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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		TcIII (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		TcIII (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

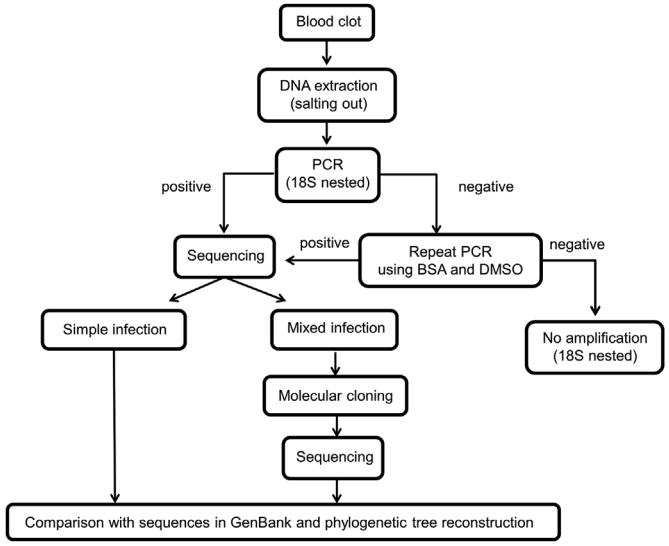

Fig. 1. Methodological algorithm employed for the identification of trypanosomes in blood clot samples.

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

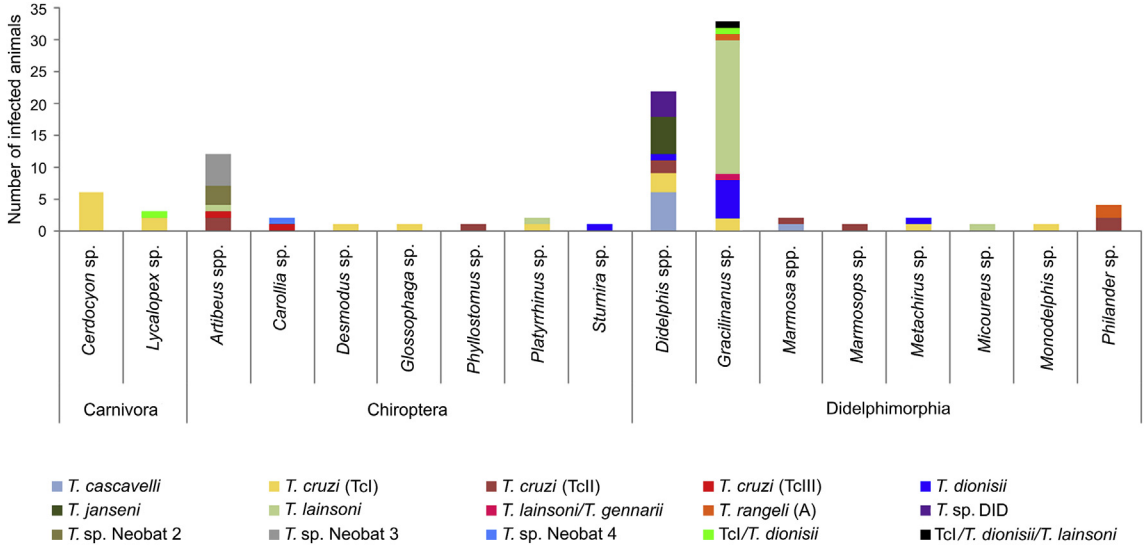

Fig. 2. *Trypanosoma* spp. identified in the blood clot of Carnivora, Chiroptera and Didelphimorphia. Each color indicates a different trypanosome species, genotype or mixed infections. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of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

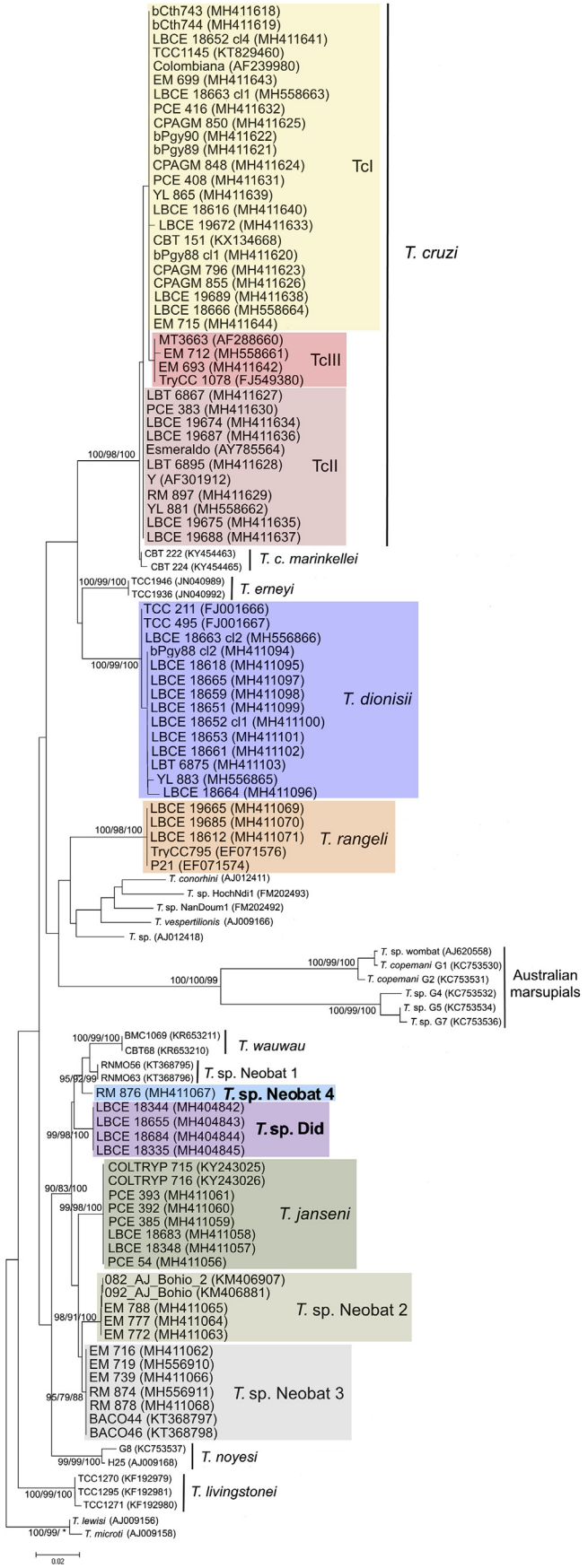

Fig. 3. *T. cruzi* clade phylogenetic tree based on 18S (SSU) gene. The tree shows ten different species and genotypes identified in the blood clot of Carnivora, Chiroptera, and Didelphimorphia: *T. cruzi* (DTUs TcI, TcII and TcIII), *T. dionisii*, *T. rangeli*, *T.* sp. Neobats 2 and 3, *T. janseni*, 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).

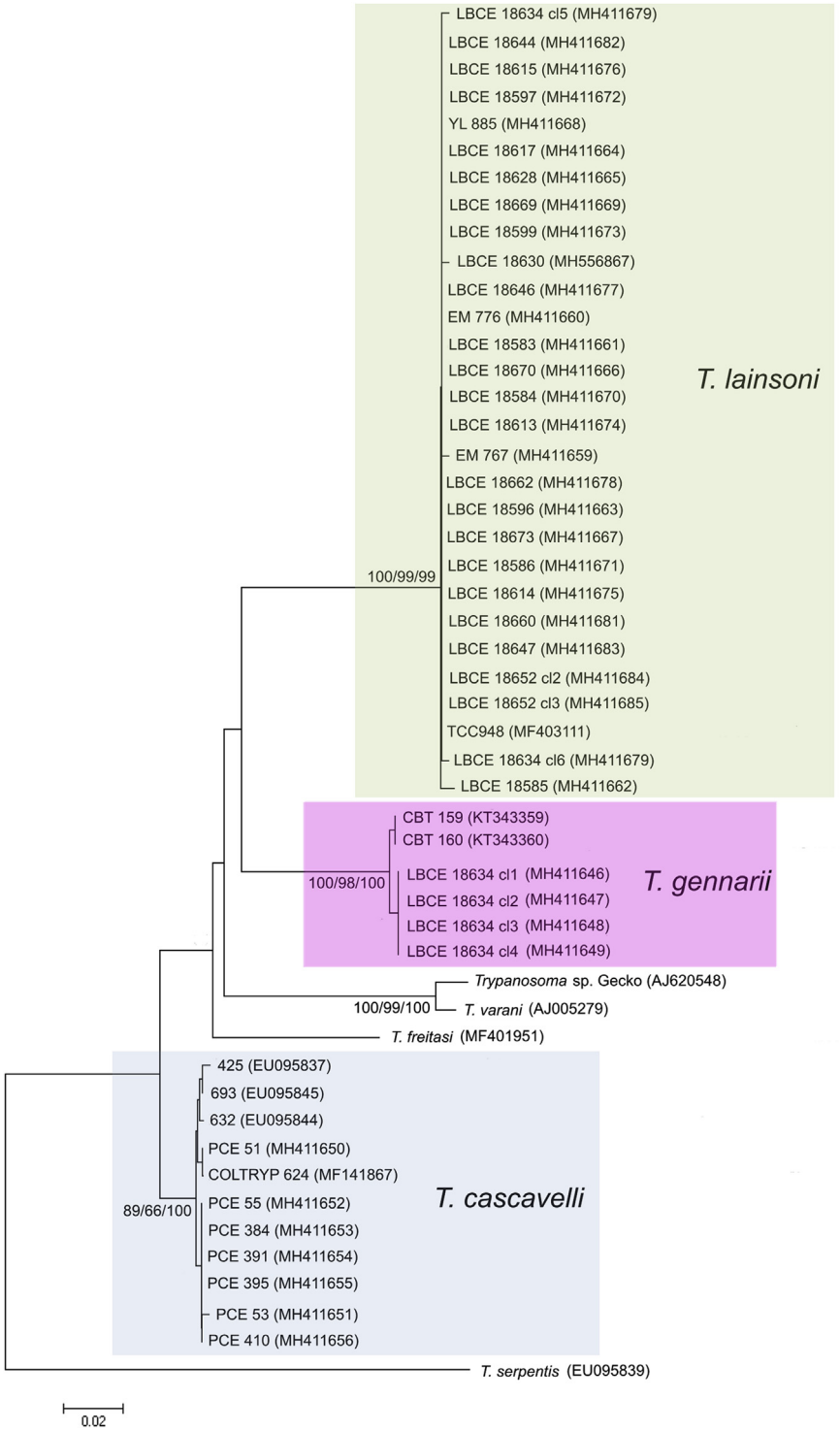

Fig. 4. Lizard/snake/rodent/marsupial clade phylogenetic tree based on 18S (SSU) gene. The tree shows the three different species from the lizard/snake/rodent/marsupial clade identified in the blood clot of Chiroptera and Didelphimorphia: *T. cascavelli*, *T. gennari*, and *T. lainsoni*. Tree inferred 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

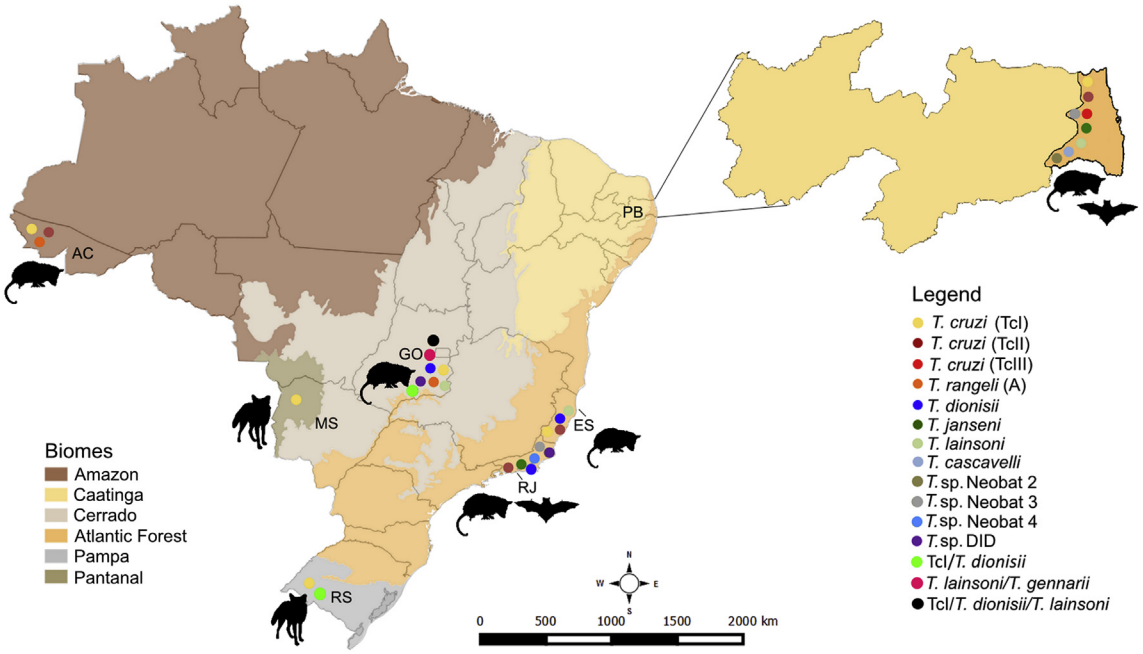

Fig. 5. Map of the distribution of the *Trypanosoma* spp. identified in this study. Thirteen different trypanosomes species/genotypes/MOTUs were identified, in single and mixed infection, in the blood clot of bats, carnivores and marsupials. The trypanosomes are distributed in five Brazilian biomes (Amazon 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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