | 3 | TcI (2); TcI/*T. dionisii* (1); TcI/*T. dionisii*/*T. lainsoni* (1); *T. dionisii* (6); *T. lainsoni* (21); *T. lainsoni*/*T. gennarii* (1); *T. rangeli* A (1) |
| *Marmosa demerarae* | PB/Atlantic Forest | 1 | 0 | *T. cascavelli* (1) |
| *Marmosa murina* | PB/Atlantic Forest | 1 | 0 | TcII (1) |
| *Marmosa paraguayana* | ES/Atlantic Forest | 1 | 0 | |
| *Marmosops incanus* | ES/Atlantic Forest | 2 | 0 | TcII (1) |
| *Metachirus nudicaudatus* | ES/Atlantic Forest | 3 | 0 | *T. dionisii* (1) |
| *Metachirus* sp. | AC/Amazon | 1 | 0 | TcI (1) |
| *Micoureus paraguayanus* | ES/Atlantic Forest | 1 | 0 | *T. lainsoni* (1) |
| *Monodelphis americana* | ES/Atlantic Forest | 1 | 0 | TcI (1) |
| *Philander* sp. | AC/Amazon | 4 | 2 | TcII (2); *T. rangeli* A (2) |

IFAT: Immunofluorescence Antibody Test.
[a] Chiroptera has not been tested for serology due to the absence of specific commercial antibodies for this group.

### 2.3. DNA extraction from blood clots

Blood clots were previously stored in absolute ethanol. DNA was extracted based on the ammonium acetate precipitation protocol used for bird blood as described previously (Garcia et al., 2018). In summary, volumes of 50, 100 or 200 µL of blood clots were used for DNA extraction. The absolute ethanol was removed and we added a step of centrifugation at 17,900g for 10 min in buffer (38 mM NaCl, 10 mM EDTA, 5 mM Tris-Cl) to remove any ethanol residue. The supernatant was removed, and the pellet was resuspended in 200 µL of Digsol buffer (120 mM NaCl, 20 mM EDTA, 50 mM Tris-Cl, 1% SDS) and 20 µL of proteinase K at 20 mg/mL (Invitrogen, California, USA). The tubes with this mixture were incubated in a thermo-shaker at 55 °C for 3 h. After incubation 400 µL of 4 M ammonium acetate were added to each tube. DNA was resuspended in 25 µL of buffer (10 mM Tris-HCl pH 7,4; 1 mM EDTA pH 8,0) and stored at −20 °C until use. DNA concentration and purity (OD260/OD280 ratio) was quantified using NanoDrop (Thermo Scientific, Waltham, Massachusetts, USA).

### 2.4. Polymerase chain reaction (PCR) and sequencing

A fragment of approximately 650 bp of the 18S (SSU) rRNA gene was amplified using the two sets of primers previously described (Noyes et al., 1999). The two rounds of the nested PCR were conducted in a final volume of 25 µL containing 8.5 µL of GoTaq MasterMix (Promega, Madison, Wisconsin, USA), 20 pmol of each primer (IDT, Coralville, Iowa, USA), 50–100 ng of DNA template and ultrapure water to reach the final volume. Ultrapure water and *T. cruzi* DNA from positive hemocultures were, respectively, used as negative and positive controls. The amplification was performed using a Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA) with the following cycle conditions: initial denaturation at 94 °C for 3 min; followed by 35 cycles at 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 90 s; and a final elongation step at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gels and stained with GelRed (Biotium, Inc., California, USA). The fragments were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit, according to the manufacturer's instructions (GE healthcare, Illinois, USA), and direct sequencing of both strands of DNA was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

Samples that did not amplify in the first attempt were again subjected to PCR in the presence of 10% of dimethyl sulfoxide (DMSO) and 5% of bovine serum albumin (BSA) at 2.0 mg/µL in the final volume of the first round of the 18S nested PCR (Fig. 1), as standardized by Farell and Alexandre (2012). The second round was performed as described above in this section.

### 2.5. Molecular cloning

Samples with electropherograms that demonstrated two or more peaks for the same position were suspected of mixed infection and were cloned (Fig. 1). Cloning was performed with pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA) following the manufacturer's protocol. Two to eight colonies were randomly collected, and minipreps were performed with Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, Wisconsin, USA) and sequenced.

### 2.6. Data analysis

The sequences were manually edited using SeqMan™ version 7.0 (DNASTAR, Madison, Wisconsin, USA) and aligned using the M-Coffee meta-multiple sequence alignment web server (Moretti et al., 2007). All

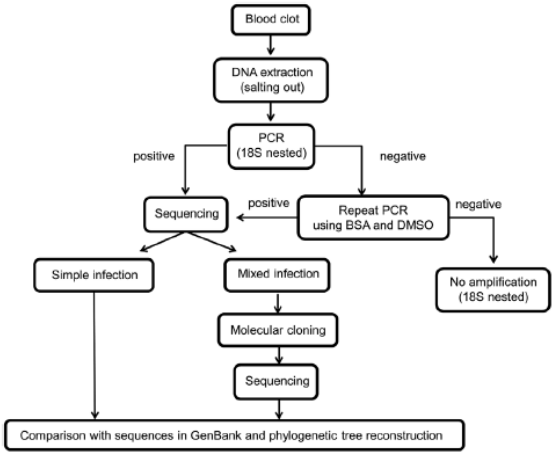


Fig. 1. Methodological algorithm employed for the identification of trypanosomes in blood clot samples.

sequences deposited in the GenBank database corresponded to consensus sequences based on overlap of both forward and reverse sequences (Supplementary Table S1).

Neighbor-joining (NJ) method and Kimura 2- parameters model were performed with MEGA version 6 (Tamura et al., 2013). For each node, bootstrap percentages (BP) were computed after 1000 resamplings. Maximum Likelihood (ML) analyses were performed using PhyML 3.0 (Guindon et al., 2010). For each node, BP was computed after 1000 resamplings. The model of nucleotide substitution that best fitted the 18S data was the general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR + Γ+ I) for the *T. cruzi* clade dataset and Tamura-Nei with gamma-distributed rate variation across sites (TN93 + Γ) for the lizard/snake/rodent/marsupial clade. The model was selected using the Akaike Information Criterion (AIC) in the Smart Model Selection in PhyML (Lefort et al., 2017). Bayesian inference (BI) was run in MrBayes v3.2.6 (Ronquist et al., 2012) with a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR + Γ+ I). The runs converged after 1,000,000 generations, by sampling every 100th generation and discarding the first 25% of the trees as burn-in. Pairwise intra- and inter-specific genetic distances were calculated using MEGA version 6 (Tamura et al., 2013).

## 3. Results

### 3.1. Diagnosis of Trypanosoma spp. from blood clots

DNA extracted directly from blood clots followed by nested PCR demonstrated to be a suitable method to identify trypanosomes, allowing the detection of these flagellates in animals that displayed negative hemocultures, (*i.e.* undetectable parasitemia), and also, the presence of new MOTUs and trypanosomes species that are non-amplifiable in axenic media. Infection by *Trypanosoma* spp. was detected in 95/120 (79.2%) samples (Tables 1 and 2; Fig. 2), including flagellates in animals that displayed negative hemocultures, (i.e. undetectable parasitemia), and also, the presence of new MOTUs.

The addition of DMSO and BSA resulted in the reversal of an earlier negative result in 12 out of 37 BC samples that were previously tested for *Trypanosoma* spp. infection in the BC PCR.

We observed positive PCR for *T. cruzi* in blood clots of 16 of 24 individuals with previously negative IFAT (Tables 1 and 2). The opposite situation was also observed in that animals with positive serology for *T. cruzi* presented negative PCR reactions (Tables 1 and 2). Moreover, the animals that displayed positive hemocultures by *T. cruzi* also tested positive by blood clot PCR (Tables 1 and 2).

**Table 2**
*Trypanosoma* spp. infection detected in DNA extracted from blood clot of Carnivora, Chiroptera and Didelphimorphia: serological test, hemoculture and molecular characterization with 18S (SSU).

| | Serology[a] | |
|---|---|---|
| | Positive (n) | Negative (n) |
| *T. cruzi* in blood clot | 8 | 16 |
| No amplification | 3 | 12 |
| | **Hemoculture** | |
| | Positive n (%) | Negative n (%) |
| Culturable trypanosomes in BC[b] | 3 (2.5) | 41 (34.2) |
| Unculturable trypanosomes in BC[c] | 0 | 51 (42.5) |
| No amplification | 0 | 25 (20.8) |

[a] Results for chiropterans were not considered.
[b] Culturable trypanosomes: *T. cruzi*, *T. dionisii* and *T. rangeli*.
[c] Unculturable trypanosomes or trypanosomes that grow poorly in axenic media: *T. cascavelli*, *T. gennarii*, *T. janseni*, *T. lainsoni*, *T.* sp. DID, *T.* sp. Neobat 2, Neobat 3 and Neobat 4.

### 3.2. Diversity of Trypanosoma spp. detected by PCR of blood clots

Thirteen *Trypanosoma* species or MOTUs were identified, among them, two new MOTUs (*T.* sp. Neobat 4 and *T. sp.* DID). All main branches had high support (> 85) for at least two methods of phylogenetic tree reconstruction (Figs. 3 and 4). PCR from blood clots showed that Didelphimorphia presented a higher infection rate (88.0%) in comparison with Chiroptera (66.7%) and Carnivora (60.0%) and also demonstrated to harbor the highest diversity of trypanosome species (Table 1; Fig. 2). *Didelphis* spp. was the taxon that displayed the highest *Trypanosoma* spp. diversity (Fig. 2). *T. cruzi* was the species found infecting the highest number of individuals (marsupials, carnivores and bats), followed by *T. lainsoni* (marsupials and bats), *T. dionisii* (marsupials, bats and carnivores) (Table 1; Fig. 2). In bats, we observed a new MOTU that we labeled as *Trypanosoma* sp. Neobat 4; further on, *Trypanosoma* sp. Neobat 2 and Neobat 3 (Fig. 3). Concerning marsupials, infections by a new MOTU that we named DID, besides infections by *T. cascavelli*, *Trypanosoma janseni*, *Trypanosoma rangeli* A, and sequences closely related to *Trypanosoma gennarii* have been observed (Fig. 4).

### 3.3. Host and geographical distribution

The most frequent and widely dispersed *T. cruzi* DTU was TcI that was identified infecting marsupials, bats and carnivores in all studied biomes (Figs. 2 and 5). The second more dispersed genotype was DTU TcII that was identified in marsupials and bats (Figs. 2 and 5). TcIII was of more restricted distribution and was found only infecting Chiroptera (Figs. 2 and 5).

*T. rangeli* lineage A was infecting two *Philander* sp., in Acre state, and one *Gracilinanus agilis*, captured in Goiás state (Fig. 2).

Concerning the recently described *T. janseni*, we detected this trypanosome in the state of Paraíba and Rio de Janeiro (Figs. 2 and 5). All marsupials infected by *T. janseni* are *Didelphis* spp. that, except one, had negative serological titers (Supplementary Table S1).

We were able to identify the so-called Neobat 2 and Neobat 3 trypanosomes (Figs. 2 and 5). in *Artibeus lituratus* and *Artibeus planirostris* of Brazilian southeastern and northern regions (Fig. 5).

We also detected infection by *T. dionisii*, a trypanosome classically

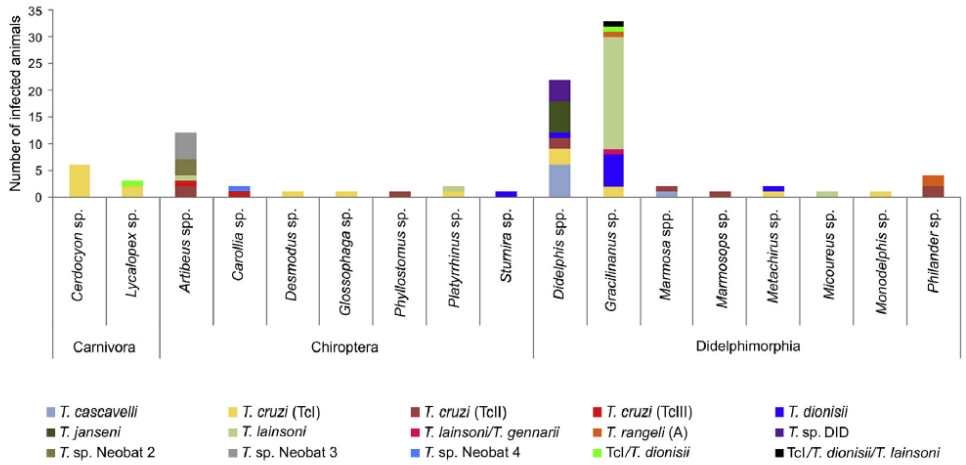


**Fig. 2.** *Trypanosoma* spp. identified in the blood clot of Carnivora, Chiroptera and Didelphimorphia. Each color indicates a different trypanosome species, genotype or mixed infections. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

associated with Chiroptera, in marsupials. Furthermore, in our samples, we found a greater number of didelphids infected with *T. dionisii* than bats. *T. dionisii* was identified, in single infection, in *Sturnira lilium* (n = 1), *Didelphis albiventris* (n = 1), *G. agilis* (n = 6) and *Metachirus nudicaudatus* (n = 1) (Table 1; Fig. 2).

*T. cascavelli* from the lizard/snake/rodent/marsupial clade was found infecting marsupials: *Marmosa* sp. and *D. albiventris* (Figs. 2 and 5; Table 1). *T. lainsoni*, from the same clade, was also found infecting the marsupials *Micoureus paraguayanus* and *G. agilis*, and the bats *A. lituratus* and *P. lineatus* (Figs. 2 and 5). One marsupial specimen (*G. agilis*) infected by *T. lainsoni* was serologically positive for *T. cruzi*, while the others (n = 23) were negative or presented titers not higher than the adopted cut-off (Supplementary Table S1). The genetic distance analyses of *T. cascavelli* sequences presented a range of 0.000–0.007. The same was observed for *T. lainsoni* (Supplementary Table S2).

### 3.4. Two novel molecular operational taxonomic units (MOTUs) identified in Didelphis spp. and Carollia perspicillata

We identified in *Didelphis* spp. nucleotide sequences from *Trypanosoma* sp. (n = 4) which did not correspond to any sequence available on GenBank, for the 18S (SSU) region. We called this molecular taxonomic unit "DID", just in reference to the host *Didelphis* spp. This MOTU was identified in three *Didelphis aurita* from Rio de Janeiro, Atlantic Forest biome, and one *D. albiventris* in Goiás, Cerrado biome (Fig. 5; Table 1). The phylogenetic analysis showed that "DID" sequences are positioned in the *T. cruzi* clade, near to *T. janseni* and *Trypanosoma* sp. Neobat 1 (Fig. 3). Interspecific genetic distance analysis confirmed this finding (Supplementary Table S3). All blood cultures and serological tests of these four marsupials were negative.

We also identified in the bat *Carollia perspicillata* captured in Rio de Janeiro a trypanosome DNA which did not have a match on GenBank, for the 18S (SSU) region. Since these sequences were similar to the other *Trypanosoma* sp. Neobat genotypes, we named this MOTU "Neobat 4". This new MOTU differs by 4 nucleotides from *T.* sp. Neobat 1 (Supplementary Fig. S1). *T.* sp. Neobat 2 and *T.* sp. Neobat 3 sequences differ by 5 nucleotides (Supplementary Fig. S1). The genetic distance between *T.* sp Neobat 2 and *T.* sp Neobat 3 was the same as observed between *T.* sp Neobat 1 and *T.* sp Neobat 4 (Supplementary Table S3). This supports "Neobat 4" as a different MOTU. Hemoculture was negative for all individuals infected with *T.* sp Neobats.

### 3.5. Mixed infection

We observed few mixed infections, but in diverse combinations. TcI and *T. dionisii* were observed in one *Lycalopex gymnocercus* and in one *G. agilis* (Figs. 2 and 3; Table 1). Triple infection with *T. cruzi* TcI, *T. dionisii* and *T. lainsoni* was observed in one marsupial (*G. agilis*) (Figs. 2–4; Table 1). Finally, a co-infection of a marsupial (*Gracilinanus* sp.) by *T. lainsoni* and a trypanosome that displayed a genetic distance of 0.000–0.005 from *T. gennarii* was also observed (Fig. 4; Table 1; Supplementary Table S2).

## 4. Discussion

The possibility of working with blood clots showed at least four advantages: i) mitigation of the selective pressures inherent to the isolation, maintenance, and amplification of the flagellates in axenic culture; ii) direct access to non-culturable parasites species; iii) the need of only small volumes of material for DNA extraction; and iv) the possibility of permanent storing of the clots in absolute ethanol for the use in retrospective studies. PCR of blood clots allowed us to increase the knowledge of the diversity of trypanosomatids of bats, canids and marsupials. The possibility of using small volumes is especially advantageous in the case of wild free-ranging small mammal species that like bats, have small body mass and, consequently, low blood volume (Hooper and Amelon, 2014). Besides these advantages blood clots were considered the most suitable tissue to be used in the detection of *T. cruzi* infection through PCR when compared to buffy coat and whole blood (Fitzwater et al., 2008).

The success in the detection and identification of a trypanosome depends on the parasite's load in the animal's circulation and not necessarily to the volume of blood collected. We were able to identify trypanosome DNA in 50 µL of blood clot of animals with low parasitemias as demonstrated by negative fresh blood smears and hemoculture.

Positive nested PCR was observed for *T. cruzi* in seronegative Carnivora and Didelphimorphia. The seronegativity in canids can be due to the use of non-specific conjugate (anti-dog IgG), but a possible explanation for both cases is the animals being still in the very initial phase of infection., when the animals did not have time to produce antibody against the parasite, especially because only IgG anti-*T. cruzi* was surveyed. Late *T. cruzi* seroconversion has been observed in *D. aurita* experimentally infected by the oral route (AMJ, personal

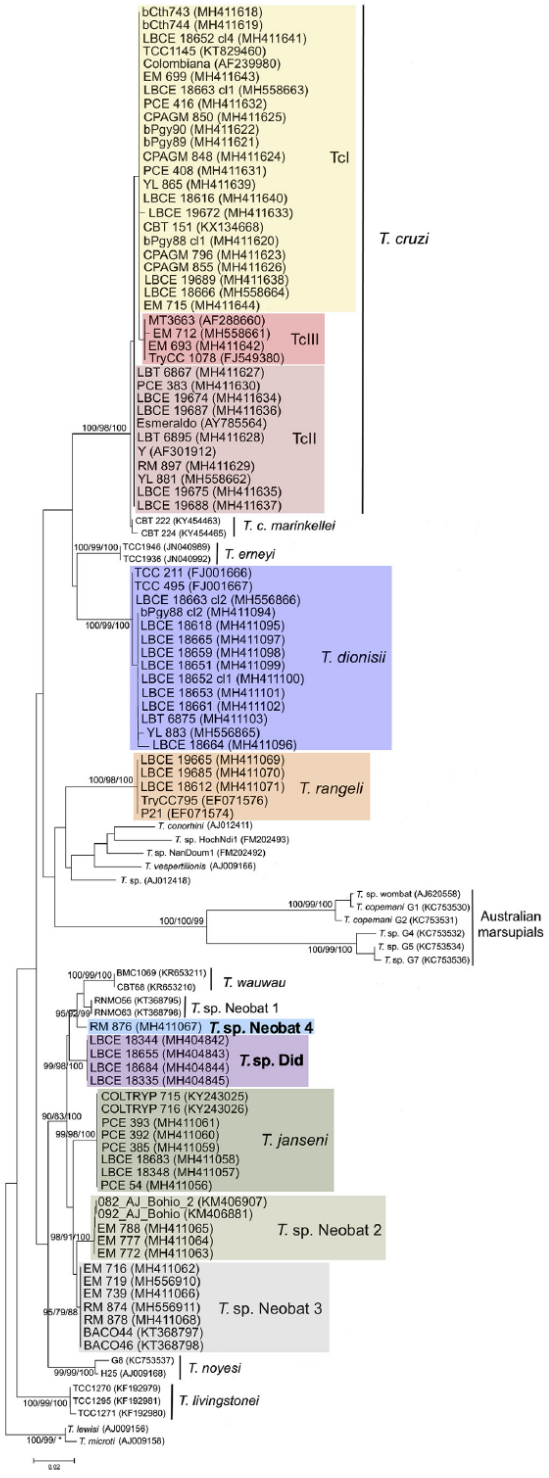


Fig. 3. *T. cruzi* clade phylogenetic tree based on 18S (SSU) gene. The tree shows ten different species and genotypes identified in the blood clot of Carnivora, Chiroptera, and Didelphimorphia: *T. cruzi* (DTUs TcI, TcII and TcIII), *T. dionisii*, *T. rangeli*, *T.* sp. Neobats 2 and 3, *T. janseni*, and two novel MOTUs (*T.* sp. DID and *T.* sp. Neobat 4). The tree was inferred with neighbor-joining. The numbers at the nodes correspond, respectively, to NJ, ML and BI support values for the main branches. The scale-bar shows the number of nucleotide substitutions per site. *Trypanosoma lewisi* and *Trypanosoma microti* were used as outgroups.

communication). *T. cruzi* detected by PCR in seronegative individuals has also been reported in humans (Gomes et al., 1999; Salomone et al., 2003; Gilbet et al., 2013).

Positive PCR for *T. cruzi* in individuals with negative hemocultures (Table 2) confirms hemocultures as a non-sensitive technique besides indicating that these hosts had, at the moment of blood collection, very low *T. cruzi* parasitemia and therefore low infectivity potential. Animals with positive serology for *T. cruzi* and negative PCR are also found by other authors and can be explained by the fact that antibodies are in solution and, therefore, distributed homogenously. Each serum sample tested is representative of the whole. DNA, on the contrary, is in suspension and, consequently, the analysis of an aliquot does not necessarily contain DNA molecules and therefore do not assure the sample negativity.

As expected, *T. cruzi* TcI was the most widely spread genotype in terms of host species and geographical distribution (Zingales et al., 2012; Brenière et al., 2016). The detection of *T. cruzi* TcII in the Amazon region confirms the broad distribution also of this DTU, previously associated to human disease below the Amazon region (Brenière et al., 2016). The DTU TcII was also already found infecting wild mammals in countries such as Bolívia, Colombia, Suriname and the United States (del Puerto et al., 2010; Ramírez et al., 2014; Lima et al., 2015a; Pronovost et al., 2018). TcIII, proposed as being associated with armadillos (Llewellyn et al., 2009; Acosta et al., 2017) was already identified in marsupials, rodents, dogs and bats, and herein confirmed infecting bats (Marcili et al., 2009; Jansen et al., 2015; Barros et al., 2017). These data reinforce that there is no evidence of any kind of association between *T. cruzi* DTU and mammal species, biome or forest strata. This seems also to be the case of the *T. rangeli* lineage A in Brazil, that was hitherto described in monkeys and bats in the states of Acre, Mato Grosso do Sul and Pará (Maia Da Silva et al., 2007, dos Santos et al., 2017; Espinoza-Álvarez et al., 2018), and *Didelphis marsupialis* and *Rhodnius robustus* in the states of Minas Gerais and Rondônia (Maia Da Silva et al., 2007). Our results demonstrated that the host and geographic distribution of this lineage are wider than assumed up to the present and that, probably, future studies will expand it even more.

*T. janseni* is demonstrating to be more widespread than formerly reported, but up to now still restricted to *Didelphis* spp. This trypanosome was first described in Rio de Janeiro (Lopes et al., 2018) and now we report it in the Paraíba state. Both areas are included in the Atlantic Forest but are 2.500 km apart. We still do not know about *T. janseni*'s ecology; however, the negative blood cultures of marsupials identified with this parasite indicate that it can probably be transmitted even during low parasitemia or the hosts display very short parasitemic period.

The finding of new bat host species of *Trypanosoma* sp. Neobat 2 and Neobat 3 widens host spectrum and geographical distribution of this group of bat trypanosomes. To date, no information is available on the morphology of these trypanosomes and their vectors are still unknown. Bats rest in habitats that are also shared with hematophagous insects, among them probably also the vectors of these trypanosomes (Froidevaux et al., 2018). Additionally, it is worth mentioning that bats ancestral diet included basically insects and except for the hematophagous, all other bats may still include insects in their diets, which means that bats can get the infection by the oral route (Carrillo-Araujo et al., 2015).

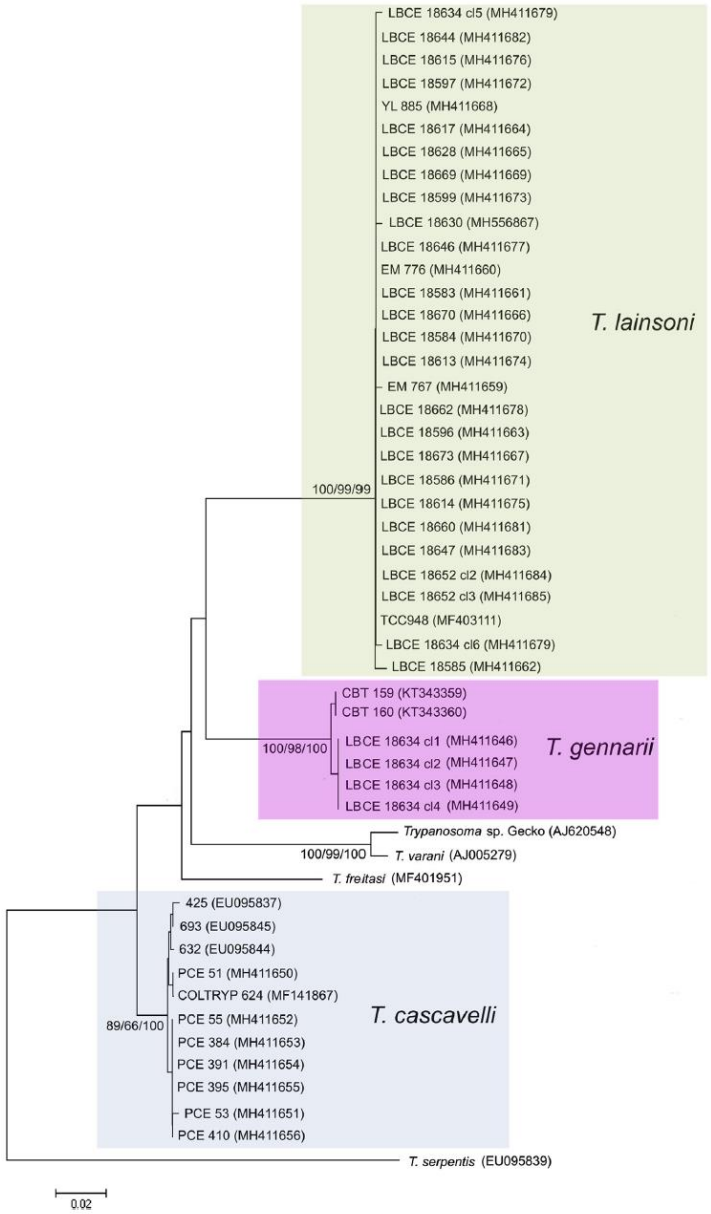


Fig. 4. Lizard/snake/rodent/marsupial clade phylogenetic tree based on 18S (SSU) gene. The tree shows the three different species from the lizard/snake/rodent/marsupial clade identified in the blood clot of Chiroptera and Didelphimorphia: *T. cascavelli*, *T. gennari*, and *T. lainsoni*. Tree inferred with neighbor-joining. The numbers at the nodes correspond, respectively, to NJ, ML and BI support values for the main branches. The scale-bar shows the number of nucleotide substitutions per site. *Trypanosoma serpentis* was used as outgroup.

Neobat groups were originally described in Panama (Cottontail et al., 2014) and *T.* sp. Neobat 3 was also found in Colombia (Lima et al., 2015b). In Brazil, the wide distribution of *T.* sp. Neobats observed here and by other authors has been suggested as the consequence of bats high dispersal capacity and lifespan (Luis et al., 2013; Lima et al., 2015b; Dario et al., 2017b; dos Santos et al., 2017).

*T. dionisii*, previously associated to bats, seems to be a generalist trypanosomatid. There are already reports of this trypanosome infecting marsupials and one human (Dario et al., 2016, 2017b). Here we observed *T. dionisii* in other marsupial species and are reporting a new host, the carnivore *Lycalopex gymnocercus*. All these mammals are generalists feeding on fruits, insects and predating small vertebrate exposing them to infection by the oral route (Cheida et al., 2011; Rossi and Bianconi, 2011; Lessa and Geisi, 2014). Very probably, both, oral

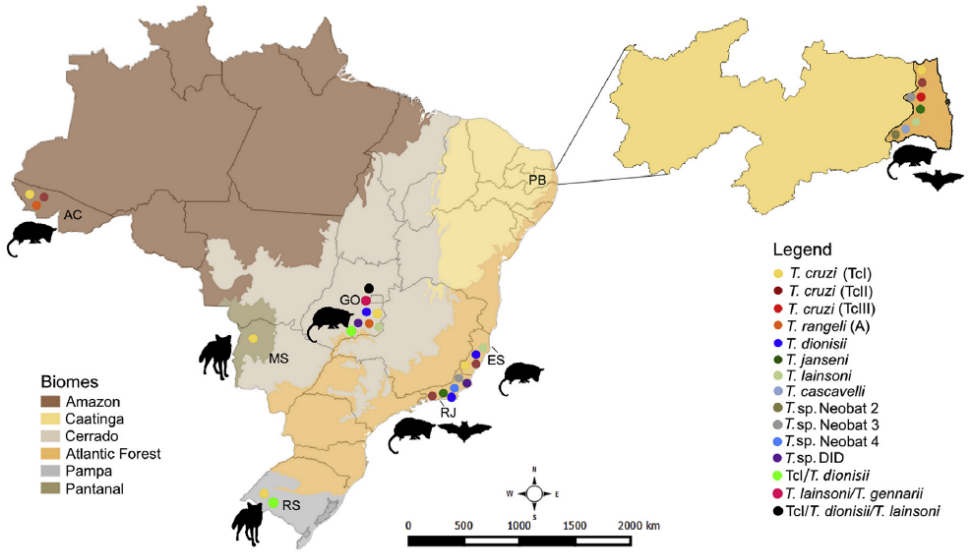


Fig. 5. Map of the distribution of the *Trypanosoma* spp. identified in this study. Thirteen different trypanosomes species/genotypes/MOTUs were identified, in single and mixed infection, in the blood clot of bats, carnivores and marsupials. The trypanosomes are distributed in five Brazilian biomes (Amazon Forest, Atlantic Forest, Cerrado, Pampa, and Pantanal). Each colored circle indicates different trypanosome species/genotypes/MOTUs. Abbreviations: Brazilian states: AC, Acre; ES, Espírito Santo; GO, Goiás; MS, Mato Grosso do Sul; PB, Paraíba; RJ, Rio de Janeiro; RS, Rio Grande do Sul.

and contaminative routes may be involved in the acquisition of *Trypanosoma* spp. infection by free-ranging wild mammals.

*T. cascavelli* in the blood of marsupials raised the following questions: which animal was the first host of *T. cascavelli*, snake or marsupial? What are the adaptive mechanisms that resulted in the ability of this huge host switch? What was the transmission route, contaminative or oral? *T. cascavelli* was described from *Crotalus durissus terrificus*, and only years later, it was isolated again from *C. d. terrificus* and from *Monodelphis americana* (Pessôa and De Biasi, 1972; Viola et al., 2008, 2009; Dario et al., 2017b).

Snakes are ectothermal animals that are submitted to large environmental temperature changes. Didelphids exhibit lower body temperature than other mammals, ranging from 25 °C to 33 °C (Dawson and Olson, 1988; Busse et al., 2014). Probably the resilience to such temperature variations represented a preadaptive trait of *T. cascavelli* to infect and survive in marsupials and snakes. Infection of a marsupial by *T. cascavelli* may also occur by predation since *Didelphis* spp. eventually includes snakes in their diet (Almeida-Santos et al., 2000; Cáceres, 2002), which is possible due to the opossums' resistance to the venom of these reptiles (Moussatché and Perales, 1989; Almeida-Santos et al., 2000; Voss and Jansa, 2012).

The finding of *T. cascavelli* in *M. demerarae*, besides broadening the range of host species of the parasite, also suggests the oral route as a source of infection since snakes could have acquired the infection by predating infected *Marmosa* spp. or another small marsupial. Trypanosomes from the reptiles' clade have already been described infecting Muridae and Chiroptera (Dobigny et al., 2011; Salzer et al., 2016; Dario et al., 2017a). All together, these data show how little is known about the ecology and host specificity of wild vertebrates trypanosomatids.

The transmission of *T. cascavelli* has been tested in *Culex* sp., *Triatoma infestans*, and leech, with negative results (Pessôa and De Biasi, 1972). The finding of flagellates closely related to snake trypanosomatids in phlebotomines led Viola et al. (2008) to propose these insects as possible vectors of representatives of the lizard/snake/rodent/marsupial clade.

*T. lainsoni* was only described twice and in these two times, only in Amazonian rodents (Naiff and Barrett, 2013; Ortiz et al., 2018). Here, using a more sensitive methodology, we enlarged the knowledge of hosts taxa and geographical distribution of this trypanosome since we found *T. lainsoni* infecting marsupials and bats in Atlantic Forest and Cerrado. The low parasitemias of the *T. lainsoni* infected animals (negative fresh blood examination and hemocultures) suggest a transmission strategy that is independent of high parasitemias and explains the rarity of the encounter of this trypanosomatid reinforces the usefulness of working with blood clots. Another aspect to emphasize is the absence of cross-reactivity in serology since only one *G. agilis*, among 23 infected, had positive serology and the mixed infection with *T. cruzi* was confirmed by PCR. Since all *G. agilis* have been collected in the same locality, they probably have been exposed to the same infection source, a fact that may explain the high infection rate of this species. Since bats use the upper forest stratum and *G. agilis* use both arboreal and terrestrial strata, *T. lainsoni* transmission very probably may occur among arboreal and terrestrial mammals, but nothing is known, up to now, about its probable vector species.

Concerning our findings of *T. dionisii*, *T. cascavelli* and *T. lainsoni* parasitizing still undescribed mammal species, there are two points supporting that these mammals are indeed acting as hosts: i) these trypanosomes species demonstrated to be able to pass through all the non-specific defense mechanisms of the host besides mechanical barriers of the intestinal tube and other tissues until finally succeeded reaching the blood; ii) once in the blood, these parasites surpassed the complement system during enough time for us to detect DNA samples in the blood. The amount of our findings is too large as to represent transitory pass-through due to consumption of vector or other host, supporting the importance of revaluating host specificity in genus *Trypanosoma*.

The herein description of new molecular taxonomic units (DID and Neobat 4), shows that the diversity of *Trypanosoma* spp. and their distribution are still underestimated. Therefore, their phylogenetic relationship as we know today is, clearly, provisional and will change with the increase of samples, host species, geographical areas studied and the use of less selective and more sensitive methodologies such as the PCR from DNA extracted directly from blood clots.

Concomitant infection in parasitism is a very common phenomenon and trypanosomes do not constitute an exception (Dario et al., 2017a; Pronovost et al., 2018). Here we observed the occurrence of *T. lainsoni* in mixed infection with *T. cruzi*, *T. dionisii* and *T. gennarii*, a little known trypanosomatid species of the lizard/snake/rodent/marsupial clade. *T. gennarii,* was first described from a *M. domestica* specimen that also was infected by *T. cruzi* (TcIII) (Ferreira et al., 2017). In the same occasion, Ferreira et al. (2017) detected *T. gennarii* in single infection also in *M. domestica*. We found *T. gennarii* infecting another didelphid species i.e. *G. agilis*. The low genetic distance between the sequences obtained in this study and the *T. gennarii* references sequences, suggests another *T. gennarii* genotype more than a new MOTU.

Our study demonstrated that *Trypanosoma* spp. diversity, as well as, their host range and geographical distribution are broader than previously recognized. Using blood clots, we were able to identify infections by trypanosomes in animals with undetectable parasitemia, not culturable trypanosomes and new MOTUs. These findings raised questions towards trypanosomatid host specificity and the evolutionary relationship between different trypanosome species/MOTUs. To conclude that a parasite is colonizing a new host species based on the encounter only of DNA is a kind of daring but we can say that this parasite succeeded in overcoming the first nonspecific defense barriers and passed into the circulatory system. This is a very important leap for acquiring a new host. Especially if we consider that circulating DNA is removed very quickly from the circulatory system, it is tempting to think that the presence of DNA signalizes that at least for a period of time the parasite remained in that new host. where we do not know how long it will remain. The acquisition of a new host is a dynamic but gradual process. Additionally, hosts are not necessarily capable of sustaining all the evolution phases of a parasite. There are several kinds of hosts as is the case of accidental host and paratenic or transport host (Bush et al., 2001).

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.02.004.

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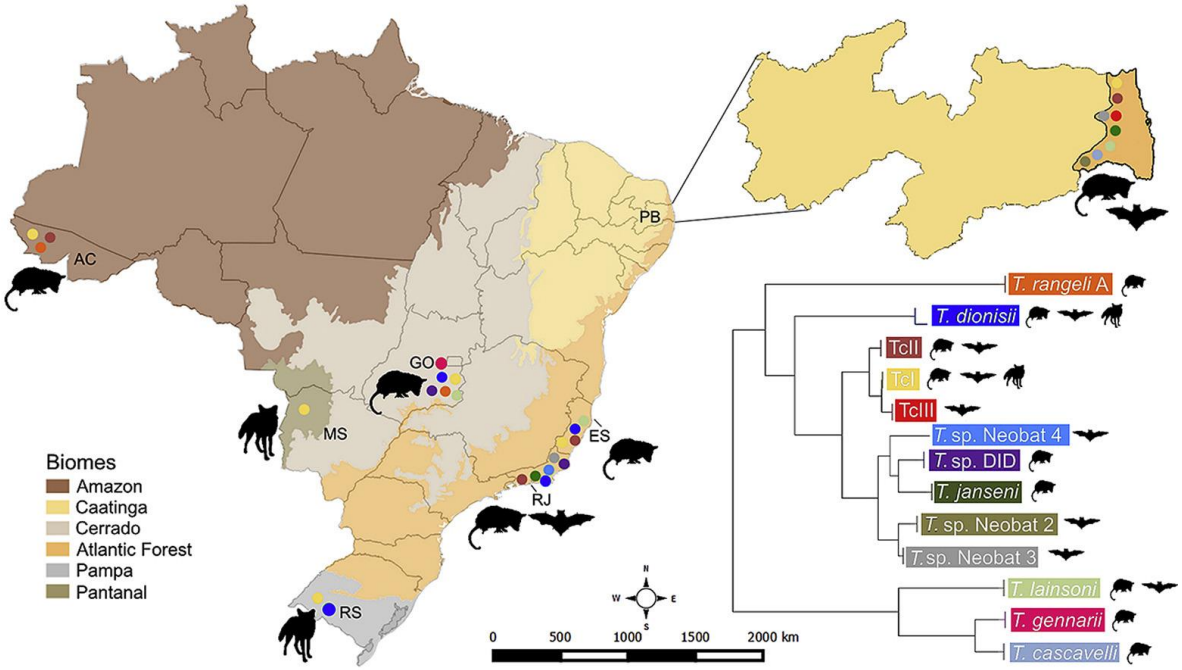
Highlights

- Blood clots are suitable as DNA source for *Trypanosoma* spp. identification
- Detection of *T. cruzi* infection in seronegative wild mammals by PCR of blood clots
- *Trypanosoma* spp. identified in individuals with negative hemoculture
- *T. dionisii*, *T. cascavelli* and *T. lainsoni* have a broader host spectrum
- Two new MOTUs were identified in *Didelphis* sp. and *Carollia* sp.

Graphical abstract



**Supplementary Table S1. Host species, geographical origin, hemoculture, serology and molecular identification of *Trypanosoma* spp. in blood clots from Carnivora, Chiroptera, and Didelphimorphia**

| Sample ID | Host order | Host species | State/Biome | Serology IFAT (*T. cruzi*) | Molecular identification (18S SSU) | GenBank accession number |
|---|---|---|---|---|---|---|
| bCth744 | Carnivora | *Cerdocyon thous* | RS/Pampa | 1/10 | *T. cruzi* TcI | MH411619 |
| CPAGM 848 | Carnivora | *Cerdocyon thous* | MS/Pantanal | 1/20 | *T. cruzi* TcI | MH411624 |
| CPAGM 850 | Carnivora | *Cerdocyon thous* | MS/Pantanal | 1/10 | *T. cruzi* TcI | MH411625 |
| CPAGM 855 | Carnivora | *Cerdocyon thous* | MS/Pantanal | Negative | *T. cruzi* TcI | MH411626 |
| CPAGM 796 | Carnivora | *Cerdocyon thous* | MS/Pantanal | 1/10 | *T. cruzi* TcI | MH411623 |
| bCth743 | Carnivora | *Cerdocyon thous* | RS/Pampa | 1/80 | *T. cruzi* TcI | MH411618 |
| EM 783 | Chiroptera | *Desmodus rotundus* | PB/Atlantic Forest | Not performed | *T. cruzi* TcI | MH411645 |
| LBCE 19689 | Didelphimorphia | *Didelphis marsupialis* | AC/Amazon | 1/20 | *T. cruzi* TcI | MH411638 |
| PCE 408 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | Negative | *T. cruzi* TcI | MH411631 |
| PCE 416[a] | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | Negative | *T. cruzi* TcI | MH411632 |
| EM 715 | Chiroptera | *Glossophaga soricina* | PB/Atlantic Forest | Not performed | *T. cruzi* TcI | MH411644 |
| LBCE 18616 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/20 | *T. cruzi* TcI | MH411640 |
| LBCE 18666 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. cruzi* TcI | MH558664 |
| bPgy89 | Carnivora | *Lycalopex gymnocercus* | RS/Pampa | 1/40 | *T. cruzi* TcI | MH411621 |
| bPgy90 | Carnivora | *Lycalopex gymnocercus* | RS/Pampa | 1/40 | *T. cruzi* TcI | MH411622 |
| LBCE 19672 | Didelphimorphia | *Metachirus* sp. | AC/Amazon | Negative | *T. cruzi* TcI | MH411633 |
| YL 865 | Didelphimorphia | *Monodelphis americana* | ES/Atlantic Forest | 1/20 | *T. cruzi* TcI | MH411639 |
| EM 699 | Chiroptera | *Platyrrhinus lineatus* | PB/Atlantic Forest | Not performed | *T. cruzi* TcI | MH411643 |
| EM 897 | Chiroptera | *Artibeus. fimbriatus* | RJ/Atlantic Forest | Not performed | *T. cruzi* TcII | MH411629 |
| LBT 6867 | Chiroptera | *Artibeus lituratus* | RJ/Atlantic Forest | Not performed | *T. cruzi* TcII | MH411627 |
| LBCE 19675[a] | Didelphimorphia | *Didelphis marsupialis* | AC/Amazon | 1/320 | *T. cruzi* TcII | MH411635 |
| LBCE 19688 | Didelphimorphia | *Didelphis marsupialis* | AC/Amazon | Negative | *T. cruzi* TcII | MH411637 |
| PCE 383 | Didelphimorphia | *Marmosa murina* | PB/Atlantic Forest | Negative | *T. cruzi* TcII | MH411630 |
| YL 881 | Didelphimorphia | *Marmosops incanus* | ES/Atlantic Forest | 1/20 | *T. cruzi* TcII | MH558662 |
| LBCE 19674 | Didelphimorphia | *Philander* sp | AC/Amazon | 1/40 | *T. cruzi* TcII | MH411634 |
| LBCE 19687 | Didelphimorphia | *Philander* sp | AC/Amazon | 1/80 | *T. cruzi* TcII | MH411636 |
| LBT 6895 | Chiroptera | *Phyllostomus hastatus* | RJ/Atlantic Forest | Not performed | *T. cruzi* TcII | MH411628 |
| EM 712 | Chiroptera | *Artibeus lituratus* | PB/Atlantic Forest | Not performed | *T. cruzi* TcIII | MH558661 |
| EM 693 | Chiroptera | *Carollia perspicillata* | PB/Atlantic Forest | Not performed | *T. cruzi* TcIII | MH411642 |
| LBCE 18612 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. rangeli* A | MH411071 |
| LBCE 19665 | Didelphimorphia | *Philander* sp | AC/Amazon | Negative | *T. rangeli* A | MH411069 |

| | | | | | | |
|---|---|---|---|---|---|---|
| LBCE 19685[a] | Didelphimorphia | *Philander* sp | AC/Amazon | 1/20 | *T. rangeli* A | MH411070 |
| LBCE 18618 | Didelphimorphia | *Didelphis albiventris* | GO/Cerrado | Negative | *T. dionisii* | MH411095 |
| LBCE 18651 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. dionisii* | MH411099 |
| LBCE 18653 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. dionisii* | MH411101 |
| LBCE 18659 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. dionisii* | Mh411098 |
| LBCE 18661 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/20 | *T. dionisii* | MH411102 |
| LBCE 18664 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. dionisii* | MH411096 |
| LBCE 18665 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. dionisii* | MH411097 |
| YL 883 | Didelphimorphia | *Metachirus nudicaudatus* | ES/Atlantic Forest | 1/20 | *T. dionisii* | MH556865 |
| LBT 6875 | Chiroptera | *Sturnira lilium* | RJ/Atlantic Forest | Not performed | *T. dionisii* | MH411103 |
| EM 772 | Chiroptera | *Artibeus planirostris* | PB/Atlantic Forest | Not performed | *T.* sp. Neobat 2 | MH411063 |
| EM 788 | Chiroptera | *Artibeus planirostris* | PB/Atlantic Forest | Not performed | *T.* sp. Neobat 2 | MH411065 |
| EM 777 | Chiroptera | *Artibeus lituratus* | PB/Atlantic Forest | Not performed | *T.* sp. Neobat 2 | MH411064 |
| EM 716 | Chiroptera | *Artibeus lituratus* | PB/Atlantic Forest | Not performed | *T.* sp. Neobat 3 | MH411062 |
| EM 739 | Chiroptera | *Artibeus lituratus* | PB/Atlantic Forest | Not performed | *T.* sp. Neobat 3 | MH411066 |
| RM 874 | Chiroptera | *Desmodus rotundus* | RJ/Atlantic Forest | Not performed | *T.* sp. Neobat 3 | MH556911 |
| RM 878 | Chiroptera | *Artibeus lituratus* | RJ/Atlantic Forest | Not performed | *T.* sp. Neobat 3 | MH411068 |
| EM 719 | Chiroptera | *Artibeus lituratus* | PB/Atlantic Forest | Not performed | *T.* sp. Neobat 3 | MH556910 |
| PCE 54 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/10 | *T. janseni* | MH411056 |
| PCE 385 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/20 | *T. janseni* | MH411059 |
| PCE 392 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/40 | *T. janseni* | MH411060 |
| PCE 393 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/10 | *T. janseni* | MH411061 |
| LBCE 18348 | Didelphimorphia | *Didelphis aurita* | RJ/Atlantic Forest | 1/10 | *T. janseni* | MH411057 |
| LBCE 18683 | Didelphimorphia | *Didelphis aurita* | RJ/Atlantic Forest | Negative | *T. janseni* | MH411058 |
| EM 776 | Chiroptera | *Artibeus planirostris* | PB/Atlantic Forest | Not performed | *T. lainsoni* | MH411660 |
| EM 767 | Chiroptera | *Platyrrhinus lineatus* | PB/Atlantic Forest | Not performed | *T. lainsoni* | MH411659 |
| LBCE 18583 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411661 |
| LBCE 18585 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411662 |
| LBCE 18596 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/20 | *T. lainsoni* | MH411663 |
| LBCE 18617 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411664 |
| LBCE 18628 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411665 |
| LBCE 18670 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411666 |
| LBCE 18673 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411667 |
| LBCE 18669 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411669 |
| LBCE 18584 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411670 |
| LBCE 18586 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/20 | *T. lainsoni* | MH411671 |

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|---|---|---|---|---|---|---|
| LBCE 18597 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411672 |
| LBCE 18599 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411673 |
| LBCE 18613 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/40 | *T. lainsoni* | MH411674 |
| LBCE 18614 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/80 | *T. lainsoni* | MH411675 |
| LBCE 18615 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411676 |
| LBCE 18644 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411682 |
| LBCE 18646 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411677 |
| LBCE 18647 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/10 | *T. lainsoni* | MH411683 |
| LBCE 18660 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411681 |
| LBCE 18662 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. lainsoni* | MH411678 |
| LBCE 18630 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/40 | *T. lainsoni* | MH556867 |
| YL 885 | Didelphimorphia | *Micoureus paraguayanus* | ES/Atlantic Forest | Negative | *T. lainsoni* | MH411668 |
| PCE 51 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/40 | *T. cascavelli* | MH411650 |
| PCE 53 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/40 | *T. cascavelli* | MH411651 |
| PCE 55 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/20 | *T. cascavelli* | MH411652 |
| PCE 384 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/40 | *T. cascavelli* | MH411653 |
| PCE 395 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/20 | *T. cascavelli* | MH411655 |
| PCE 391 | Didelphimorphia | *Marmosa demerarae* | PB/Atlantic Forest | 1/20 | *T. cascavelli* | MH411654 |
| PCE 410 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | Negative | *T. cascavelli* | MH411656 |
| LBCE 18655 | Didelphimorphia | *Didelphis albiventris* | GO/Cerrado | Negative | *T.* sp. DID | MH404843 |
| LBCE 18344 | Didelphimorphia | *Didelphis aurita* | RJ/Atlantic Forest | Negative | *T.* sp. DID | MH404842 |
| LBCE 18684 | Didelphimorphia | *Didelphis aurita* | RJ/Atlantic Forest | Negative | *T.* sp. DID | MH404844 |
| LBCE 18335 | Didelphimorphia | *Didelphis aurita* | RJ/Atlantic Forest | Negative | *T.* sp. DID | MH404845 |
| RM 876 | Chiroptera | *Carollia perspicillata* | RJ/Atlantic Forest | Not performed | *T.* sp. Neobat 4 | MH411067 |
| LBCE 18663 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. cruzi* TcI | MH558663 |
| | | | | | *T. dionisii* | MH556866 |
| bPgy88 | Carnivora | *Lycalopex gymnocercus* | RS/Pampa | 1/80 | *T. cruzi* TcI | MH411620 |
| | | | | | *T. dionisii* | MH411094 |
| LBCE 18652 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | Negative | *T. cruzi* TcI | MH411641 |
| | | | | | *T. dionisii* | MH411100 |
| | | | | | *T. lainsoni* | MH411684 |
| | | | | | *T. lainsoni* | MH411685 |

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|---|---|---|---|---|---|---|
| LBCE 18634 | Didelphimorphia | *Gracilinanus agilis* | GO/Cerrado | 1/20 | *T. gennarii* | MH411646 |
| | | | | | *T. gennarii* | MH411647 |
| | | | | | *T. gennarii* | MH411648 |
| | | | | | *T. gennarii* | MH411649 |
| | | | | | *T. lainsoni* | MH411679 |
| | | | | | *T. lainsoni* | MH411680 |
| EM 701 | Chiroptera | *Carollia perspicillata* | PB/Atlantic Forest | Not performed | NA | |
| EM 724 | Chiroptera | *Artibeus lituratus* | PB/Atlantic Forest | Not performed | NA | |
| RM 873 | Chiroptera | *Artibeus lituratus* | RJ/Atlantic Forest | Not performed | NA | |
| PCE 52 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/20 | NA | |
| PCE 399 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/10 | NA | |
| CPAGM 832 | Carnivora | *Cerdocyon thous* | MS/Pantanal | 1/40 | NA | |
| CPAGM 840 | Carnivora | *Cerdocyon thous* | MS/Pantanal | 1/20 | NA | |
| CPAGM 852 | Carnivora | *Cerdocyon thous* | MS/Pantanal | Negative | NA | |
| CPAGM 853 | Carnivora | *Cerdocyon thous* | MS/Pantanal | Negative | NA | |
| CPAGM 856 | Carnivora | *Cerdocyon thous* | MS/Pantanal | Negative | NA | |
| CPAGM 857 | Carnivora | *Cerdocyon thous* | MS/Pantanal | Negative | NA | |
| EM 726 | Chiroptera | *Artibeus planirostris* | PB/Atlantic Forest | Not performed | NA | |
| EM 778 | Chiroptera | *Artibeus planirostris* | PB/Atlantic Forest | Not performed | NA | |
| PCE 50 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/20 | NA | |
| PCE 68 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/10 | NA | |
| PCE 415 | Didelphimorphia | *Didelphis albiventris* | PB/Atlantic Forest | 1/40 | NA | |
| YL 894 | Didelphimorphia | *Marmosa paraguayana* | ES/Atlantic Forest | 1/10 | NA | |
| YL 900 | Didelphimorphia | *Metachirus nudicaudatus* | ES/Atlantic Forest | 1/10 | NA | |
| YL 882 | Didelphimorphia | *Metachirus nudicaudatus* | ES/Atlantic Forest | 1/20 | NA | |
| YL 889 | Didelphimorphia | *Marmosops incanus* | ES/Atlantic Forest | 1/10 | NA | |
| RM 883 | Chiroptera | *Desmodus rotundus* | RJ/Atlantic Forest | Not performed | NA | |
| LBT 6891 | Chiroptera | *Artibeus lituratus* | RJ/Atlantic Forest | Not performed | NA | |
| LBT 6892 | Chiroptera | *Phyllostomus hastatus* | RJ/Atlantic Forest | Not performed | NA | |
| LBT 6896 | Chiroptera | *Sturnira lilium* | RJ/Atlantic Forest | Not performed | NA | |
| EM 790 | Chiroptera | *Platyrrhinus lineatus* | PB/Atlantic Forest | Not performed | NA | |

[a]*positive hemoculture*

[b]NA: no amplification in the nested PCR

Serology: cut-off value 1/20 (carnivores) and 1/40 (marsupials

Chiroptera has not been tested for serology due to the absence of specific commercial antibodies for this group

IFAT: Immunofluorescence Antibody Test

**Supplementary Table S2.** Inter- and intraspecific genetic distance[a] in the Lizard/snake/rodent/marsupial clade based on 18S sequences.

| Groups | Min | Max | Mean |
|---|---|---|---|
| **Interspecific** | | | |
| *T. cascavelli* X *T.gennarii* | 0.084 | 0.092 | 0.087 |
| *T.cascavelli* X *T. lainsoni* | 0.107 | 0.113 | 0.108 |
| *T. gennarii* X *T. lainsoni* | 0.118 | 0.123 | 0.120 |
| **Intraspecific** | | | |
| *T. cascavelli* | 0.000 | 0.007 | 0.003 |
| *T. gennarii* | 0.000 | 0.005 | 0.001 |
| *T. lainsoni* | 0.000 | 0.007 | 0.003 |

[a]Inter- and intraspecific distances were calculated only for *Trypanosoma* spp. from the lizard/snake/rodent/marsupial clade identified in this study (*T. cascavelli*, *T. gennarii* and *T. lainsoni*).

**Supplementary Table S3.** Inter- and intraspecific genetic distance[a] in the *T. cruzi* clade based on 18S sequences.

| Groups | Min | Max | Mean |
|---|---|---|---|
| **Interspecific** | | | |
| *T. cruzi* X *T. dionisii* | 0.057 | 0.070 | 0.061 |
| *T. cruzi* X *T. rangeli* | 0.089 | 0.099 | 0.092 |
| *T. cruzi* X *T. janseni* | 0.077 | 0.087 | 0.079 |
| *T. cruzi* X *T.* sp. Neobat 1[b] | 0.073 | 0.080 | 0.076 |
| *T. cruzi* X *T.* sp. Neobat 2 | 0.077 | 0.086 | 0.079 |
| *T. cruzi* X *T.* sp. Neobat 3 | 0.067 | 0.077 | 0.070 |
| *T. cruzi* X *T.* sp. Neobat 4 | 0.070 | 0.073 | 0.072 |
| *T. cruzi* X *T.* sp. DID | 0.067 | 0.070 | 0.069 |
| *T. dionisii* X *T. rangeli* | 0.076 | 0.080 | 0.077 |
| *T. dionisii* X *T. janseni* | 0.083 | 0.087 | 0.084 |
| *T. dionisii* X *T.* sp. Neobat 1 | 0.097 | 0.100 | 0.097 |
| *T. dionisii* X *T.* sp. Neobat 2 | 0.083 | 0.087 | 0.084 |
| *T. dionisii* X *T.* sp. Neobat 3 | 0.080 | 0.083 | 0.081 |
| *T. dionisii* X *T.* sp. Neobat 4 | 0.090 | 0.093 | 0.090 |
| *T. dionisii* X *T.* sp. DID | 0.087 | 0.087 | 0.087 |
| *T. rangeli* X *T. janseni* | 0.087 | 0.087 | 0.087 |
| *T. rangeli* X *T.* sp. Neobat 1 | 0.100 | 0.100 | 0.100 |
| *T. rangeli* X *T.* sp. Neobat 2 | 0.077 | 0.077 | 0.077 |
| *T. rangeli* X *T.* sp. Neobat 3 | 0.077 | 0.077 | 0.077 |
| *T. rangeli* X *T.* sp. Neobat 4 | 0.090 | 0.090 | 0.090 |

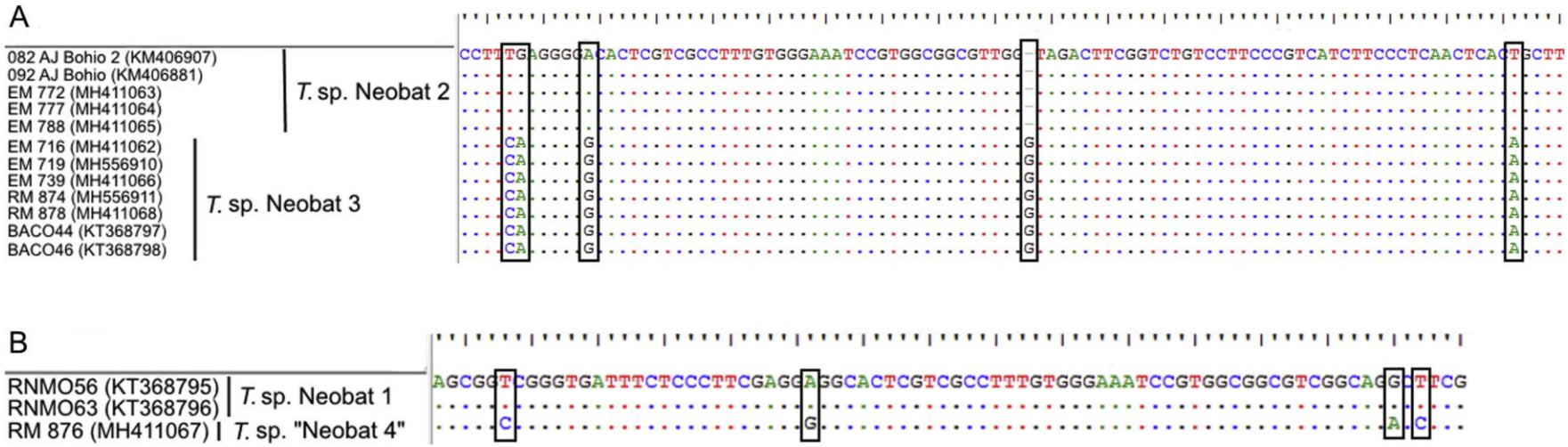
| | | | |
|---|---|---|---|
| *T. rangeli* X *T.* sp. DID | 0.096 | 0.096 | 0.096 |
| *T. janseni* X *T.* sp. Neobat 1 | 0.021 | 0.021 | 0.021 |
| *T. janseni* X *T.* sp. Neobat 2 | 0.024 | 0.024 | 0.024 |
| *T. janseni* X *T.* sp. Neobat 3 | 0.018 | 0.018 | 0.018 |
| *T. janseni* X *T.* sp. Neobat 4 | 0.027 | 0.027 | 0.027 |
| *T. janseni* X *T.* sp. DID | 0.024 | 0.024 | 0.024 |
| *T.* sp. Neobat 1 X *T.* sp. Neobat 2 | 0.027 | 0.027 | 0.027 |
| *T.* sp. Neobat 1 X *T.* sp. Neobat 3 | 0.021 | 0.021 | 0.021 |
| *T.* sp. Neobat 1 X *T.* sp. Neobat 4 | 0.012 | 0.012 | 0.012 |
| *T.* sp. Neobat 1 X *T.* sp. DID | 0.015 | 0.015 | 0.015 |
| *T.* sp. Neobat 2 X *T.* sp. Neobat 3 | 0.012 | 0.012 | 0.012 |
| *T.* sp. Neobat 2 X *T.* sp. Neobat 4 | 0.027 | 0.027 | 0.027 |
| *T.* sp. Neobat 2 X *T.* sp. DID | 0.024 | 0.024 | 0.024 |
| *T.* sp. Neobat 3 X *T.* sp. Neobat 4 | 0.021 | 0.021 | 0.021 |
| *T.* sp. Neobat 3 X *T.* sp. DID | 0.018 | 0.018 | 0.018 |
| *T.* sp. Neobat 4 X *T.* sp. Did | 0.015 | 0.015 | 0.015 |

| | | | |
|---|---|---|---|
| **Intraspecific** | | | |
| *T. cruzi* | 0.000 | 0.009 | 0.002 |
| *T. dionisii* | 0.000 | 0.003 | 0.001 |
| *T. rangeli* | 0.000 | 0.000 | 0.000 |
| *T. janseni* | 0.000 | 0.000 | 0.000 |
| *T.* sp. Neobat 1 | 0.000 | 0.000 | 0.000 |
| *T.* sp. Neobat 2 | 0.000 | 0.000 | 0.000 |
| *T.* sp. Neobat 3 | 0.000 | 0.000 | 0.000 |
| *T.* sp. Neobat 4 | NC[c] | NC[c] | NC[c] |
| *T.* sp. DID | 0.000 | 0.000 | 0.000 |

[a]Inter- and intraspecific distances were calculated for *Trypanosoma* spp. species identified in this study (*T. cruzi*, *T. dionisii*, *T. rangeli*, *T. janseni*, *T.* sp. Neobat 2, *T.* sp. Neobat 3, *T.* sp. Neobat 4 and *T.* sp. DID).

[b]Inter- and intraspecific distances were calculated for *T.* sp. Neobat 1 reference sequences due to their genetic closeness to *T.* sp. Neobat 4 and *T.* sp. DID.

[c]NC = not calculated. Low number of sequences available for calculation.




Supplementary Fig. S1. Comparison between *T*. sp. Neobats nucleotide sequences generated with 18S (SSU). (A) Sequence alignment shows five nucleotide changes differentiating the already well-established genotypes *T*. sp. Neobat 2 and *T*. sp. Neobat 3. (B) Sequence alignment shows four nucleotide changes differentiating *T*. sp. Neobat 1 reference sequences from the novel MOTU, which we named *T*. sp. 'Neobat 4" (RM 876)

# 5 DISCUSSÃO

Nas últimas décadas, diferentes métodos moleculares e regiões gênicas têm sido utilizados para diagnosticar e avaliar diversidade intra e interespecífica dos organismos vivos. Em 2003 Hebert et al. propuseram o gene mitocondrial COI como código de barras universal para identificação de espécies de animais. Além disso, desde 2008 o consórcio internacional de código de barras da vida (iBOL) tem estimulado a identificação de todas as formas de vida para a formação de bibliotecas de referência e conhecimento da biodiversidade do planeta. Até o momento foram publicados mais de 12 mil trabalhos com código de barras de DNA e mais de 500 mil espécies foram identificadas utilizando esta metodologia (iBOL, 2018).

Para avaliar o gene COI como código de barras para identificação e caracterização de espécies de tripanosoma, utilizamos *T. cruzi* como modelo por este ser um parasita multi-hospedeiro, conhecidamente heterogêneo e com ampla distribuição no território brasileiro (Zingales et al., 2012).

COI demonstrou ser filogeneticamente informativo ao reconhecer o gênero *Trypanosoma,* distinguir quatro espécies do clado *T. cruzi* (*T. c. cruzi*, *T. c. marinkellei*, *T. dionisii* e *T*. *rangeli*) e identificar variabilidade intraespecífica em três das quatro espécies estudadas. COI distinguiu cinco das sete DTUs de *T. cruzi* (TcI, TcII, TcIII, TcIV e Tcbat). Embora TcV e TcVI tenham sido propostos como sendo heterozigotos híbridos derivados de trocas genéticas entre TcII e TcIII (Westenberger et al., 2005), na nossa experiência essas DTUs foram agrupadas com TcIV. Esse posicionamento também foi relatado por Tomasini & Diosque (2015) e Barnabé et al. (2016) com os genes mitocondriais citocromo b (cytb), citocromo c oxidase subunidade II (COII) e NADH desidrogenase subunidade 1 (ND1). De acordo com a proposta de Tomasini & Diosque (2015), o posicionamento de TcV e TcVI junto com TcIV se deve a eventos de introgressão mitocondrial entre TcIV e TcIII que também foram relatados por Lewis et al. (2011). O posicionamento das DTUs ainda é controverso. Barnabé et al. (2016) propuseram uma divisão de *T. cruzi* em três clados mitocondriais (mtTcI, mtTcII e mtTcIII) em vez das sete DTUs, afirmando que as DTUs TcIII, TcIV, TcV e TcVI são equivalentes quando analisaram somente o DNA mitocondrial, não podendo ser distinguidas umas das outras. Contudo, nossos resultados confirmam COI como um marcador confiável das DTUs TcI, TcII, TcIII e TcIV, além de Tcbat.

Em relação aos genótipos de *T. cruzi*, COI identificou diversidade intra-DTU em TcI, TcII e TcIII. A DTU TcI apresentou a maior heterogeneidade, resultado consistente com outros autores e que pode ser explicado por TcI ser uma linhagem multi-hospedeira com ampla distribuição geográfica e, consequentemente, maior número de amostras estudadas (Guhl & Ramírez et al., 2017; Roman et al., 2018). TcII é a segunda DTU mais amostrada no território brasileiro e diversidade também já demonstrada com a utilização de marcadores mitocondriais e nucleares (Lima et al., 2014; Barnabé et al., 2016). Em relação à TcIII, identificamos três haplótipos diferentes mesmo caracterizando apenas quatro amostras, indicando uma ampla diversidade desta DTU. Além disso, cada amostra era proveniente de uma espécie de mamífero ou triatomíneo diferente coletados em três biomas brasileiros distintos, mostrando a ampla distribuição geográfica e de hospedeiros de TcIII.

Com relação às outras espécies do gênero *Trypanosoma*, COI também mostrou ser um marcador de heterogeneidade em *T. c. marinkellei* e *T. rangeli* mesmo com a genotipagem de poucas amostras. Diversidade em *T. c. marinkellei* já foi relada por Barnabé et al. (2003) que propuseram uma subdivisão deste táxon em dois grupos. A heterogeneidade de *T. rangeli* é bem conhecida, com proposta de divisão em cinco linhagens (Maia da Silva et al., 2004; 2007; 2009). Não observamos diversidade em *T. dionisii* provavelmente porque as amostras foram coletadas de morcegos da mesma área e no mesmo período.

Concomitantemente ao trabalho sobre COI realizamos o diagnóstico molecular de tecido cardíaco, já emblocado em parafina, proveniente de um menino de 2 anos com laudo *post mortem* de doença de Chagas aguda, no Estado do Espírito Santo. Esse caso aconteceu por via oral em um único contato com um espécimen de *Triatoma vitticeps* infectado. Nosso objetivo era diagnosticar a DTU (ou DTUs) de *T. cruzi* responsável (eis) pelo caso, porque anteriormente Dario et al. (2017a) haviam identificado as DTUs TcI, TcII, TcIII/TcV e TcIV sendo transmitidas no ciclo silvestre daquela região.

Naquele momento optamos por uma abordagem com o gene GPI, utilizado na discriminação das principais DTUs de *T. cruzi*, e da região V7V8 do gene 18S (SSU). Com GPI identificamos a DTU TcIV e com 18S (SSU) identificamos infecção mista por *T. dionisii* e quatro DTUs de *T. cruzi* (TcI, TcII, TcIII e TcIV). O achado de *T. dionisii*, até aquele momento descrito somente em morcegos, mostra o pouco que sabemos sobre especificidade parasitária.

A maior parte dos casos de doença de Chagas que chegam ao sistema de saúde já encontram-se na fase crônica. Na fase aguda, devido aos seus sintomas inespecíficos, testes para diagnóstico da doença não são realizados de imediato. Não sabemos ao certo como seria a patogenicidade dessa infecção mista *T. cruzi-T. dionisii*. Nas poliinfecções os parasitas podem interagir entre si de forma sinérgica ou antagônica (Perez et al., 2014). Infecções por diferentes subpopulações ou diferentes espécies de parasitas podem estar associadas com mudanças na virulência e desencadear diferentes respostas imunes do hospedeiro. Além disso, infecções mistas podem passar por seleção no hospedeiro ou de métodos como o hemocultivo e, por isso, *T. dionisii* ou algumas das DTUs talvez não fossem detectadas na fase crônica. Essa seleção de parasitas poderia também, por exemplo, justificar TcII ser a DTU, que com algumas exceções, é classicamente associada aos casos humanos crônicos no Brasil (Brenière et al., 2016; Rodrigues-dos-Santos et al., 2018).

O trabalho seguinte aprofundou a avaliação da diversidade de *Trypanosoma* spp. utilizando coágulos sanguíneos. Nós queríamos ver o grau de subestimação de infecções por tripanosomas nos indivíduos que apresentavam hemocultura e sorologia negativas. Analisamos DNA de coágulos provenientes de marsupiais, morcegos e carnívoros de vida livre. Marsupiais e morcegos são reconhecidos como os hospedeiros mais antigos de tripanosomas do clado *T. cruzi* (Stevens et al., 1999, Hamilton et al., 2012; Lopes et al., 2018), e carnívoros são animais de topo de cadeia e propostos como bioacumuladores de parasitas (Cleaveland, 2006; Rocha et al., 2013).

O uso de coágulos sanguíneos como fonte de DNA para identificar diversidade de *Trypanosoma* spp. mostrou ser uma metodologia menos seletiva do que hemocultivo, e permitiu identificar tripanosomas em animais com baixas parasitemias, tripanosomas descritos como de difícil cultivo e novas MOTUs. Hemoculturas positivas foram observadas apenas em três marsupiais. Dois foram confirmados como estando infectados por *T. cruzi* e um por *T. rangeli* através de PCR de DNA de coágulo e sequenciamento. A sensibilidade deste método foi demonstrada pela identificação de *T. cruzi* em 26,6% dos morcegos, 60,0% dos carnívoros e 18,05% dos marsupiais com hemocultivos negativos.

Infecção por *T. cruzi* também foi identificada em 50% (4/8) dos carnívoros com reação de imunofluorescência indireta (RIFI) negativa. Em marsupiais foi observado que 18,75% (12/64) testados como negativos por RIFI estavam na

verdade infectados por *T. cruzi*. Nos morcegos não foi realizada sorologia. Sorologia negativa e PCR positiva para *T. cruzi* é observada há mais de duas décadas (Ávila et al., 1993; Wincker et al., 1994; Batista et al., 2010; Tahir et al., 2018). As hipóteses levantadas para explicar esse resultado foram: 1) trata-se de infecção recente e não houve tempo suficiente para uma resposta humoral detectável (Wincker et al., 1994), 2) carga parasitária insuficiente, ou seja, poucos parasitas intracelulares para desencadear resposta imune e produção de anticorpos.

*T. rangeli* também foi identificado em indivíduos com hemoculturas negativas e apresentou uma distribuição geográfica e espectro de hospedeiros mais amplos da linhagem A (Maia da Silva et al., 2007, dos Santos et al., 2017; Espinoza-Álvarez et al., 2018). A identificação de DNA a partir de coágulos sanguíneos mostrou ser uma ferramenta para rastrear a distribuição de genótipos de tripanosomas geneticamente heterogêneos como *T. cruzi* e *T. rangeli*.

Neste trabalho identificamos um maior espectro de hospedeiros de *T. dionisii*. Além do encontro em tecido cardíaco humano, também identificamos *T. dionisii* infectando marsupiais e um carnívoro. Todos os animais apresentaram hemocultura negativa, o que sugere que *T. dionisii* pode ser mantido em mamíferos silvestres, bem como em outras espécies, em baixas parasitemias. A proximidade filogenética entre *T. dionisii* e *T. cruzi* sugere que esses parasitas podem explorar estratégias semelhantes para completar seu ciclo de vida (Oliveira et al., 2009). Assim como *T. cruzi*, *T. dionisii* pertence ao subgênero *Schizotrypanum* e tem a capacidade de invadir e se desenvolver em células de mamíferos (Maeda et al., 2012).

Nós também observamos um maior espectro de hospedeiros para *T. lainsoni*, descrito até o momento somente em roedores na Amazônia (Naiff & Barrett, 2013; Ortiz et al., 2018). Identificamos este tripanosoma infectando morcegos na Paraíba, marsupiais em Goiás e no Espírito Santo. O encontro de marsupiais infectados por *T. cascavelli*, reforça o possível caráter generalista desta espécie, como proposto por Dario et al. (2017a). No entanto, mesmo que esses parasitas sejam capazes de infectar grupos taxonômicos distantes ainda não sabemos o curso destas infecções e o papel desses hospedeiros na manutenção desses tripanosomas na natureza.

A especificidade parasitária é um traço difícil de ser mensurado e avaliado, principalmente em animais silvestres de vida livre devido às dificuldades de acompanhamento do animal na natureza, além de ser uma condição multifatorial determinada pela ecologia, possibilidade de encontro do hospedeiro e encontro do vetor para que haja dispersão do parasita (Poulin et al., 2011).

Nosso trabalho mostrou que o espectro de hospedeiros que diferentes tripanosomas infectam estava sendo subavaliado. O conhecimento dessa diversidade de hospedeiros ainda é parcial porque mamíferos silvestres de vida livre são pouco estudados devido a dificuldades de captura e manuseio.

O encontro de tamanha diversidade de tripanosomas infectando morcegos e marsupiais sugere que esses animais podem exercer um papel importante na dispersão de tripanosomas entre animais silvestres. A caracterização de *Trypanosoma* spp. aliada às informações sobre ecologia de seus hospedeiros mamíferos silvestres é fundamental na compreensão da diversidade, amplitude de hospedeiros e da distribuição geográfica de *Trypanosoma* spp.

Os genótipos *T*. sp. Neobats, até o momento, permanecem considerados como sendo restritos a morcegos seja no Brasil, Colômbia ou Panamá (Cottontail et al., 2014; Lima et al., 2015b; Dario et al., 2017b). Do mesmo modo, *T. gennarii* e *T. janseni* também podem ser considerados restritos a marsupiais (Ferreira et al., 2017; Lopes et al., 2018). Contudo, nossos resultados ampliaram o conhecimento da distribuição geográfica dessas espécies.

No nosso estudo, infecções simples se mostraram mais comuns do que infecções mistas. Das 16 amostras que clonamos por apresentarem eletroferogramas com múltiplos picos, somente quatro apresentaram de fato infecção mista, por duas ou três espécies de tripanosoma. Esse predomínio de infecções simples também foi relatado por Ihle-Soto et al. (2019) ao analisarem DNA de sangue total de mamíferos.

Além da diversidade relatada acima, identificamos duas novas unidades taxonômicas operacionais moleculares (MOTUs) que nomeamos *T*. sp. Neobat 4, por termos identificado em um morcego neotropical, e *T*. sp. DID, identificado em espécimes de *Didelphis* spp. O encontro dessas MOTUs indica que a diversidade de *Trypanosoma* spp. ainda é subestimada. Os resultados deste trabalho confirmam que a diversidade dentro do gênero *Trypanosoma* vai além do que seja possível cultivar.

# 6 CONCLUSÕES

- O gene COI demonstrou ser um marcador confiável na identificação de *T. cruzi*, *T. c. marinkellei*, *T. dionisii* e *T. rangeli*;
- O gene COI é adequado para a caracterização de DTUs de *T. cruzi*, separando cinco delas (TcI, TcII, TcIII, TcIV e Tcbat);
- COI mostrou diversidade genética intra-DTU em TcI, TcII e TcIII.
- COI é adequado para identificar diversidade intra-específica em *T. c. marinkellei* e *T. rangeli*;
- *T. dionisii* invade células cardíacas humanas;
- PCR de DNA de coágulo sanguíneo mostrou ser uma técnica sensível para o diagnóstico parasitológico de *Trypanosoma* spp.
- O uso de DNA extraído diretamente de coágulo permitiu aumentar o conhecimento da diversidade, espectro de hospedeiros e dispersão de espécies e genótipos de *Trypanosoma* spp.;
- Sorologia por RIFI subestima a real taxa de infecção por *T. cruzi* em mamíferos silvestres de vida livre;
- O genótipo TcII de *T. cruzi* apresenta maior distribuição na região Amazônica do que relatado até o momento;
- A linhagem A de *T. rangeli* apresenta maior distribuição geográfica e espectro de hospedeiros do que previamente descrito;
- O encontro de *T. janseni* em marsupiais na Paraíba aumenta o conhecimento da área de distribuição geográfica deste tripanosoma;
- A caracterização diretamente de coágulo mostrou que o espectro de hospedeiros de algumas espécies de *Trypanosoma* spp. está sendo subestimado;
- O encontro de duas novas unidades taxonômicas do gênero *Trypanosoma*, nomeadas *T*. sp. DID e *T*. sp. Neobat 4 mostra que a diversidade de *Trypanosoma* spp. é subestimada.

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