

Geographical distribution of *Trypanosoma cruzi* in triatomine vectors in the State of Mato Grosso do Sul, Brazil

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ABSTRACT

Introduction: This work presents the initial findings of a molecular epidemiological investigation of *Trypanosoma cruzi* in triatomine insects in State of Mato Grosso do Sul. **Methods:** A total of 511 triatomines from different regions of the state were examined. Deoxyribonucleic acid (DNA) was extracted from the intestinal contents of the insects using phenol-chloroform-isoamyl alcohol (25:24:1). Polymerase chain reaction (PCR) using primers 121/122 targeting DNA kinetoplast (kDNA) was then performed to identify *T. cruzi*, and positive samples were subjected to PCR using the primer pair TcSC5D-F/R followed by restriction fragment length polymorphism (RFLP) with the restriction enzymes SphI and HpaI (1 U/reaction), cloning and sequencing. **Results:** One hundred samples were positive for *T. cruzi*, and three discrete typing units (DTUs) were identified (TcI, TcII, and TcBat). *Triatoma sordida* had the highest *T. cruzi* occurrence (83.3%), and DTUs were found in three samples: 58.3% of the samples were TcI, 33.3% were TcII and 8.3% were TcBat. There was a clear geographical distribution of the DTUs throughout the state, with TcI, TcII and TcBat located in the center, TcI located in the east, and TcII located in the west. **Conclusions:** This study showed the occurrence of overlapping DTUs in State of Mato Grosso do Sul. The distributions of the DTUs were different, with TcI, TcII and TcBat in the center of the state, TcI predominantly in the east, and TcII in the west. Further studies may reveal a more defined mosaic distribution of DTUs in MS.

Keywords: *Trypanosoma cruzi* DTUs. PCR. Restriction fragment length polymorphism. Microscopy examination.

INTRODUCTION

Trypanosoma cruzi, a protozoan flagellate belonging to the order Kinetoplastida and family Trypanosomatidae, is the etiologic agent of Chagas disease, which is one of the most important parasitic infections in Latin America, surpassed only by malaria. Over 10 million people are infected with this parasite. The disease is a complex zoonosis, with mammals serving as reservoirs and hosts¹, and is endemic to South and Central America² as well as some states in the United States of America³.

Transmission is primarily vectorial and occurs through parasite penetration of the host through injured skin or mucosa. The invertebrate vectors of *T. cruzi* are the triatomine insects Hemiptera, subfamily Reduviidae. Among the 138 described

species of triatomines, only four play a direct role in the epidemiology of the parasite: *Triatoma brasiliensis* (Neiva, 1911), *Panstrongylus megistus* (Burmeister, 1835), *Triatoma pseudomaculata* (Corrêa e Espínola, 1964) and *Triatoma sordida* (Stal, 1859). In a study of triatomines in the State of Mato Grosso do Sul (MS), *T. sordida* was the most frequently observed species parasitized by flagellate protozoa⁴. The presence of three major of triatomine species was confirmed in MS: *Triatoma brasiliensis* (Neiva, 1911), *P. megistus* (Burmeister, 1835) and *T. sordida* (Stal, 1859), with significant infestation rates in domestic and peridomestic areas only for *T. sordida* (9.3% and 86.6%, respectively). *T. brasiliensis* and *P. megistus* exhibited less than 0.2% infestation⁴.

Infection by *T. cruzi* is maintained in cycles of wild transmission over a broad range of mammalian reservoir hosts⁵. Human infection occurs due to the natural distribution of *T. cruzi* in triatomines that are adapted to the domestic environment or are peridomestic. However, infection also occurs orally through the ingestion of triatomines or food contaminated with the parasite, which is the most common form of transmission among wild animals, as well as through blood transfusion, organ transplantation and congenital infection⁶.

The population structure of the parasite is predominantly clonal, suggesting that recombination events are rare

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in nature⁷, although there are complex sexual processes in *T. cruzi*⁸. Nonetheless, the parasite presents considerable genetic diversity⁹⁻¹¹.

Although intraspecific polymorphisms occur, an analysis of the isoenzyme patterns of *T. cruzi* isolates revealed three groups that are classified as zymodemes: Z1, Z2 and Z3^{12,13}. Subsequent studies using markers based on ribosomal gene and mini-exon (spliced leader) sequences revealed two major lineages, *T. cruzi* I (TcI) and *T. cruzi* II (TcII), and indicated the existence of hybrid lines (TcI/II)^{7,9,13-16}. DNA sequencing analysis revealed that the TcI group is a relatively homogeneous clade, whereas TcII is divided into five subgroups (a-e), with two or three distinct phylogenetic clades (IIa-c) and two hybrid strains (IId and IIe) that are derived from the clades of the IIb and IIc subtypes^{13,17-20}. A new classification of the types and subtypes divides *T. cruzi* into six strains called discrete typing units (DTUs), i.e., TcI, TcII, TcIII, TcIV, TcV, and TcVI²¹, wherein TcI corresponds to the group TcI, TcII to subgroup TcIIb, TcIII to TcIIc, TcIV to TcIIa, TcV to TcIId, and TcVI to TcIIe. More recently, a new DTU has been described, TcBat²².

The objective of this study was to investigate the distribution of *T. cruzi* DTUs from samples of triatomines collected from entomology cores of the State Secretariat of Health of MS using molecular methods.

METHODS

Study area

The State of Mato Grosso do Sul is located in the Midwest region of Brazil and has an area of 357,145,532km² with 79 municipalities, an estimated population of 2,619,657 inhabitants, and a population density²³ of 6.86 inhabitants/km². Approximately two-thirds of the state is a part of the Cerrado, a heterogeneous, floristic savannah that covers more than 2 million km² and extends from Central Brazil to parts of Bolivia and Paraguay²⁴. In the western area of the state lies the Pantanal, one of the richest floodplains in the world, with an abundance and diversity of wildlife, in addition to habitats with a complex mosaic of resources²⁵.

All insects were collected from the municipalities of Jaraguari (May to August 2009 and September 2011), *Rochedo*, *Caarapó*, *Douradina*, *Antônio João*, *Dourados*, *Terenos*, *São Gabriel do Oeste*, *Aparecida do Taboado*, *Paranaíba*, *Rio Verde de Mato Grosso*, *Corumbá*, *Miranda* and *Aquidauana* (August 2011 to November 2012) in the State of Mato Grosso do Sul using the method described by Cominetti et al.²⁶. The selection of municipalities was based on the collection of material by the regional units of the *Coordenadoria de Controle de Vetores do Estado de Mato Grosso do Sul* (CCV-MS).

Survey of triatomine fauna

Insects were collected by teams from regional units of the *Coordenadoria de Controle de Vetores do Estado de Mato Grosso do Sul* (CCV-MS). When found, the insects were collected and identified using the methodology described by Cominetti et al.²⁶

The state is currently divided into five Vector Technician cores, as established by the *Coordenadoria de Controle de Vetores* (CCV) of the *Secretaria Estadual de Saúde* (SES). These cores were responsible for the collection and identification of insects as well as for parasitological examinations, i.e., both fresh and thin-layer smear preparations.

After the tests, the collected triatomines were placed in tubes containing 70% alcohol and sent to the Animal Health unit of Embrapa Beef Cattle for molecular tests, as described by Cominetti et al.²⁷

Identification of triatomines and microscopic examination

Triatomines were identified using the dichotomous keys proposed by Carcavallo et al.²⁸ Flagellated protozoa were detected using the method described by Souza²⁹.

DNA extraction and PCR

DNA was extracted from insects as described by Westenberger et al.³⁰. The integrity of the DNA samples was determined via electrophoresis on a 0.8% agarose gel that was subsequently stained with ethidium bromide (0.5µg.mL⁻¹) and examined under ultraviolet light.

The DNA was quantified using a spectrophotometer (GeneQuantTM *pro*; Biochrom). An A260nm/A280nm ratio above 1.8 was established as ideal, and the concentration of each sample was adjusted to 20ng.µL⁻¹.

The following primers, described by Wincker et al.³¹, were used for the molecular identification of *T. cruzi*: 121 (5'-AAATAATGTACGGG(T/L)GAGATGCATGA-3') and 122 (5'-GGTTCGATTGGGGTTGGTGTAATATA-3'). This primer pair permitted the amplification of a 330-bp fragment of the *T. cruzi* kinetoplast (kDNA). The amplification scheme that was used was previously described by Schijman et al.³².

Under natural conditions, triatomines are often co-infected with *T. cruzi* and *Trypanosoma rangeli*. Therefore, the samples were also subjected to PCR for *T. rangeli* using the primers TRF3 (5'-CCCCATACAAAACACCCTT-3') and TrR8 (5'-TGGAATGACGGTGC GGCGAC-3'), which target the conserved subtelomeric region of this species (SubTr, GenBank accession number AF426020). The amplification protocol was previously described by Chiurillo et al.³³

Trypanosoma cruzi-positive samples were submitted to a second PCR targeting the TcSC5D gene (genome CL-Brener loci: TcCLB.473111.10, TcCLB.507853.10). To this end, the primers TcSC5D-fwd (5'-GGACGTGGCGTTTGATTTAT-3') and TcSC5D-rev (5'-TCCCATCTTCTTCGTTGACT-3') were used, which amplify an 832-bp fragment. The amplification protocol that was used was previously described by Cosentino and Agüero³⁴.

The PCRs were performed in an Eppendorf AG 22331 thermocycler, and the DNA controls (*T. cruzi* as a positive control and *T. rangeli* as a negative control, as well as controls for the DTUs – TcI-VI and TcBat) were from a laboratory of the University of São Paulo (USP) and were provided by Dra. Marta M.G. Teixeira. Ultrapure water was used instead of template as an additional negative control.

The reactions were performed in a final volume of 25µL containing 1X PCR buffer (Tris-HCl 10mM, pH 8.3; 50mM KCl), 1.5mM MgCl₂, 0.2mM dNTPs, 0.2pmol each primer, 1U of Taq DNA polymerase (Platinum®, Invitrogen) and 20ng of genomic DNA.

After electrophoresis in an agarose gel (2%) and staining with ethidium bromide, the amplification products were visualized under UV light.

Identification of DTUs using restriction enzymes

All restriction enzymes were purchased from Promega (Southampton, UK). A 20-µL aliquot of the amplification product of the TcSC5D gene was digested in a single reaction with 1U of HpaI (R6305) and 1U of SphI (R6265) and heated to 37°C for 1h. The resulting restriction fragments were visualized under ultraviolet (UV) light after electrophoresis on an agarose gel (2%) and staining with ethidium bromide.

The process for identifying the DTUs followed the protocol described by Cosentino and Agüero³⁴ and is shown in a simplified form in **Figure 1**.

Sequencing

The amplified gene product was purified using a TcSC5D Purelink™ Kit (Invitrogen), followed by cloning into the pGEM-Teasy plasmid (Promega) according to the recommendations of the manufacturer. Sequencing was then performed using the method described by Sanger³⁵ and an ABI 3730 DNA Analyzer (Applied Biosystems). The sequencing reactions were performed using the universal T7 primer for sequencing (5'-AATACGACTCACTATAG-3') and the BigDye® Terminator

v3.1 Cycle Sequencing Kit. The races were performed in 36-cm capillary tubes using POP7 polymer. The obtained sequences were analyzed, and the plasmid sequences were identified and removed using BioEdit software³⁶. The obtained sequences were compared with the sequences in the GenBank database, and a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed to determine the sequence identity³⁷. Phylogenetic analyses were performed using the Geneious v.4.8.5 (Biomatters) software package³⁸.

RESULTS

DNA samples from 511 triatomines from 14 different regions of State of Mato Grosso do Sul were examined. Of these, 100 (19.6%) from eight municipalities were confirmed positive using PCR with primers 121/122, which target the kinetoplast deoxyribonucleic acid (kDNA) of the parasite. Of the 100 positive samples, 12 (12%) were amplified with primers TcSC5D-fwd/TcSC5D-rev, which target the TcSC5D gene. It was not possible to amplify the samples from the municipalities of Corumbá and Dourados using the TcSC5D-fwd/TcSC5D-rev primers (**Table 1**).

Trypanosoma cruzi-positive triatomines were uncovered in samples from six municipalities. It was possible to identify *T. cruzi* DTUs in 12 of the samples. Three DTUs were found in *T. sordida*, which was the most commonly infected species (83.3%), followed by *T. matogrossensis* and *P. megistus* (both with 8.3% infection); 58.3% of the 12 samples were TcI, 33.3% were TcII, and 8.3% were TcBat. There was a clear geographical distribution of DTUs in the state, with

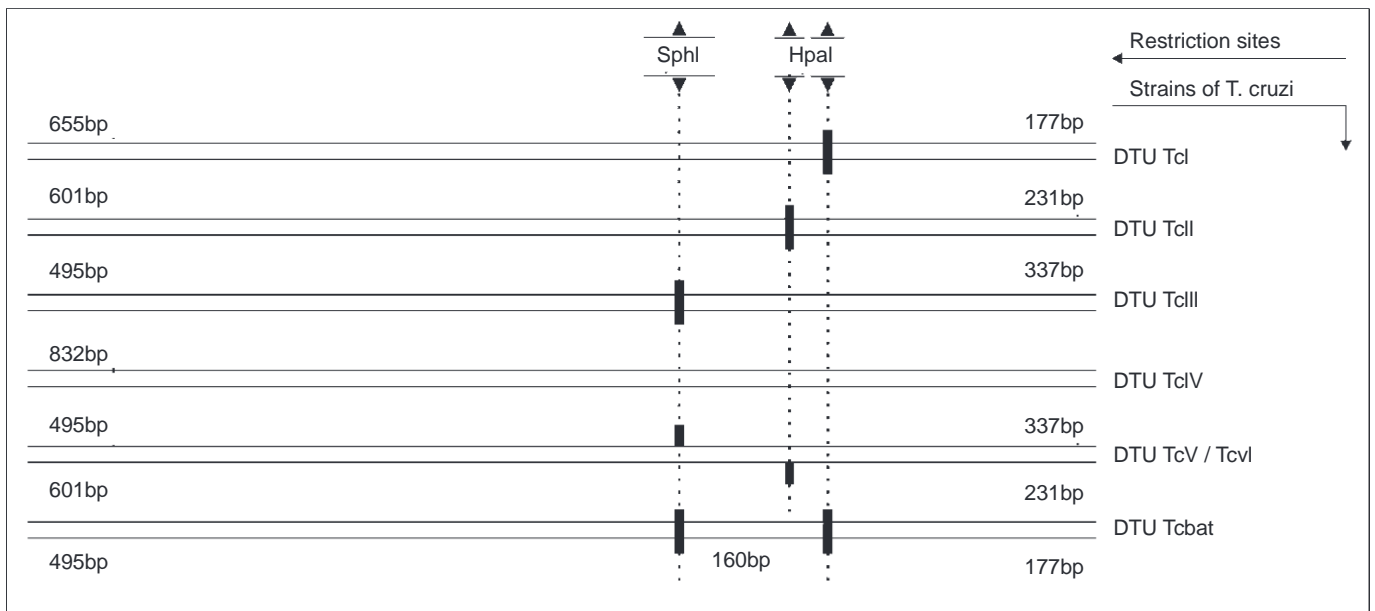


FIGURE 1 - Marker lineage using the TcSC5D gene. Simplified schematic representation of the TcSC5D amplicon and the polymorphic restriction sites HpaI/SphI, showing the presence/absence of *T. cruzi* in each strain (modified from Cosentino and Agüero³⁴). DTU: discrete typing units; T.: *Trypanosoma*.

TABLE 1 - Molecular identification of *Trypanosoma cruzi* using primers 121/122 and the DTUs identified using primers TcSC5D-fwd/TcSC5D-rev in triatomines found in municipalities of the State of Mato Grosso do Sul, Brazil.

Municipality	Triatomine	Number*	Positive		DTU
			121/122	TcSC5D-fwd/rev	
Jaraguari	<i>Triatoma sordida</i>	260	76	5	TcI
Terenos	<i>Triatoma sordida</i>	48	1	1	TcII
	<i>Triatoma matogrossensis</i>	9	5	1	TcII
Aquidauana	<i>Triatoma sordida</i>	12	2	1	TcII
	<i>Rhodnius</i> sp.	1	-	-	-
Aparecida do Taboado	<i>Triatoma sordida</i>	54	10	2	TcI
Rochedo	<i>Triatoma sordida</i>	64	3	1	TcBat
Caarapó	<i>Panstrongylus megistus</i>	1	1	1	TcII
Dourados	<i>Panstrongylus megistus</i>	1	1	-	-
Corumbá	<i>Triatoma sordida</i>	5	1	-	-
Miranda	<i>Triatoma sordida</i>	7	-	-	-
Rio Verde de Mato Grosso	<i>Triatoma sordida</i>	17	-	-	-
São Gabriel do Oeste	<i>Triatoma matogrossensis</i>	8	-	-	-
Paranaíba	<i>Triatoma sordida</i>	22	-	-	-
Antônio João	<i>Triatoma sordida</i>	1	-	-	-
Douradina	<i>Triatoma sordida</i>	1	-	-	-
Total		511	100	12	

*number of triatomines captured. DTU: discrete typing units.

TcI, TcII and TcBat located in the center, TcI in the east, and TcII in the west (**Figure 2**).

The PCR product generated from the TcSC5D gene was cloned and sequenced. After alignment and analysis of the nucleotide sequence, TcI, TcII and TcBat were found in 98-100% of the samples (**Figure 3**). It was not possible to sequence some of the samples; however, identification via RFLP revealed that DTUs were present in triatomines in those samples.

The reactions using primers for *T. rangeli* (data not shown) did not produce overlapping data, confirming that the amplicons were specific for *T. cruzi*.

DISCUSSION

The most commonly captured triatomine was *T. sordida*, which was similar to what was observed in previous studies, showing that this species, which is usually considered secondary, is the most frequently encountered species in MS^{4,26,27}. *Triatoma sordida* is native to the Cerrado, the biome in which State of Mato Grosso do Sul as well as the transition areas of Maranhão, Piauí, Bahia, the Pantanal and the eastern Chaco are located³⁹. This was also the species that harbored the greatest variety of DTUs (TcI, TcII and TcBat). As this species was the most frequently encountered, the likelihood of finding the largest

number of infected insect species as well as a greater variety of DTUs was higher.

Insect vectors usually occupy wild environments. However, if their habitat is degraded, they can relocate near human habitations, such as corrals, sties or chicken coops as well as other peridomiciliary constructions. Although all captured triatomines were found in peridomiciles, their mere coexistence with an insect vector increases the chances of infection⁴⁰. One notable factor is that TcII was associated with the severe forms of Chagas disease that were found south of the Amazon region^{41,42}; however, other factors are also associated with a higher or lower pathogenicity of the parasite⁴³, and its presence near residences is therefore reason to focus attention on the control of triatomines, even though this DTU was found less frequently in this study.

Trypanosoma cruzi is extremely successful, as observed by its continental distribution and its broad host range, which was replicated in our State of Mato Grosso do Sul data. The DTUs found in this work are common in MS, and other studies have identified their presence in the Pantanal region^{22,44}. **Figure 2** shows that the central region of State of Mato Grosso do Sul had the largest variety of DTUs, presenting TcI, TcII and TcBat (first report of natural infection in triatomines).

Although TcI was also found in the western area of the state^{22,44}, this DTU was found to infect triatomines in only

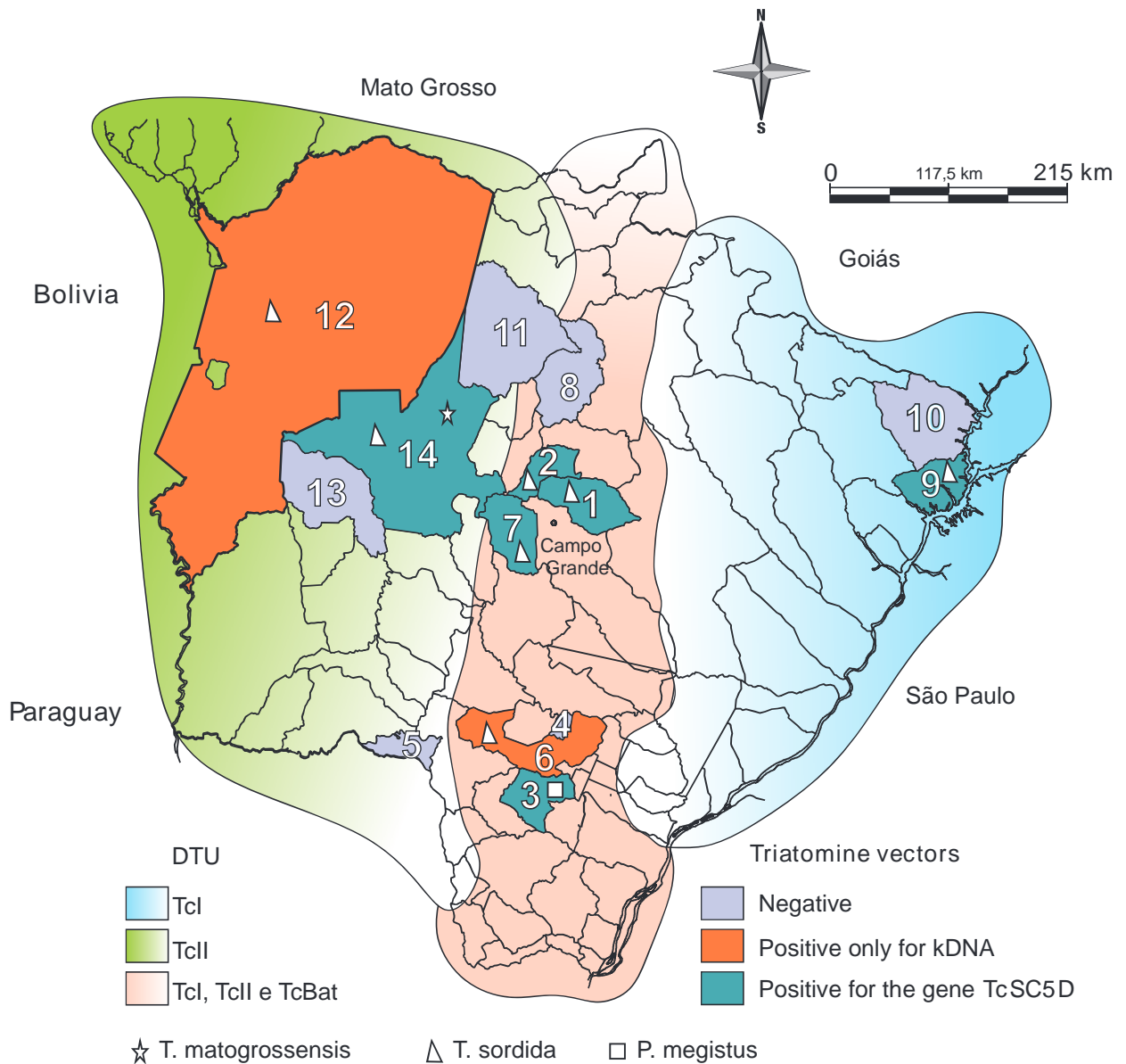


FIGURE 2 - Map of the State of Mato Grosso do Sul, Brazil, highlighting the cities where the triatomines were captured and the distribution of the DTUs found in infected insects. 1: Jaraguari; 2: Rochedo; 3: Caarapó; 4: Douradina; 5: Antônio João; 6: Dourados; 7: Terenos; 8: São Gabriel do Oeste; 9: Aparecida do Taboado; 10: Paranaíba; 11: Rio Verde de Mato Grosso; 12: Corumbá; 13: Miranda; 14: Aquidauana. DTU: discrete typing units; kDNA: kinetoplast deoxyribonucleic acid. *T.*: *Triatoma*; *P.*: *Panstrongylus*.

the central and eastern areas of the state. Other studies have demonstrated the presence of TcI in São Paulo (SP)⁴⁵ and Minas Gerais (MG)⁴⁶, neighboring states to the East of MS, and in the City of Aparecida do Taboado. However, the occurrence of more than one DTU was observed in SP and MG. One of the factors that may be associated with finding only one TcI in the eastern area of MS may be that it is the most anthropized portion of the state⁴⁷, with large agropastoral formations that reduce the availability of meat for triatomines. As *T. sordida* was the most abundant species, is predominantly ornithophilous and was obtained from henhouse peridomiciles, finding other DTUs in the studied sites might be difficult. The incidence of TcII was higher in the west,

which is similar to the results of previous work with vectors and other wild animals, but this DTU was found mainly in the Pantanal region^{44,48}. One possible explanation for this localization is that the region is preserved (in relation to the eastern state), leading to an increased supply of hosts as food sources for the insect vectors, thus facilitating the movement of the parasite. Studies indicate the occurrence of at least approximately 124 species of mammals in the Pantanal region alone⁴⁹⁻⁵², but this is only an estimate, and further studies may confirm or refute this hypothesis. However, as the dispersion and isolation of *T. cruzi* is limited by the dispersion of their hosts, which include several orders of mammals and triatomines⁵³, this idea deserves more attention.

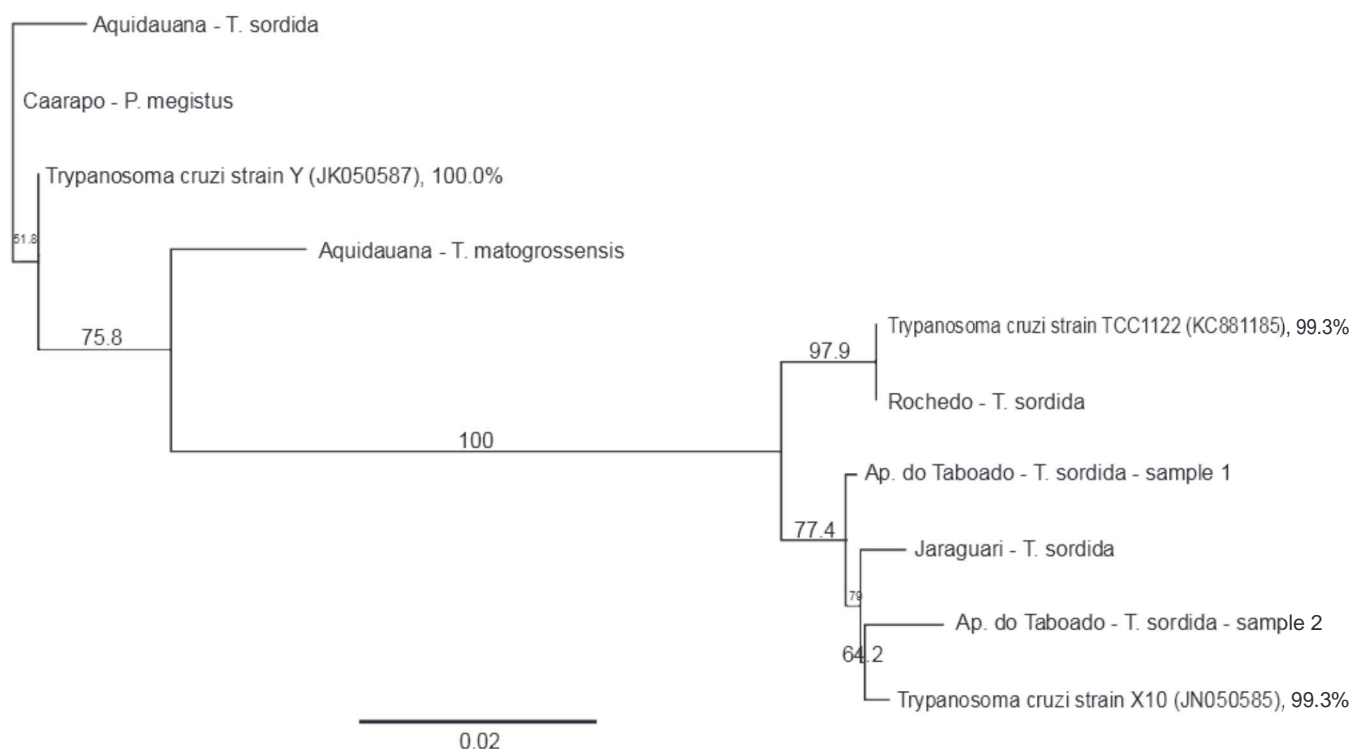


FIGURE 3 - Partial sequence of the TcSC5d gene. Phylogenetic position of the samples of different genotypes in relation to other DTUs of *Trypanosoma cruzi*. The analysis was performed with 1,000 bootstrap replicates. The number in parentheses next to the accession number is the GenBank. The scale bar indicates the nucleotide substitutions per site. DTU: discrete typing units. *T.*: *Triatoma*; *P.*: *Panstrongylus*.

Other studies have demonstrated that TcIII is present in central MS⁴⁵, which is the same area where TcI is prevalent, more specifically, in the community of Furnas do Dionísio, Jaraguari. This is a rural Quilombola community located 45km from Campo Grande, the capital of MS. The community comprises approximately 1,031ha, with modest brick houses that are often near wood sties, chicken coops and pens. However, although Marcili et al.⁴⁵ found TcIII circulating in dogs, the present study showed that only TcI circulated in triatomines and TcII circulated in bats (data not shown) in this community. TcI is a DTU that is widely distributed from South to North America, with most of the isolates from the Amazon region⁵⁴⁻⁵⁷. Although TcI is known to be predominantly wild and the source of more frequent, serious infections in humans in Central America, Venezuela⁵⁸ and even in the Amazon region, studies have shown that TcI can manifest severe forms of Chagas disease, especially when co-infection occurs in humans with different DTUs^{42,59}. In this case, the epidemiological situation of the studied community has drawn attention because it is located between hills, resulting in a high frequency of infected insects and a large number of natural and artificial ecotopes, as described by Cominetti et al.²⁶; it is therefore an ideal environment for triatomine colony infestations. Moreover, the observed TcII infection of bats leads to the question of whether there is overlap between peridomestic and sylvatic cycles that leads to an exchange of the *T. cruzi* types found in the area. Eco-epidemiological and molecular

studies are needed in this region and are now being performed to elucidate this question.

The same types found in the central areas of MS, except for TcBat, were also found in the State of Paraná⁶⁰, which is a route of passage for DTUs (**Figure 2**). However, it can be inferred from our data, in contrast to other studies^{44,45,48}, that it is not possible to delimit DTUs to a specific region. The overlap of DTUs can result from the variety of intricate types of dispersion of the parasite due to the peculiar mechanisms of vector reinfestation⁶¹ the wide variety of hosts, which are abundant and conspicuous in the Cerrado¹⁹, and from environmental heterogeneity⁶².

Despite the fact that we did not demonstrate mixed infection by TcI and TcII in insect vectors, as reported by Abolis et al.⁶⁰, this phenomenon may also occur in MS because the conditions for these mixed infections in triatomines are present in this state. Three DTUs were identified in the center of the state and in nearby municipalities. In addition, the Cerrado biome, which covers a large portion of MS, has significant horizontal variation, with open fields, capons, woods, forests and wetlands that potentially coexist in the same region¹⁹. These data add more strength to the idea that the distribution of the parasite is very wide and dynamic.

TcBat, a trypanosome that, until now, had been found only in bats and a child⁶³, has been identified in *T. sordida*. This

finding extends the distribution of this DTU in the state, as it was previously described only in the Pantanal region of MS^{22,48}. Additionally, this is the first report of this DTU in a naturally infected triatomine, which had not been previously described, even after attempts at laboratory infection²². However, it is expected that the incidence of this DTU is more frequent because it is most closely related to TcI, the most widely distributed DTU in MS, and has finally been identified to infect a triatomine. In the same region, TcII was found in a sample of DNA extracted from the blood of an armadillo (*Euphractus sexcinctus*) (data not shown).

This study demonstrates the occurrence of overlapping DTUs in the State of Mato Grosso do Sul. The geographical distributions of the DTUs follow different patterns, with TcI, TcII and TcBat in the center, TcI predominantly in the east, and TcII in the west. Furthermore, TcI was the most frequent DTU in triatomines, followed by TcII and TcBat. This study provides greater insight into the distribution of *T. cruzi* in Brazil, but further studies may reveal a more defined mosaic distribution of DTUs in MS over the long term.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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