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Trypanosoma cruzi and *Trypanosoma rangeli* in Acre, Brazilian Amazonia: Coinfection and Notable Genetic Diversity in an Outbreak of Orally Acquired Acute Chagas Disease in a Forest Community, Wild Reservoirs, and Vectors

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Abstract: Acute Chagas disease (ACD) caused by *Trypanosoma cruzi* has emerged as a major food-borne disease in Brazilian Amazonia. For the first time, we characterized an outbreak of orally acquired ACD in Acre, in the forest community of Seringal Miraflores, affecting 13 individuals who shared the pulp of açai palm berries: 11 adults and two children (one newborn), all diagnosed by thick-drop blood smears. The fluorescent fragment length barcoding method, which simultaneously identifies species/genotypes of trypanosomes in blood samples, uncovered an unprecedented genetic diversity in patients from a single outbreak of ACD: *T. cruzi* TcI in all patients, mostly concomitantly with the non-pathogenic *Trypanosoma rangeli* of genotypes TrA or TrB, and TcI, TcIV, and TrB in the child. The patients presented persistent fever, asthenia, myalgia, edema of the face and lower limbs, hepatosplenomegaly and, rarely, cardiac arrhythmia. The clinical symptoms were not correlated to gender, age, or to trypanosome species and genotypes. The inferred SSU rRNA phylogenetic analyses of trypanosomes from humans, triatomines and sylvatic hosts included the first sequences of *T. cruzi* and *T. rangeli* from humans in southwestern (Acre and Rondônia) Amazonia, and the first TcI/TcIV sequences from *Rhodnius* spp. from Acre. The sylvatic transmission cycles of genetically different trypanosomes in landscapes changed by deforestation for human settlements and increasing açai production is a novel scenario favoring trypanosome transmission to humans in Acre.

Keywords: emerging chagas disease; food-borne disease; zoonotic disease; Amazon Forest; family outbreak; oral infection; molecular diagnosis; genotyping

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1. Introduction

Chagas disease (CD) epidemiology has changed drastically in Brazil, where currently acute Chagas disease (ACD) prevails in Amazonia, differing from the former endemic areas where CD was mostly a chronic disease acquired before the control of the domestic vectorial transmission. In the last two decades, more than 70% of ACD cases in Brazil

occurred in Amazonia, mostly as outbreaks of oral transmission [1–8]. In 2012, recognizing the importance of oral transmission of CD, the United Nations Food and Agriculture Organization (FAO) and the World Health Organization (WHO) classified CD as the tenth among 24 food-borne parasitoses of epidemiological and clinical relevance in the world [9,10].

Trypanosoma cruzi, the etiological agent of CD, circulates among a wide variety of wild mammalian and triatomine vectors through the Amazon region. Nevertheless, Brazilian Amazonia was considered free of autochthonous ACD until Shaw et al. (1969) reported the first family outbreak affecting four people [11]. After about three decades of sporadic reports, a succession of studies in the states of Pará (PA), Amapá, and Amazonas (AM) evidenced widespread ACD due to oral and, occasionally, vectorial transmission [1–3,8,12–15]. Even though ACD became considered emergent in Brazilian Amazonia two decades ago, the disease remained underreported until 2007, when it became of obligatory notification to SINAN (the Notifiable Diseases Information System of the Brazilian Ministry of Health). Though still underestimated, the number of confirmed cases of ACD in Brazilian Amazonia has nearly tripled in the last decade, increasing from 136 in 2010 to 386 cases in 2019, most times attributed to oral transmission by the consumption of pulp of açai and bacaba palm berries (SINAN, available at: http://tabnet.datasus.gov.br/cgi/deftohtm.exe?sinannet/cnv/chagasac.def, accessed on 14 November 2021). *T. rangeli* is a non-pathogenic species that, as with *T. cruzi*, is found in vertebrate hosts and vectors (*Rhodnius* spp.) in central and South America. In Brazil, this species is widespread in sylvatic transmission cycles, but very rare in humans, with just three cases reported in Brazilian Amazonia (AM) [16,17].

The remarkable increase in the number of ACD reports in the Amazon region, predominantly in areas of high production and daily consumption of fresh traditionally processed açai, can be attributed to a combination of more effective surveillance, the improved diagnosis of ACD (particularly by Malaria services), the training of public health personnel, and compulsory notification to SINAN. To date, the number of ACD cases reported from southwestern Amazonia in Acre (AC) [18] and Rondônia (RO) [19] is relatively small compared to those reported from PA in eastern [1,5,12,20–22] and AM in western [3,6,8,13,14,23] Amazonia. In AC, the first report of autochthonous ACD was in a child of a seven-member family in the municipality of Plácido de Castro. Vectorial transmission was assumed, due to the discovery of *T. cruzi*-infected *Rhodnius robustus* in palm trees near the house [24]. In 1993, three children of the same family showed fever, dyspnea, abdominal distention, and edema of the face and lower limbs, as well as trypanosome-positive thick-blood drop tests; two recovered after treatment, and one died of acute pan-carditis [25].

Epidemiological studies conducted by SESACRE (Secretaria de Saúde do Acre, Divisão de Vigilância e Epidemiologia: Doença de Chagas e Leishmanioses) have indicated an increasing number of ACD cases in AC, where oral infection is becoming of increasing concern. From 2009 to 2016, 42 cases were identified, while in 2019, the number reached 60 cases (Figure 1). Most cases (~78%) were associated with oral infection, ~20% with vectorial transmission, and ~2% of cases were of unascertained transmission route [18] (SESACRE). Small outbreaks of ACD (1–6 cases) have been reported annually in AC, except for two large outbreaks, both associated with açai consumption (Figure 1). The last one occurred in 2019, affecting 12 individuals from three families in a rural locality (Marechal Thaumaturgo) (SINAN). The first occurred in 2016 in the forest community of Seringal Miraflores, affecting 13 persons from eight families of one family group (Table 1). Seringal Miraflores belongs to the municipality of Feijó (Figure 1), the most important producer of açai in AC, where 21 cases of ACD occurred, most associated with oral transmission. The ACD data in AC likely present a low estimate, considering the unspecific symptoms of ACD, the inaccessibility of population to medical services, and the low sensitivity of the thick-drop blood smears used to diagnose ACD.

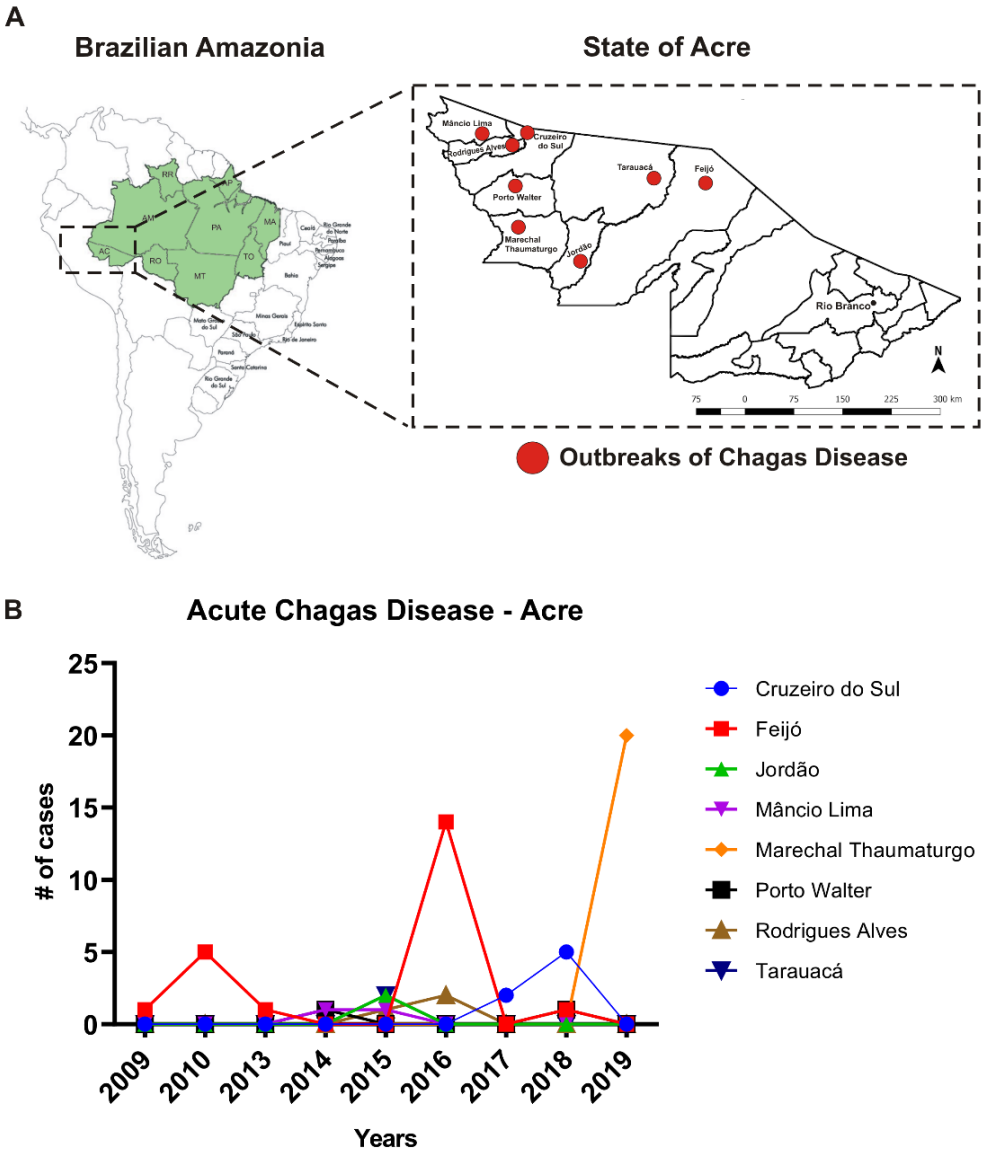

Figure 1. (**A**) Map of the Acre state in southwestern Brazilian Amazonia indicating the municipality of Feijó, where the Seringal Miraflores is situated, and other localities where outbreaks of acute Chagas disease (ACD) occurred. (**B**) Retrospective data (2009 to 2019) of outbreaks, including the number of ACD cases, that occurred in 8 municipalities in Acre, and were notified to SINAN (Notifiable Diseases Information System of Brazilian Ministry of Health).

Comprehensive studies of ACD in AC are required to understand risk factors, disease manifestations, and the genetic diversity, vectors, and reservoirs of trypanosomes. Here, we describe the most plausible epidemiological scenario, symptomatology, and molecular characterization of the trypanosomes present in the ACD patients of the Seringal Miraflores outbreak. In addition, we report on the genetic diversity of *T. cruzi* and *T. rangeli* in the wild mammals and triatomine vectors that maintain the enzootic cycle and that may serve as sources of parasites for human infections in southwestern Amazonia.

Table 1. Outbreak of acute Chagas disease in the Seringal Miraflores, Feijó, Acre: patients, families, diagnosis (blood smears), symptoms, and clinical signs.

Patient	Sex	Age	House	Blood Smear	Fever	Chagoma of Inoculation	Edema on Face and Lower Limbs	Asthenia	Splenomegaly	Hepatomegaly	Cardiac Arrhythmia	Severe Cardiac Involvement
M1	F	20	1	POS	Yes	No	Yes	Yes	Yes	Yes	No	No
M2	M	28	1	POS	Yes	No	No	Yes	Yes	Yes	Yes	No
M3	F	26	2	POS	Yes	No	No	Yes	Yes	Yes	No	Yes
M4	M	4	2	POS	Yes	No	No	No	No	No	No	No
M5	M	27	3	POS	Yes	No	Yes	Yes	Yes	Yes	No	No
M6	F	27	3	POS	Yes	No	Yes	Yes	Yes	Yes	Yes	No
M7	F	25	4	POS	Yes	No	Yes	Yes	Yes	No	Yes	No
M8	M	30	4	POS	Yes	No	No	Yes	No	No	No	No
M9	M	16	5	POS	Yes	No	No	Yes	Yes	Yes	No	No
M10	M	31	5	POS	Yes	No	Yes	Yes	Yes	Yes	No	No
M11	F	20	6	POS	Yes	No	No	Yes	Yes	Yes	Yes	No
M12	M	3 m	6	POS	Yes	No	No	Yes	Yes	Yes	No	No
M13	M	23	7	POS	Yes	No	Yes	Yes	Yes	Yes	No	No

2. Material and Methods

2.1. Study Area, Açai Consumption, and the Community of Seringal Miraflores

Acre is located within the southwest Amazon moist forest region, where much of the forest remains near-intact as drastic deforestation has been limited to areas near urban centers and the BR-364 National Road, which has drastically threatened the forest due to logging and the agricultural and pastoral frontier expansion along this major road. Acre is the fourth national producer of açai in Brazil; the municipality of Feijó is the leading producer of AC and is a distance of 90 km from Rio Branco, the AC capital (Figure 1). Feijó harbors around 35,000 inhabitants with a demographic density of 1.16 habitants per square km, most living in rural communities (IBGE, Instituto Brasileiro de Geografia e Estatística available at: https://www.ibge.gov.br/cidades-e-estados/ac/feijo.html, accessed on 15 November 2021). The Seringal Miraflores in Feijó is a small community located at the 60 km marker of the BR-364, formed by local people living in houses constructed in areas deforested for subsistence agriculture and bordered by the forest. The main activity in Seringal Miraflores is the extraction of açai berries (*Euterpe oleracea*). This economic activity greatly substituted the traditional collection of both Brazilian nuts (*Bertholetia excelsa*), and the latex of *Hevea brasiliensis* in the originally well-preserved forest.

Acre adopted the Brazilian National Health Surveillance Agency program to prevent and manage orally transmitted ACD. This program established good practices for handling açai, aiming to eliminate *T. cruzi* contamination, which included the "bleaching" of the açai, a thermal shock treatment consisting of soaking the berries in water at 80 °C and then in cold water [26]. This procedure is obligatory in fresh açai pulp markets, whereas pasteurization is obligatory for the industrial processing of açai. However, rural communities consume açai processed at home, either manually or by employing domestic electrical devices to crush the berries, and generally without following sanitary recommendations. Rural Amazonian people preserve the traditional habit of preparing the pulp at night, when the light attracts triatomines that fly from palms and can fall into the containers of açai pulp to be consumed on the same night and early in the morning.

2.2. Parasitological and Serological Diagnosis of Acute Chagas Disease

People from the Miraflores locality were screened (6–14 July 2016) by thick-blood drop tests in the Feijó Hospital, which also collected a blood sample from each patient in heparin-containing tubes. After the transfer of the sera to new tubes (preserved at −20 °C), the blood clots remaining in each tube were covered with ethanol (99.5%) and preserved at the biobank of the Chagas disease Laboratory at the Institute Evandro Chagas (IEC), in Belém, a reference center for ACD in Brazilian Amazonia. The serological diagnoses of ACD were carried out at the IEC by indirect hemagglutination assay (IHA), and the indirect immunofluorescence assay for the detection of IgM against *T. cruzi* (IFA-IgM); for both

tests, a cut-off of 1:40 was accepted as positive. IFA-IgG (cut-off 1:80) was simultaneously performed to search for chronic cases, as described in [12,20,21].

2.3. Clinical Data and Field Epidemiological Investigations

Before and after treatment, clinical exams of the Miraflores patients were performed in the Feijó Hospital. The ACD diagnosis and the most plausible transmission route were established following the protocol recommended by the Health Ministry of Brazil, including information from epidemiological and entomological investigations, the laboratory tests of the blood samples, and clinical exams (Table 1).

2.4. Molecular Diagnosis and Trypanosome Genotyping

The molecular diagnosis of ACD was performed by the FFLB (fluorescent fragment length barcoding) method, which simultaneously detects and distinguishes with high sensitivity and accuracy trypanosome species and genotypes, using DNA obtained from blood clots preserved in ethanol. The visual inspection of FFLB profiles permitted the simultaneous identification of the DTUs of *T. cruzi* (except for very similar profiles of TcII and TcVI) and the genotypes (TrA-TrE) of *T. rangeli* [19,27–30]. In addition, FFLB was used to assess trypanosomes in the digestive tract of *Rhodnius* spp. captured in palm trees from peridomestic environments in AC.

Selected samples from blood (human and wild mammals) and triatomines showing different trypanosome species/genotypes by FFLB were submitted for the nested PCR amplification of polymorphic SSU rRNA sequences. The amplicons were cloned, and sequences of 5–10 clones were determined for each sample, as described previously [27,31]. The sequences were deposited in GenBank (accession numbers in Supplementary Table S1). Two alignments were created with sequences of *T. cruzi* or *T. rangeli* from Brazilian Amazonia herein determined and available in GenBank, and sequences from reference isolates previously reported [32–37]. The two alignments were submitted for parsimony analysis with 500 bootstrap replications on PAUP software version 4.0b10 [38]. The host species, geographical origin, and GenBank accession numbers of all sequences included in our analyses are found in Supplementary Table S1.

3. Results

3.1. Parasitological, Serological, and Molecular Diagnosis of Acute Chagas Disease

Individuals of the Seringal Miraflores sought medical care within one-week intervals in the Feijó hospital (presumably on days 7 to 14 of infection), referring to fever, asthenia, and myalgia. The microscopy of Giemsa-stained thick-drop blood smears revealed trypomastigote forms typical of *T. cruzi* in 13 individuals, thus prompting the diagnosis of ACD in all patients, 7 males and 6 females. These patients included 11 young adults (between 16 and 31 years old), one child (4 years old), and one newborn child (3 months old) (Table 1). The diagnosis of ACD by thick-drop blood smears was corroborated by positive IHA and IFA-IgM serological tests, and by FFLB using DNA from the blood clots of the patients (Table 2). All patients were negative for IFA-IgG, thus excluding the possibility of previous infections by *T. cruzi*.

3.2. Transmission Route, Outbreak Description, Clinical Data, and Treatment

The most likely source of the simultaneous infection of all patients from the Seringal Miraflores outbreak was indicated by patient interviews, revealing that only people that consumed açai pulp at a community meeting got sick. The 13 diagnosed patients dwelled in 8 separate houses, cohabited by another 19 family members, 3 to 8 people per house, who all tested negative on thick-drop blood smears. Only one to three individuals per house consumed açai in the reunion, and only these individuals yielded *T. cruzi*-positive blood smears (Table 1). Although the whole community consumes açai practically daily, none of the other individuals examined presented fever or referred to any sickness during the 60-day follow-up period, and all had negative blood smears. No external signs of parasite

entry (chagoma of inoculation) were observed in any patient. Moreover, no triatomines were found inside the patients' houses when the outbreak occurred.

The Miraflores ACD patients present symptoms and signs typical of acute infection due to the oral route in Brazilian Amazonia (Table 1). Besides unspecific fever, asthenia, and myalgia, physical examination evidenced a set of clinical signs typical of ACD in the Amazon region: edema of the face and lower limbs (7 patients), moderated hepatosplenomegaly established by ultrasonography (10 patients), and moderated cardiac arrhythmia (4 patients), with severe cardiac involvement observed in only one adult patient (M3, female, 26 years old) also showing hepatosplenomegaly and edema. The young adult exhibiting the most severe cardiac involvement was diagnosed exclusively with TcI (Table 1).

All the Miraflores patients were successfully treated with benznidazole (60 days), as recommended by the Brazilian Ministry of Health, and all were clinically healthy, and showed negative thick-drop blood tests at the end of the treatment. Unfortunately, the follow-up did not include serology because of the logistical troubles faced in managing this first (2016) large outbreak of ACD in Acre. Most patients were examined for up to three years, and none ever showed any signs suggestive of CD- nor *T. cruzi*-positive blood smears. Nowadays, SESACRE can support the whole protocol for ACD diagnosis, treatment, and follow-up post-treatment recommended by the Brazilian Ministry of Health.

Table 2. FFLB genotyping of *Trypanosoma cruzi* (DTUs Tc and TcIV) and *Trypanosoma rangeli* (TrA and TrB genotypes) from southwestern Amazonia, including Acre and Rondônia: hosts, geographic origin, and respective DTUs/genotypes.

Sample [a]	Isolate [b] Species	Host Species		Geographic Origin	FFLB Genotype	
					T. cruzi [c]	*T. rangeli* [d]
BS	AcM1	human	*Homo sapiens*	Acre	**TcI**	-
BS	AcM2	human	*Homo sapiens*	Acre	**TcI**	-
BS	AcM3	human	*Homo sapiens*	Acre	**TcI**	-
BS	AcM4	human	*Homo sapiens*	Acre	**TcI and IV**	**TrB**
BS	AcM5	human	*Homo sapiens*	Acre	**TcI**	**TrB**
BS	AcM6	human	*Homo sapiens*	Acre	**TcI**	**TrB**
BS	AcM7	human	*Homo sapiens*	Acre	**TcI**	-
BS	AcM8	human	*Homo sapiens*	Acre	**TcI**	**TrB**
BS	AcM9	human	*Homo sapiens*	Acre	**TcI**	**TrA**
BS	AcM10	human	*Homo sapiens*	Acre	**TcI**	**TrA**
BS	AcM11	human	*Homo sapiens*	Acre	**TcI**	**TrB**
BS	AcM12	human	*Homo sapiens*	Acre	**TcI**	-
BS	AcM13	human	*Homo sapiens*	Acre	**TcI**	**TrA**
DT	Rro1AC	triatomine	*Rhodnius robustus*	Acre	**TcI**	-
DT	Rro2AC	triatomine	*Rhodnius robustus*	Acre	**TcI**	-
DT	Rro3AC	triatomine	*Rhodnius robustus*	Acre	**TcI**	-
DT	Rro4AC	triatomine	*Rhodnius robustus*	Acre	**TcI**	-
DT	Rro5AC	triatomine	*Rhodnius robustus*	Acre	**TcI and IV**	-
DT	Rsp1AC	triatomine	*Rhodnius* sp.	Acre	**TcI**	-
DT	Rsp2AC	triatomine	*Rhodnius* sp.	Acre	**TcI**	-
BS	LBT7074	bat	*Artibeus lituratus*	Acre	TcI	-
Cult 262	AEAAB	non-human primate	*Cebuella pygmaea*	Acre	TcI	-
Cult 331	AM-ANV	non-human primate	*Sapajus apella*	Acre	TcI	-
BS	LBT7097	bat	*Phyllostomus discolor*	Acre	TcI	-
Cult 338	fusciolis 15	non-human primate	*Saguinus fuscicollis*	Acre	TcIV	-
Cult 338	labiatus 17	non-human primate	*Saguinus labiatus*	Acre	TcIV	-
BS	LBT5009	bat	*Artibeus lituratus*	Acre	TcIV	-
BS	LBT5060	bat	*Phyllostomus hastatus*	Acre	TcIV	-
DT	Rsp1Ac	triatomine	*Rhodnius* sp.	Acre	**TcIV**	-
Cult 353	Maloch-05	non-human primate	*Callicebus cupreus*	Acre	-	TrA
BS	LBT 5428	bat	*Artibeus planirostris*	Acre	-	TrA
BS	LBT 5472	bat	*Carollia perspicillata*	Acre	-	TrA

Table 2. *Cont.*

Sample [a]	Isolate [b] Species	Host Species		Geographic Origin	FFLB Genotype	
					T. cruzi [c]	*T. rangeli* [d]
BS	C750	dog	*Canis familiaris*	Acre	-	TrA
Cult 232	11841	non-human primate	*Saguinus labiatus*	Acre	-	TrB
Cult 235	46388	non-human primate	*Saguinus fuscicollis*	Acre	-	TrB
Cult 233	11049	non-human primate	*Saguinus labiatus*	Acre	-	TrB
Cult 207	AE-AAA	non-human primate	*Cebuella pygmaea*	Acre	-	TrB
Cult 194	AE-AAB	non-human primate	*Cebuella pygmaea*	Acre	-	TrB
Cult 1042	ROi 309	triatomine	*Rhodnius robustus* II	Rondônia	TcI	-
Cult 649	Rr649	triatomine	*Rhodnius robustus* II	Rondônia	TcI	-
DT	Rm13	triatomine	*Rhodnius montenegrensis*	Rondônia	TcI	-
Cult 363	Roma06C	didelphid	*Didelphis marsupialis*	Rondônia	TcI	-
Cult 640	640	bat	*Carollia perspicillata*	Rondônia	TcI	-
Cult 642	642	bat	*Carollia perspicillata*	Rondônia	TcI	-
BS	cujubim	human	*Homo sapiens*	Rondônia	TcIV	-
Cult 661	Rr661	triatomine	*Rhodnius robustus* II	Rondônia	TcIV	-
Cult 698	Rr698	triatomine	*Rhodnius robustus* II	Rondônia	TcIV	-
DT	Rm17	triatomine	*Rhodnius montenegrensis*	Rondônia	TcIV	-
DT	cujubim	triatomine	*Panstrongylus geniculatus*	Rondônia	TcIV	-
Cult 704	ROR-85	triatomine	*Rhodnius robustus* II	Rondônia	-	TrA
Cult 667	ROR-20	triatomine	*Rhodnius robustus* II	Rondônia	-	TrA
DT	Rm23	triatomine	*Rhodnius montenegrensis*	Rondônia	-	TrA
Cult 369	Roma01	opossum	*Didelphis marsupialis*	Rondônia	-	TrA
Cult 382	Roma06	opossum	*Didelphis marsupialis*	Rondônia	-	TrA

[a], BS, blood sample; DT, triatomine digestive tract; Cult number = TCC code numbers of cultures cryopreserved in the Trypanosomatid Culture Collection, Department of Parasitology, University of São Paulo, Brazil; [b], field-codes. [c], DTU, Discrete Typing Unit; [d], TrA and TrB genotypes of *T. rangeli*. Bold = sequences determined in the present study.

3.3. Genotyping of Trypanosoma cruzi and Trypanosoma rangeli Detected in Blood Samples and Triatomine Digestive Tracts from Acre

The molecular characterization of trypanosomes in blood samples from the Miraflores patients was carried out via the FFLB method. The resulting profiles evidenced a complex genetic diversity of trypanosomes (Figure 2). By comparing the FFLB profiles of trypanosomes from the blood samples of Miraflores patients with those of reference-isolates of TcI-TcVI DTUs and TrA-TrE genotypes, we identified two genotypes each, of *T. cruzi* (TcI and TcIV) and *T. rangeli* (TrA and TrB). Infection by more than one trypanosome species/genotype presented double or triple peak profiles (Figure 2).

T. cruzi TcI was identified in all patients concomitantly with TrA or TrB in three and five patients, respectively. TcI alone occurred in five patients, including the baby (Table 2, Figure 2). Notably, the baby (M12, male, three months old, son of M11) infected with TcI exhibited fever and hepatosplenomegaly, whereas its mother (M11, 20 years old), infected with TcI and TrB, presented fever and edema of the face and lower limbs. The child (M4, female, four years old, daughter of M3) infected with mixed TcI, TcIV, and TrB was the last to show symptoms and exhibited only fever, thus presenting relatively much better clinical conditions than some of the young adults exhibiting exclusively TcI (Table 1). Therefore, symptoms and clinical signs could not be related to DTUs, gender, or age. The presence of *T. rangeli*, of both the TrA and TrB genotypes, in different combinations with *T. cruzi* did not induce visible clinical changes in the early phases in ACD patients (Table 1).

In addition to blood samples, FFLB was used to assess trypanosomes in the digestive tracts of 14 specimens of *Rhodnius* spp. collected in peridomestic and domestic environments in the periphery of the Rio Branco and Cruzeiro do Sul municipalities in AC. The

results revealed four specimens of *R. robustus* infected with TcI and one with TcI plus TcIV, two *Rhodnius* sp. infected with TcI, and one *Rhodnius* sp. infected with TcIV (Table 2).

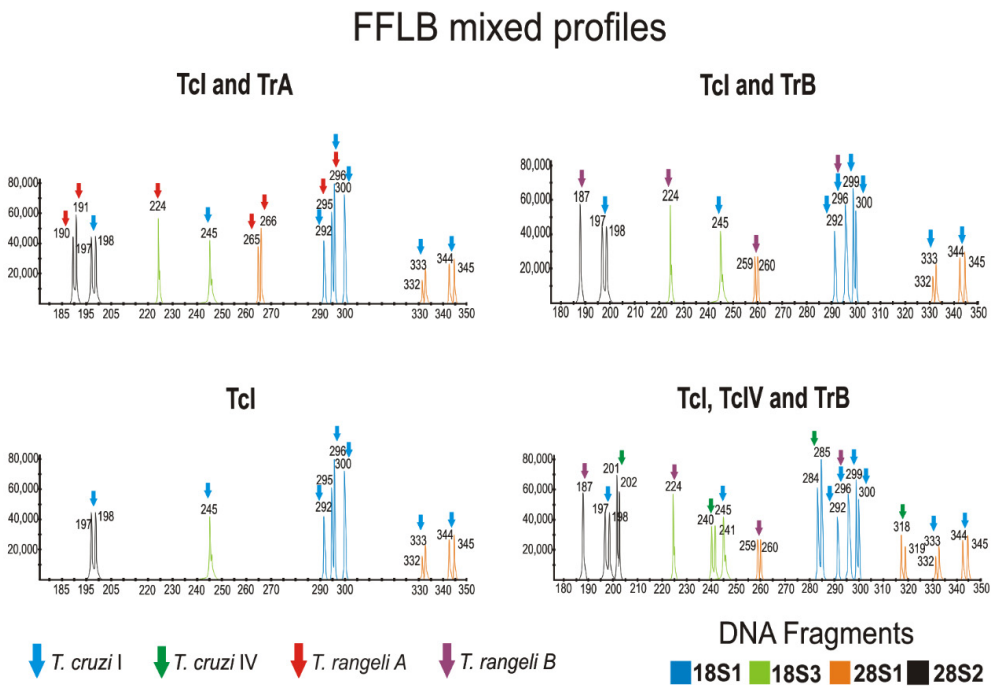

Figure 2. FFLB profiles obtained using DNA from blood samples of patients diagnosed with acute Chagas disease, Miraflores outbreak, Acre. Different combinations of FFLB profiles were selected to illustrate the different co-infections in the patients: M9, M10, and M13 showed TcI and TrA, M5, M6, M8, and M11 showed TcI and TrB; M4, TcI, TcIV, and TrB; M1, M2, M3, M7, and M12 showed exclusively TcI (Table 2).

3.4. Phylogenetic Relationships between T. cruzi and T. rangeli from Brazilian Amazonia

The genetic diversity of the trypanosomes identified by FFLB in the blood of the Miraflores patients (Figure 2, Table 2) was corroborated by SSU rRNA sequences. To illustrate the phylogenetic relationships of *T. cruzi* isolates from AC and RO (southwestern Amazonia) with those reported in other Amazon regions, an alignment of SSU rRNA sequences was created comprising TcI and TcIV from humans and triatomines from AC, which were herein characterized, TcI and TcIV from monkeys, dogs, and bats from AC (Genbank), and sequences of isolates from humans, monkeys, opossums, bats, and triatomines from RO from this study and the previous studies [17,19,32–37]. For comparison, we included sequences representative of the whole genetic diversity of *T. cruzi* from AM, PA, and AP (northwestern and eastern Amazonia) (Table 1; Supplementary Table S1).

Our *T. cruzi* phylogenetic analysis was the first including isolates from ACD cases in southwestern Brazilian Amazonia (Table 2): TcI and TcIV from AC were from the outbreak characterized herein, whereas TcIV from RO was from the first characterized ACD case in this state [19]. AC and RO are contiguous states sharing landscapes, mammalian and triatomine fauna, and a trypanosome repertoire, as herein demonstrated. TcI isolates from ACD cases formed two clusters, one comprising TcI from AC and RO more closely phylogenetically related to TcI from AM compared to TcI from PA and AP (Figure 3A). This finding indicated genetic differences among the TcI causing ACD in western and eastern Amazonia. The analysis of TcIV isolates showed that the sequence obtained from the Miraflores child's blood was identical to that of TcIV from the ACD case associated with vectorial transmission by *Panstrogylus geniculatus* in RO. In addition, TcIV sequences from AC and RO were highly similar to those from isolates from humans, wild mammals, and triatomines from AM, PA, and AP (Figure 3A).

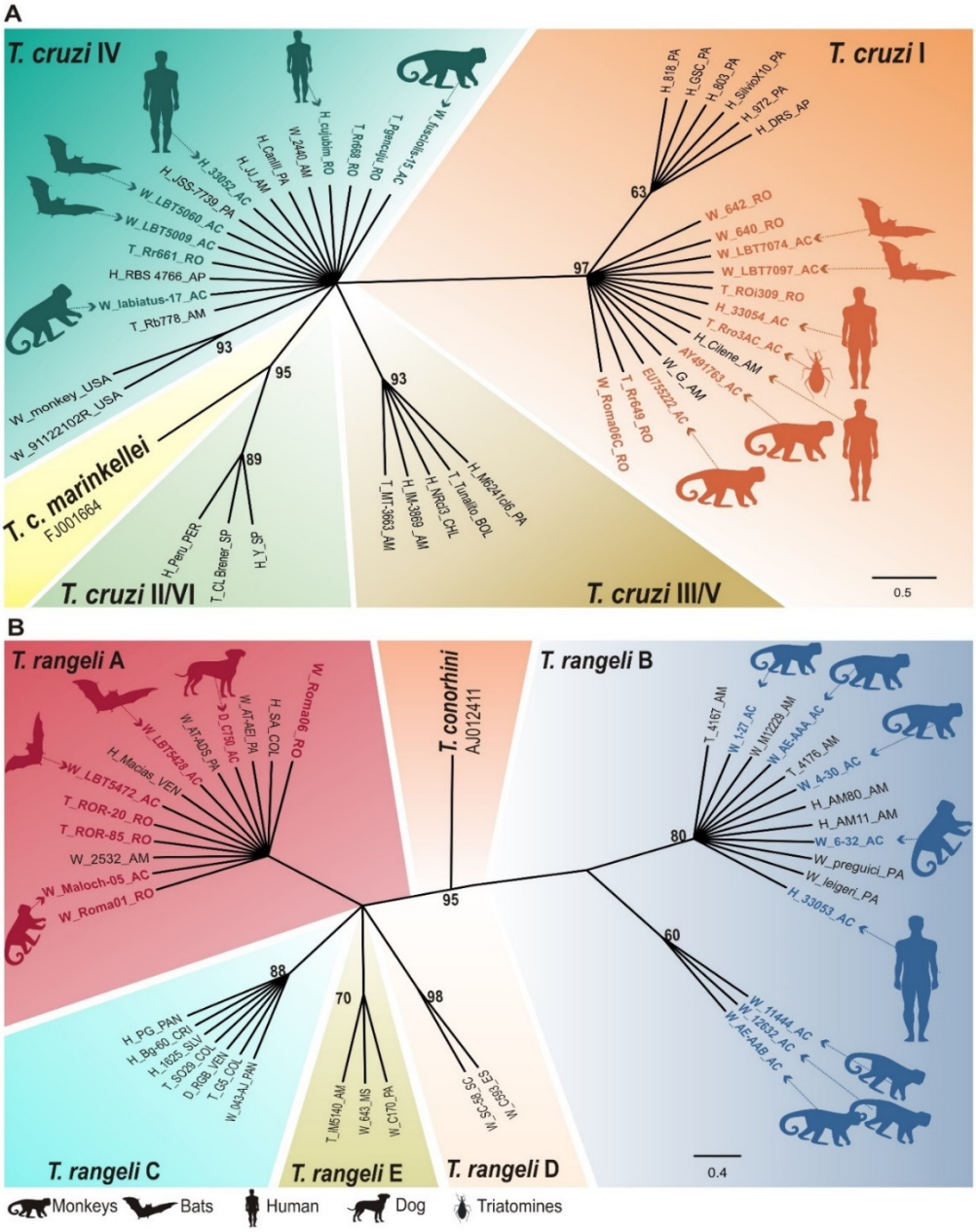

Figure 3. Phylogenetic relationships of (**A**) *T. cruzi* and (**B**) *T. rangeli* isolates from Acre (AC) and Rondônia (RO) in southwestern Brazilian Amazonia, compared with isolates from Pará and Amapá (eastern), and Amazonas (northwestern) states. (**A**) *T. cruzi* isolates (DTUs TcI and TcIV) of humans from AC (Miraflores) and RO, and from wild hosts and triatomines. (**B**) *T. rangeli* isolates from one Miraflores patient (TrB), and from monkeys (TrA and TrB), bats, dogs, and *Rhodnius* spp. (TrA) from AC and RO. Reference-isolates of *T. cruzi* DTUs (TcI- TcVI) and *T. rangeli* (TrA-TrE) were included in A and B, respectively. The inferences were based on SSU rRNA sequences using parsimony; the numbers at nodes correspond to the bootstrap values derived from 1000 replicates. Geographic origin of each isolate is indicated by the last letters in the sequence code: Brazilian States: AC, Acre; AM, Amazonas; AP, Amapá; PA, Pará; RO, Rondônia; SC, Santa Catarina; SP, São Paulo; ES, Espírito Santo; MS, Mato Grosso do Sul. Countries—BOL, Bolivia; COL, Colombia; CHL, Chile; CRI, Costa Rica; PAN, Panamá; SLV, El Salvador; VEN, Venezuela. The host origin of each isolate is indicated by the first letter in the sequence code: H, humans; W, wild hosts; T, triatomines. Figures indicate isolates from AC, and human isolates from RO.

The phylogenetic relationships of *T. rangeli* isolates included *T. rangeli* sequences of isolates from AC: TrB from the Miraflores patient, TrA and TrB from monkeys, and TrA from opossums, bats, and dogs. Additionally, TrA from opossums and *Rhodnius* spp. from RO were included in the analysis (Table 2). For comparison, we included sequences of TrA, TrB, and TrE from humans, wild mammals, and *Rhodnius* spp. from AM and PA, and sequences of TrA-TrE from different countries (Figure 3B; Supplementary Table S1). The analysis supported considerable genetic diversity, with two main phylogenetic lineages of *T. rangeli*. One lineage was formed exclusively by TrB isolates formed by two clusters; one comprising human isolates from AC and AM, and isolates from monkeys, sloths, anteaters, and *Rhodnius* spp. from other regions in Amazonia; the other cluster was formed exclusively by monkey isolates from AC. The lineage exclusive of TrB was positioned distantly from the second major phylogenetic lineage comprising four clusters: one cluster corresponding to the TrA genotype, two small clusters representing the TrE and TrD genotypes that are very closely related to TrA, and the cluster comprising the genotype TrC, never reported in Brazil. We could not obtain sequences of the TrA identified by FFLB in Miraflores patients, most likely due to very low parasitemia. Nevertheless, in our phylogenetic inference, all TrA isolates from Brazilian Amazonia shared identical sequences regardless of hosts and geographic origin. The analysis tightly clustered together TrA isolates from monkeys, bats, opossums, and domestic dogs from AC and RO with isolates from humans, monkeys, and *Rhodnius* spp. from PA and AM (Figure 3B).

4. Discussion

In the present study, we explored an outbreak of ACD that occurred in 2016 in the locality of Seringal Miraflores, a forest community in AC that depends economically on the extraction of açai. The simultaneous infection with *T. cruzi* and *T. rangeli* of 13 individuals of a family group that shared açai during a community reunion, the absence of triatomines inside the houses, and no signs of any chagoma of inoculation altogether disfavor vectorial transmission and support oral transmission. Our epidemiological investigations suggest oral infection with two genotypes of both *T. cruzi* and *T. rangeli* of Miraflores patients during the reunion, when açai brought by different families were shared. However, while the oral transmission of *T. cruzi* is very well-documented in experimental studies [39], to our knowledge, oral transmission of *T. rangeli* remains undocumented. In Miraflores, peridomicile palms are colonized by *Rhodnius* spp.; therefore, we could not exclude the possibility of vectorial transmission of both *T. cruzi* and *T. rangeli* before the outbreak.

In Amazonian Forest communities, açai pulp is prepared and ingested daily, and the pulp is consumed from the early months of life. The baby herein diagnosed with ACD may have been infected through the oral route by ingesting açai. However, the possibility of transmission through breastfeeding cannot be excluded because of the mother's acute infection, even though this route does not appear to be effective for humans [40].

This is the first time that *T. cruzi* infecting humans from AC was genotyped, revealing a predominance of TcI and one case of TcI mixed with TcIV. In AC, we previously reported TcI and TcIV in monkeys [34]; additionally, TcI was detected in bats and dogs [34,41]. The different profiles of trypanosomes in the Miraflores patients (Figure 2, Table 2) may be due to different sources of parasites contaminating the preparations of açai consumed. However, we cannot discard the possibility of very low parasitemia undetectable by FFLB, and the different levels of susceptibility of individuals to multiple trypanosome infections, as suggested by the triple infection detected exclusively in one four-year-old child. Notably, our phylogenetic analysis showed that TcI isolates from AC and RO were more closely phylogenetically related compared to TcI isolates from PA and AP. Understanding the intra-TcI genetic diversity of human isolates from Amazonia requires additional research. Multilocus approaches revealed that TcI from wild hosts and vectors from Amazonia clustered separately from those of other regions in Brazil, and quite distantly from TcI from other countries in South America, Central and North America [42,43]. The phylogenetic analyses in previous studies of TcIV from humans, wild hosts, and triatomines uncovered

the relevant genetic diversity of isolates from different Brazilian regions, suggesting clusters related to geographical origin. All the isolates from Amazonia clustered tightly together in the present study, and were deeply distant from North American isolates. Our findings corroborated previous studies showing relevant differences among TcIV isolates from North and South America [34,44].

The disease severity, symptoms, and clinical signs of ACD in the Miraflores patients could not be related to gender, age, or combinations of trypanosome species and genotypes in mixed infections. All patients recovered promptly with a specific treatment, possibly because they were diagnosed and treated at the early stages of infection. In AC, death due to ACD, probably due to delayed diagnosis and treatment, was reported in a child and young adults (SESACRE). The existence of virulent *T. cruzi* strains in AC, unfortunately not available for genotyping, was previously demonstrated by the death of one child with acute pan-carditis, and its high lethality was demonstrated by the severe cardiac and liver parasitism in mice infected with *T. cruzi* isolated from the first ACD case in AC [24,25].

Throughout Amazonia, sylvatic triatomines pose a significant risk of CD transmission to humans, especially the palm-dwelling *Rhodnius* spp. that are frequently infected with *T. cruzi* [45–47]. In AC, *Rhodnius* spp. have been found in peridomiciles and inside homes, but domestic colonies have never been found [41,48–51]. Here, we provided the first TcI and TcIV sequences obtained from *Rhodnius* spp. from AC. Previous studies in AC revealed TcI in *Rhodnius* sp. from the Seringal Miraflores [41] and in *R. pictipes,* and *R. montenegrensis* from Cruzeiro do Sul [50]. The genotyping of trypanosomes from triatomines captured in RO revealed TcI and TcIV in *R. robustus* and *R. montenegrensis,* and TcIII and TcIV in *P. geniculatus* [19,34]. AC and RO are contiguous states sharing landscapes, mammalian fauna, triatomines and, as we demonstrated herein, a trypanosome repertoire.

In addition to *T. cruzi*, the highly sensitive method of FFLB uncovered *T. rangeli* of two genotypes in the Miraflores patients, TrA and TrB, which are widespread in wild mammals and *Rhodnius* spp. in Amazonia. Our study was the first to detect *T. rangeli* in humans in AC, providing the first report of TrA and the second of TrB infecting humans in Brazil. Previously, three cases of asymptomatic human infection by *T. rangeli* in AM were revealed by hemocultures and PCR in 9-, 51-, and 71-year-old individuals that reported that they had been bitten several times by *R. brethesi* [16]; one isolate (AM80) from this study was genotyped as TrB [16]. In AC, before the present study revealing TrA and TrB in humans, we previously reported TrA and TrB in monkeys [32], and TrA was identified in bats, opossums, and domestic dogs from AC [35–37].

The only confirmed vectors of *T. rangeli* are *Rhodnius* spp. [52,53]. A previous phylogenetic study of *T. rangeli* from Amazonia revealed TrA in *R. robustus* II (=*R. montenegrensis*) from RO, TrA in *R. robustus* from PA, TrB in *R. brethesi* from AM, and TrE in *R. pictipes* from AM [32]. Systematic and comprehensive molecular surveys are required to better understand the vectors of *T. rangeli* genotypes in *Rhodnius* spp. from AC and across Brazilian Amazonia [32,33,37,54].

Our discovery that 8 of 13 patients from Miraflores harbored *T. rangeli* indicates an underestimated level of human infection and suggest that the presence of *T. rangeli* concomitantly with *T. cruzi* did not induce specific changes in the ACD clinical profiles. Humans infected by *T. rangeli* by vectorial transmission are common in Central America and northwestern South America (Colombia and Venezuela), where the infection is transient and non-pathogenic [51,52]. Nevertheless, the effects of *T. rangeli* on human health must be re-examined taking into consideration the possible oral infection suggested by the present study, parasite accumulation in lymphoid organs, and the immune protection against the harmful effects of acute infection by *T. cruzi* [55,56]. The well-known cross-reactivity of *T. rangeli* with *T. cruzi* requires differential serological and molecular diagnoses for the prompt treatment of *T. cruzi,* but not *T. rangeli* infections [57,58].

The trypanosomes from AC herein characterized in humans were identical to (or share high sequence similarity with) those detected in wild mammals and triatomines, thus supporting dynamic and overlapped sylvatic transmission cycles and the risk of human

infection by different genotypes of both *T. cruzi* and *T. rangeli.* The uncovered notable trypanosome genetic diversity in Miraflores patients is consistent with preserved Amazon Forest sustaining a rich mammal fauna harboring a diversity of trypanosomes transmitted by triatomines. The landscape in the forest community of Seringal Miraflores has been modified by ecological changes influencing the dynamic of *T. cruzi* transmission by an assembly of ACD risk factors: economic activity dependent on expanding açai production; the consumption of poorly sanitized homemade processed açai; houses bordering the forest and surrounded by palms harboring *Rhodnius* spp.; wild reservoirs of trypanosomes such as bats, rodents, and marsupials invading domestic habitats due to deforestation triggering the loss of habitats; and domestic animals that serve as reservoirs and blood sources for triatomines. The ability of *T. cruzi* to infect various wild and domestic hosts, and to circulate in sylvatic, peridomestic, and domestic environments are risk factors for its emergence as an important human pathogen in Amazonia. In Seringal Miraflores, houses constructed bordering the forest and within proximity of the hosts and vectors of trypanosomes, and the daily ingestion of homemade fresh açai, altogether facilitate human oral infections.

5. Conclusions

Our molecular epidemiological study of the first molecularly characterized outbreak of orally acquired ACD in AC, and the first in Southwestern Brazilian Amazonia, describe the greatest ever known genetic diversity of trypanosomes infecting humans in a single outbreak: *T. cruzi* (TcI/TcIV) and *T. rangeli* (TrA/TrB), present concomitantly in single, double, and triple infections. Corroborating oral infection, only individuals that shared açai in a community meeting became sick. Symptoms and clinical signs of ACD patients could not be related to gender, age, or to trypanosome species and genotypes. Integrated knowledge of novel socio-economic and ecological aspects is crucial to designing practical and sustainable measures to reduce the risk of ACD considering Amazonian peculiarities. Public health education and sanitary and entomological surveillance must be more effectively adopted in AC and throughout Brazilian Amazonia to reduce the risk of orally acquired ACD, and to facilitate the diagnosis and prompt treatment of vulnerable populations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/parasitologia2040029/s1. Table S1: *Trypanosoma cruzi* and *Trypanosoma rangeli* isolates, host and geographic origin, genotypes, and SSU rRNA sequences used for phylogenetic inferences (GenBank accession numbers).

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and following the guidelines and standards recommended by the Brazilian National Health Council, which regulates research involving humans. The Ethical Committee on research involving humans from the Federal University of Acre approved the study (Protocol code CAAE: 53407321.3.0000.5010), and ICB-USP Ethical Committee (Protocol code CEP-ICB nº 880/2017) approved the molecular characterization. To guarantee the confidentiality of data obtained from SESACRE, the name of each patient was encrypted.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All retrospective data on ACD reported in this study came from public domain databases of SINAN (Notifiable Diseases Information System of Brazilian Ministry of Health) web site that did provide individually identifiable information. The SSU rRNA sequences determined herein were deposited in GenBank under the accession numbers shown in Supplementary Table S1.

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