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SHORT COMMUNICATION

Molecular study of *Trypanosoma caninum* isolates based on different genetic markers

Juliana H. S. Barros · Helena K. Toma · Maria de Fatima Madeira

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Abstract *Trypanosoma caninum* is a parasite recently described in dogs, whose life cycle is rather unknown. Here, we performed a genetic study with *T. caninum* samples obtained in different Brazilian regions. The study was based on PCR assays target to small and large subunit ribosomal DNA (rDNA) (18S rDNA and 24Sα rDNA), cytochrome B (Cyt b), and internal transcribed spacer 1 rDNA (ITS1 rDNA) following by the sequence analysis. Additionally, we used primers for the variable regions of kinetoplast DNA (kDNA) minicircles and endonucleases restriction in the ITS1 rDNA amplification product. *T. caninum* samples displayed the same patterns. Tree construction confirmed the close relationship between *T. caninum* samples, regardless of the molecular target used and endonuclease restriction digestion revealed that all samples have the same restriction profile. Therefore, *T. caninum* seems to be a genetically homogeneous specie. In the kDNA assay, *T. caninum* possessed a different molecular size profile with respect to others trypanosomes, 330 and 350 bp. This study provides nucleotide sequences from different regions of the genome of *T. caninum* that certainly facilitate future studies.

J. H. S. Barros
Programa de Pós-Graduação em Pesquisa Clínica em Doenças Infecciosas, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, RJ 21040-900, Brazil

H. K. Toma
Laboratório de Diagnóstico Molecular e Hematologia, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

M. de Fatima Madeira (✉)
Laboratório de Vigilância em Leishmanioses, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, RJ 21040-900, Brazil
e-mail: fatima.madeira@ipec.fiocruz.br

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Introduction

Flagellates of the *Trypanosoma* genus are parasites that belong to the Trypanosomatidae family, and this group includes species that infect a variety of vertebrate hosts (Hoare 1972). *Trypanosoma caninum* has been reported in dogs from different Brazilian states (Madeira et al. 2009; Pinto et al. 2010; Almeida et al. 2011), and little is known about genetic data of this parasite. To date, partial 18S ribosomal DNA (rDNA) PCR following sequencing has been the only genetic approach used aiming to identify the various isolates. In these studies, *T. caninum* isolates were genetically identical or closely similar. Moreover, all collected samples from different Brazilian regions grouped into a single clade and were substantially different from others within this genus (Barros et al. 2012). The molecular marker have suggested that could be a useful diagnostic tool, particularly for distinguishing between *T. caninum* and *Leishmania chagasi* infections in areas where these two parasites coexist (Madeira et al. 2014).

Several genetic markers have been employed for the molecular study of parasites from the *Trypanosoma* genus and each one is chosen for a specific reason. Targets for the conserved regions of the genome, such as genes of the small and large subunit ribosomal DNA (18S rDNA and 24Sα rDNA), are frequently used for diagnostic approach, mainly for identification of new species (Lima et al. 2012; Simo et al. 2013; Villareal et al. 2013), and phylogenetic studies of related species (Garcia et al. 2011; Votýpka et al. 2012; Arenas et al. 2012; Fisher et al. 2013; Ramirez et al. 2014). Moreover, targets for the variable regions of the genome, such as, internal

transcribed spacers (ITS rDNA) and mitochondrial genes are extremely useful for studies involving aspects of intraspecific and interspecific variability due to high degree of mutations in these genetic regions (Auty et al. 2012; García et al. 2012; Silva-Iturriza et al. 2013; Nakayima et al. 2013; Roelli et al. 2013).

Understanding *T. caninum* at the molecular level is important for a variety of reasons, such as, further study of identification, genetic variability, and phylogenetic analyses. In this study, we analyzed distinct regions of the *T. caninum* genome by using different molecular markers, thus providing a comprehensive analysis of this recently discovered parasite.

Materials and methods

T. caninum samples

All *T. caninum* samples studied were obtained from culture of intact skin fragments of naturally infected dogs from different geographic regions of Brazil as Rio de Janeiro, São Paulo, Minas Gerais, Goiás, and Mato Grosso states.

PCR assay of multiple genes

The assays were performed with cultured trypanosomes. DNA were extracted using DNAzol kit (Invitrogen, Life Sciences, USA) according to the manufacturer's instructions and were stored at −20 °C until use. Partial sequences of variable regions of small subunit ribosomal DNA gene (18S rDNA) were amplified using a nested-PCR (n=21 *T. caninum* isolates) with external primers TRY927F/TRY927R and internal primers SSU561F/SSU561R (Smith et al. 2008). The D75/D76 primers were performed on 48 samples to amplify conserved partial sequence of the large subunit of the ribosomal DNA gene (24Sα rDNA) within the Trypanosomatidae genome according to Souto et al. (1999). The primers used to amplify the 5′-half of the cytochrome b (Cyt b) gene region encoded in the maxicircle DNA of the mitochondrial genome were P18/P20 (n=45 samples) (Barnabé et al. 2003). PCR targeting the minicircle kinetoplast DNA (kDNA) region was performed using 49 samples by amplifying the variable regions of minicircle using primers 121/122 that anneal to the constant regions present in all minicircles (Wincker et al. 1994). The PCR primers employed in 47 samples to amplify the kinetoplastid internal transcribe spacer 1 (ITS1 rDNA) sequences were KIN1/KIN2, (Desquesnes et al. 2001). These primers anneal to the conserved regions of the 18S and 5.8S rDNA to amplify the variable region of ITS1. The descriptions of molecular targets used in this study are listed in Table 1. Negative controls (no DNA) and positive controls (*T. caninum* DNA—MCAN/BR/03/stock A27) was used in all assays.

Table 1 Molecular targets used for amplification of different genomic regions of *Trypanosoma caninum*

Target	Primers	Sequence 5′–3′
18S rDNA	TRY927F	GAAACAAGAAACACGGGAG CTACTG
	TRY927R	GGCAGCTTGGA TGGGATAACAAAG
	SSU561F	GAGCA CTGAGACTGTAACCTCAAA
	SSU561R	GC
24Sα rDNA	D75	GCAGATCTTGGTTGGCGTAG
	D76	GGTTCTCTGTTGCCCCTTTT
Cytochrome b	P18	GACAGGATTGAGAAGCGAGAGAG
	P20	CAAACCTATCACAAAAAGCATCTG
kDNA	121	AAATAATGTACGGGKGAGATGCATGA
	122	GGTTCGATTGGGGTTGGTGTATA TA
ITS1 rDNA	KIN1	GCGTTCAAAGATTGGGCAAT
	KIN2	CGCCCGAAAGTTCACC

All PCR products were analyzed by electrophoresis in 2 % agarose gels and were visualized by staining with ethidium bromide. Furthermore, all PCR products, except those tested for minicircle kDNA assay, were purified using the QIAquick Gel Extraction Kit (Qiagen, Crawley, Reino Unido) following the manufacturer's instructions for PCR-RFLP and sequenced.

Restriction fragment length polymorphism PCR

The amplification products of ITS1 rDNA PCR were digested with *Hae*III, *Rsa*I, *Hinf*I, *Mbo*I and *Bsh*I enzymes (Invitrogen; Life Sciences, USA; Fermentas). Five microliters of the PCR product was subjected to enzymatic digestion with 1 U of enzyme for 3 h at 37 °C in the appropriate buffer. The generated fragments were electrophoresed run on 3 % agarose gel, stained with 0.5 g/ml ethidium and visualized under ultraviolet light. The restriction fragment polymorphism lengths were estimated by comparison with molecular weight marker (100-bp DNA ladder).

Cloning

Purified PCR products from the ITS1 rDNA PCR reaction was ligated into an pGEM®-T Vector System I Kit (Promega, Madison, USA). The ligation reaction mixture, containing 3 μl purified PCR, 1 μl TVector (cloning vector [50 ng/μl]), 5 μl buffer solution (T4 DNA ligase), and 1 μl de T4 DNA ligase, was incubated overnight at 4 °C. After transformation into chemically competent BL21 *Escherichia coli* cells, positive clones were identified by blue/white screening using X-Gal followed by amplification with KIN forward and reverse oligonucleotides. Eight representative clones were inoculated into LB broth overnight at 37 °C, and plasmid DNA was purified using PureLinkTM Quick Plasmid Miniprep

Table 2 *Trypanosoma caninum* samples, geographic origin, and GenBank accession numbers sequences of different genes employed for analyses

Sample code	Geographical origin	GenBank acession numbers			
		18S rRNA	24Sα rDNA	Cyt b	ITS1 rDNA
RJA27	RJ/Rio de Janeiro	GU385824[a]	FJ801040[a]	KF805529	KF805452
RJ002	RJ/Rio de Janeiro	JF907507[a]	KF805481	KF805530	nd
RJ016	RJ/Rio de Janeiro	JF907508[a]	KF805482	KF805531	nd
RJ058	RJ/Rio de Janeiro	JF907509[a]	KF805483	KF805532	nd
RJ066	RJ/Rio de Janeiro	JF907510[a]	KF805484	KF805533	nd
RJ071	RJ/Rio de Janeiro	JF907531[a]	KF805485	KF805534	KF805453
RJ134	RJ/Rio de Janeiro	JF907523[a]	KF805486	KF805535	nd
RJ4814	RJ/Rio de Janeiro	JF907517[a]	nd	nd	nd
RJ4052	RJ/Niterói	JF907516[a]	KF805487	KF805536	nd
RJ8150	RJ/Maricá	JF951431[a]	KF805488	KF805537	nd
RJ003	RJ/Rio de Janeiro	KF805460	KF805489	KF805538	nd
RJ020	RJ/Rio de Janeiro	GU385826[a]	KF805490	KF805539	nd
RJ019	RJ/Rio de Janeiro	KF805461	KF805491	KF805540	nd
RJ042	RJ/Rio de Janeiro	KF805462	KF805492	KF805541	nd
RJ059	RJ/Rio de Janeiro	KF805463	KF805493	KF805542	nd
RJ060	RJ/Rio de Janeiro	KF805464	KF805494	nd	nd
RJ053	RJ/Rio de Janeiro	KF805465	KF805495	KF805543	nd
RJ062	RJ/Rio de Janeiro	KF805466	KF805496	KF805544	nd
RJ120	RJ/Rio de Janeiro	KF805467	KF805497	KF805545	nd
RJ118	RJ/Rio de Janeiro	GU385825[a]	KF805498	KF805546	nd
RJ176	RJ/Rio de Janeiro	KF805468	KF805499	KF805547	nd
RJ194O	RJ/Rio de Janeiro	KF805469	KF805500	KF805548	nd
RJ194E	RJ/Rio de Janeiro	KF805470	KF805501	KF805549	nd
RJ201	RJ/Rio de Janeiro	KF805471	KF805502	KF805550	nd
RJ249	RJ/Rio de Janeiro	KF805472	KF805503	KF805551	nd
RJ259	RJ/Rio de Janeiro	KF805473	KF805504	KF805552	nd
RJ271	RJ/Rio de Janeiro	KF805474	KF805505	nd	nd
RJ310	RJ/Rio de Janeiro	KF805475	KF805506	KF805553	nd
RJ265	RJ/Rio de Janeiro	KF805476	KF805507	nd	nd
RJ331	RJ/Rio de Janeiro	KF805477	KF805508	KF805554	nd
SP137	SP/Bauru	JF907518[a]	KF805509	KF805555	KF805454
SP269	SP/Bauru	JF907519[a]	KF805510	nd	nd
MG771	MG/Belo Horizonte	JF907521[a]	KF805511	KF805556	KF805455
MG764	MG/Belo Horizonte	JF907520[a]	KF805512	KF805557	nd
MG610	MG/Belo Horizonte	JF907524[a]	nd	nd	nd
GO718	GO/Brasília	JF907522[a]	KF805513	KF805558	KF805456
MT527	MT/Cuiabá	JF907511[a]	KF805514	KF805559	nd
MT531	MT/Cuiabá	JF907513[a]	KF805515	KF805560	nd
MT534	MT/Cuiabá	JF907532[a]	KF805516	KF805561	nd
MT576	MT/Cuiabá	JF907514[a]	KF805517	KF805562	nd
MT577	MT/Cuiabá	JF907515[a]	KF805518	KF805563	nd
MT604	MT/Cuiabá	JF907512[a]	KF805519	KF805564	KF805457
MT669	MT/Cuiabá	JF907533[a]	KF805520	KF805565	nd
MT732	MT/Cuiabá	JF907534[a]	KF805521	KF805566	nd
MT769	MT/Cuiabá	JF907535[a]	KF805522	KF805567	nd
MT798	MT/Cuiabá	JF907536[a]	KF805523	KF805568	nd
MT799	MT/Cuiabá	JF907537[a]	KF805524	KF805569	KF805458

Table 2 (continued)

Sample code	Geographical origin	GenBank acession numbers			
		18S rRNA	24Sα rDNA	Cyt b	ITS1 rDNA
MT808	MT/Cuiabá	JF907538[a]	KF805525	KF805570	KF805459
MT1561	MT/Cuiabá	KF805478	KF805526	KF805571	nd
MT1567	MT/Cuiabá	KF805479	KF805527	KF805572	nd
MT1671	MT/Cuiabá	KF805480	KF805528	KF805573	nd

nd not done

[a] Reference sequences retrieved from GenBank (in addition to JF907525-JF907530 sequences)

(Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

DNA sequencing and analysis

All nucleotide sequences were determined using an automatic sequencer (3730 DNA Analyzer, Applied Biosystems) and were initially analyzed with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cg). All nucleotide sequences obtained for each molecular target were compared using Mega version 5.1 program (Tamura et al. 2011) with *T. caninum* sequences determined in our previous studies and with sequences of genus *Trypanosoma* available in GenBank. The alignment results were processed using the same software and the neighbor-joining (NJ) method that was used to construct the phylogenetic tree. We calculated models of sequence evolution and their parameters using jModelTest in Mega version 5.1 (Posada 2008; Tamura et al. 2011). To evaluate the robustness of the nodes in the resulting phylogenetic tree, 1000 bootstrap replications were performed. The same analysis was performed in eight recombinant clones derived from products of the ITS1 rDNA PCR amplification.

Results

After amplification of partial sequence of 18S rDNA, the samples showed a 900-bp molecular pattern after the first round and a 700-bp pattern after the second round. For the 24Sα rDNA targets, the samples yielded a 250-bp product, and amplification of the Cyt b mitochondrial gene resulted in a 700-bp product. The PCR results performed targeting the variable region of the ribosomal gene (ITS1 rDNA) yielded a 950-bp amplified product. Amplification of the minicircle kDNA region yielded two products, one with 350 bp and other with 330 bp. Some samples were not analyzed by PCR assays and sequencing for the following reasons: (a) some stocks already been performed in other studies (Madeira et al. 2009; Pinto et al. 2010; Barros et al. 2012), (b) due to lost by secondary contamination, or (c) reduced growth through the subcultures of the initial isolate (Table 2).

Endonuclease restriction digestion of the ITS rDNA revealed that all samples have the same restriction profile. Among those endonucleases studied, the *Bsh*I digested PCR product resulted in six fragments 320 and 160 bp and four fragments about 100 bp. The *Mbo*I enzyme digested the ITS rDNA into three fragments about 430, 380 and 90 bp. The enzymes *Hae*III, *Rsa*I, and *Hin*fI did not digest any PCR product.

Molecular analyses revealed clear separation of *T. caninum* isolates from other samples of the *Trypanosoma* genus, regardless of the target used (Fig. 1 a–c). Investigation of the 18S rDNA across all samples studied revealed 100 % query coverage and clustered with all samples of *T. caninum* retrieved from GenBank. The same result was achieved with the sequences obtained from the partial region of the 24Sα rDNA, in which all samples clustered together with the first isolate of *T. caninum* (stock A27—accession number FJ801040). Regarding the sequences obtained from the partial region of the mitochondrial Cyt b gene, all samples aligned and grouped in the same cluster but were separate from other parasites of the *Trypanosoma* genus.

By ITS1 rDNA, all selected clones showed a 100 % identity. However, despite the high similarity observed, multiple sequence alignment of ITS1 rDNA revealed 40 polymorphic sites among the studied clones.

All sequences obtained in this study were deposited in GenBank (Table 2).

Discussion

In this study, we utilized various molecular markers to help clarify some issues about *T. caninum*, a new parasite described in Brazil. First, the species status of all samples were confirmed by sequencing of the 18S rDNA gene, since this marker is already been considered an acceptable molecular criterion for taxonomic identification (Hamilton and Stevens 2010; Pinto et al. 2012). Using this target, we identified three new

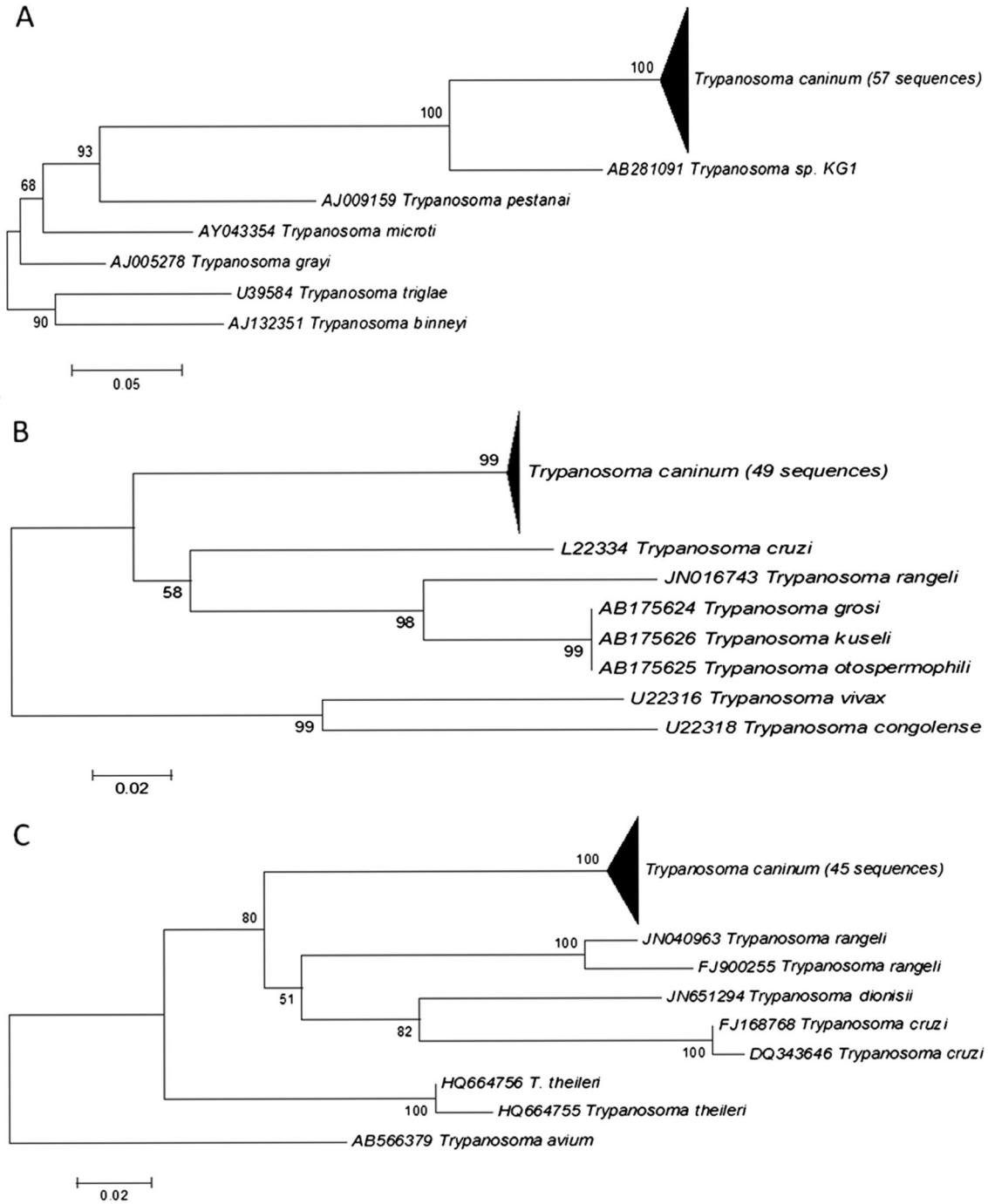

Fig. 1 Phylogenetics analyses demonstrating clear separation of *T. caninum* isolates of others samples of *Trypanosoma* sp. Neighbor-joining phylogenetic tree with the Kimura-2-parameter model of partial sequences of **a** 18S rRNA, **b** 24Sα rDNA and with the Tamura-Nei model of partial sequences of **c** cytochrome b genes of *T. caninum* from different states and others parasites of genus *Trypanosoma* aligned with Mega version 5.1 program. Outgroups: *T. binneyi* and *T. triglae* for 18S rRNA, *T. vivax* and *T. congolense* for 24Sα rDNA and *T. avium* for cytochrome b. Bootstrap test (1000 replicates) are shown next to the branches. GenBank accession numbers of *T. caninum* are shown in Table 2 in this paper and other *Trypanosoma* species in front of its name on the tree

T. caninum samples from the Mato Grosso state and confirmed the identity of the 18 isolates from Rio de Janeiro state, previously identified by other tools (Pinto et al. 2010). Based on phylogenetic analysis using this molecular target, all *T. caninum* samples were grouped into the same cluster with sequences determined in our previous studies (Pinto et al. 2010; Barros et al. 2012). Indeed, there are many advantages in using this marker: it is a highly conserved region in the genome, it is present in thousands of copies, and its DNA sequence is widely available in public databases. Moreover,

several species have been reliably identified using this marker (Lima et al. 2012; Votýpka et al. 2012; Fisher et al. 2013; Simo et al. 2013). Using this marker, our group confirmed a total of 57 cases of natural infection by *T. caninum* in different geographic areas of Brazil. Interestingly, the results obtained using 24Sα rDNA and Cyt b markers support these findings and demonstrated that *T. caninum* displays a high degree of similarity among them. All studied samples were located in the same cluster, regardless of geographic precedence and the genetic marker used.

Based on ITS rDNA analysis, high genetic homogeneity was also detected in *T. caninum* isolates, and the restriction pattern proved it is not a gold standard for detecting genetic heterogeneity in this specie. For this reason, we selected eight representative samples of different geographical regions, and by cloning and sequencing, confirmed this observation. All cloned ITS rDNA sequences showed 100 % query coverage to the isolates sequence sharing the highest identity. However, multiple sequence alignment indicates some degree of heterogeneity in *T. caninum* isolates demonstrated by some polymorphic sites among the studied clones.

The low level of variability observed in our study must be considered with caution because the samples were isolated from limited geographical surroundings. It is important to note that Brazil is a country with an extensive geographic area with different epidemiological patterns and that *T. caninum* has been isolated only from dogs in household and peridomestic environments. Additionally, *T. caninum* seems to have tropism for specific anatomic sites, the intact skin (Madeira et al. 2009, 2014). The genetic homogeneity found in *T. caninum* might be associated with these factors; however, the specific host–parasite association must also be considered. Taking this into account, investigation of *T. caninum* in other hosts such as wild canids and synanthropic animals, which are exposed to more complex epidemiological patterns, should also be investigated. Furthermore, additional isolates from dogs should be investigated to accurately evaluate the level of genetic diversity within this species.

Phylogenetic trees were constructed using the GenBank sequences of organisms related to *T. caninum*. However, based on the molecular targets analyzed, we find different related trypanosomes sequences. If more DNA sequences in public databases become available, greater confidence will be inferred to the studies, especially in identification with new species, strains, or isolates (Hamilton and Stevens 2010). Thus, the availability of nucleotide sequences from different regions of the genome of *T. caninum* certainly facilitate future studies, including the design or choice of specific molecular targets that could be used for their diagnosis.

Some studies have reported differences in the lengths of minicircles from several trypanosomatid species (García et al. 2012; Brewster and Barker 2002), and the length of the variable region of the minicircles in *T. caninum* is different from other trypanosomes.

Finally, *T. caninum* is an intriguing parasite of which very little is known and our results provide further information concerning the genetic profile of all isolates described to date.

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