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Research paper

Phylogenetic analysis of the *Trypanosoma* genus based on the heat-shock protein 70 gene☆

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ABSTRACT

Trypanosome evolution was so far essentially studied on the basis of phylogenetic analyses of small subunit ribosomal RNA (SSU-rRNA) and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) genes. We used for the first time the 70 kDa heat-shock protein gene (*hsp*70) to investigate the phylogenetic relationships among 11 *Trypanosoma* species on the basis of 1380 nucleotides from 76 sequences corresponding to 65 strains. We also constructed a phylogeny based on combined datasets of SSU-rDNA, gGAPDH and *hsp*70 sequences. The obtained clusters can be correlated with the sections and subgenus classifications of mammal-infecting trypanosomes except for *Trypanosoma theileri* and *Trypanosoma rangeli*. Our analysis supports the classification of *Trypanosoma* species into clades rather than in sections and subgenera, some of which being polyphyletic. Nine clades were recognized: *Trypanosoma carassi*, *Trypanosoma congolense*, *Trypanosoma cruzi*, *Trypanosoma grayi*, *Trypanosoma lewisi*, *T. rangeli*, *T. theileri*, *Trypanosoma vivax* and *Trypanozoon*. These results are consistent with existing knowledge of the genus' phylogeny. Within the *T. cruzi* clade, three groups of *T. cruzi* discrete typing units could be clearly distinguished, corresponding to TcI, TcIII, and TcII + V + VI, while support for TcIV was lacking. Phylogenetic analyses based on *hsp*70 demonstrated that this molecular marker can be applied for discriminating most of the *Trypanosoma* species and clades.

1. Introduction

Parasites from the genus *Trypanosoma* (Kinetoplastida: Trypanosomatidae) are ubiquitous protozoans that infect a wide range of animals, including leeches, insects, fish, amphibians, reptiles, birds, and mammals, and are the causative agents of some of the most neglected human and animal diseases. The genus *Trypanosoma* is considered a monophyletic group, sharing a common ancestor that dates back about 100 million years ago (Barrett et al., 2003; Stevens and Gibson, 1999).

The classification established by Hoare was based on morphology and development in the vector (Hoare, 1972) (Table 1). Mammal-infecting *Trypanosoma* species were separated into sections: Salivaria and Stercoraria. The salivarian trypanosomes were further subdivided into four subgenera: *Trypanozoon*, *Duttonella*, *Nannomonas* and *Pycnomonas*. The Stercoraria comprised three subgenera: *Schizotrypanum*, *Megatrypanum*, and *Herpetosoma* (Vickerman, 1976). Since some of these groups are polyphyletic and lack evolutionary and taxonomic relevance, more recently some authors suggested classifying the genus into clades, based on the small subunit rRNA (SSU-rRNA) and glycosomal glyceraldehyde 3-phosphate dehydrogenase (gGAPDH) genes (Simpson et al., 2006; Stevens, 2008; Stevens and Gibson, 1999; Stevens and Rambaut, 2001) (Table 1): the '*Trypanosoma brucei* clade' with *T. brucei* and relatives, which typically are transmitted by tsetse flies (with exceptions); the '*Trypanosoma cruzi* clade' with the predominantly New World species of *T. cruzi* and relatives (typically transmitted by triatomine bugs); a 'rodent clade' (presumably transmitted primarily by fleas); an 'avian clade' (transmitted primarily by black flies and hippoboscid flies); and an 'aquatic clade' (transmitted by leeches and insects).

In spite of the information provided by DNA sequence analysis, trypanosomatid phylogeny and evolutionary relationships have not been firmly established (Hughes and Piontkivska, 2003; Kelly et al., 2014; Votýpka et al., 2015). Phylogenies among the *Trypanosoma* have varied depending on the gene sequences analyzed, the number of taxa included, the choice of outgroup, and the phylogenetic methodology employed (Leonard et al., 2011). Especially the monophyly and clade classification of *Trypanosoma* have been subject of debate. Two

☆ Nucleotide sequence data reported in this paper are available in GenBank, EMBL and DDBJ databases under the accession numbers KC959988-KC960011; KP208734-KP208748; KP257564-KP257565.

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Table 1
Taxonomy of the *Trypanosoma* genus.

Classical classification (Hoare, 1972)			Clade classification (Simpson et al., 2006)[b]
Section	Subgenus	Species and subspecies[a]	
Salivaria	*Trypanozoon*	*T. brucei brucei*	*T. brucei* (*T. brucei* and related species mostly transmitted by tsetse flies)
		T. brucei gambiense	
		T. brucei rhodesiense	
		T. evansi	
		T. equiperdum	
	Nannomonas	*T. congolense*	
		T. simiae	
		T. godfreyi	
	Duttonella	*T. vivax*	
		T. uniforme	
	Pycnomonas	*T. suis*	
Stercoraria	*Schizotrypanum*	*T. cruzi*	*T. cruzi* (*T. cruzi* and related species transmitted by triatomine bugs)
		T. cruzi marinkellei	
		T. vespertilionis	
		T. dionissi	
	Herpetosoma	*T. rangeli*	
		T. lewisi	Rodent (presumably transmitted by fleas)
		T. musculi	
		T. nabiasi	
		T. microti	
	Megatrypanum	*T. theileri*	Other *Trypanosoma* clades
		T. melophagium	
		T. conorhini	
		T. minasense	
		T. pestanai	
		T. cyclops	
		T. theodori	
-	-	*T. carassi*, *T. cobitis*, *T. granulosum*, *T. sinipercea*, *T. ophiocephali*, *T. chelodinae*, *T. nudigobii*, *T. blennidini*, *T. haploblephari*, *T. boissoni*, *T. capigobii*, *T. semegalense*, *T. murmanense*, *T. pleuronectidium*, *T. triglae*, *T. rotatorium*, *T. ranarum*, *T. chattoni*, *T. binneyi*, *T. therezieni*, *T. mega*, *T. neveulemairei*, *T. fallisi*	Aquatic (transmitted by insects and leeches, also includes trypanosomes of platypuses and aquatic tortoises)
-	-	*T. avium*, *T. bennetti*, *T. corvi*, *T. culicavium*, *T. gallinarum*, *T. polygranularis*, *T. anguiformis*	Avian (transmitted primarily by black flies and hippoboscid flies)

[a] Listing contains only the most frequently phylogenetically studied species.
[b] Species listed with each clade were not specified in this paper.

molecular markers, the SSU-rRNA and gGAPDH genes, have been extensively used for phylogenetic studies within the entire *Trypanosoma* genus, and for establishing relationships with other genera of the Trypanosomatidae family (Hamilton et al., 2004, 2007; Hughes and Piontkivska, 2003; Stevens and Gibson, 1999).

Another promising evolutionary marker, the 70 kDa heat-shock protein (HSP70), has so far not been applied to *Trypanosoma*, even though it was proven useful to study various parasites such as *Cryptosporidium* spp. (Langkjaer et al., 2007; Sulaiman et al., 2000), *Babesia* spp. (Yamasaki et al., 2002, 2007), *Giardia* spp. (Arisue et al., 2002b), *Entamoeba* spp. (Arisue et al., 2002b), *Microsporidium* spp. (Arisue et al., 2002b), *Blastocystis hominis* (Arisue et al., 2002a), and the kinetoplastid *Leishmania* genus (Fraga et al., 2010, 2013). HSP70 proteins are highly conserved across prokaryotes and eukaryotes both in sequence and function, and have great importance as molecular chaperones and in protein folding and transport (Hartl and Hayer-Hartl, 2002). They are a subfamily of the larger HSP70 family (Folgueira and Requena, 2007), which could play a major role in environmental adaptations of trypanosomes (Drini et al., unpublished results). The *hsp*70 subfamily genes used here are arranged as a tandem repeat array in a head to tail manner, of which in *T. cruzi* at least seven copies are present (Requena et al., 1988). Genes encoding cytoplasmic HSP70s were among the first kinetoplastid genes to be cloned and characterized because of their conserved nature (Folgueira and Requena, 2007). In this study we investigate the phylogenetic relationships among several species of the *Trypanosoma* genus using partial coding sequences of the *hsp*70 gene, and compare the results with those obtained with other markers.

2. Material and methods

2.1. Strains and isolates

Fig. 1 presents the geographical origin and the species of the strains and isolates used in our study (full details in Table S1). Our main goal was to cover the species of particular relevance to human and animal health, while only including other strains that were available in our lab at the time of analysis, or from which the sequence could be retrieved from public repositories (see further). The analysis included 11 species of *Trypanosoma*. Among these were the mammal infecting *Trypanosoma* species from the subgenus and sections described by Hoare (1972), and trypanosomes isolated from fish and reptiles. Parasite isolates and DNA were obtained from different laboratories and collections acknowledged at the end of this article. The reference species identification and genotype was based on various data such as multilocus sequence typing (MLST), multilocus enzyme electrophoresis (MLEE), multilocus microsatellite typing (MLMT), restriction fragment size polymorphism analysis, and sequencing.

2.2. PCR amplification of hsp70

A PCR product of 1422 bp, corresponding to positions 435–1856 of the complete *T. cruzi* CL Brener gene (2034 bp, GenBank accession XM_812645) was amplified from 41 strains listed in Table S1, using primers HSP70sen and HSP70ant (Table 2) from Garcia et al. (2004). The reaction mix (50 μL) contained 1× standard PCR buffer including 1.5 mM $MgCl_2$, 1× Q-buffer, 200 μM of each deoxynucleoside

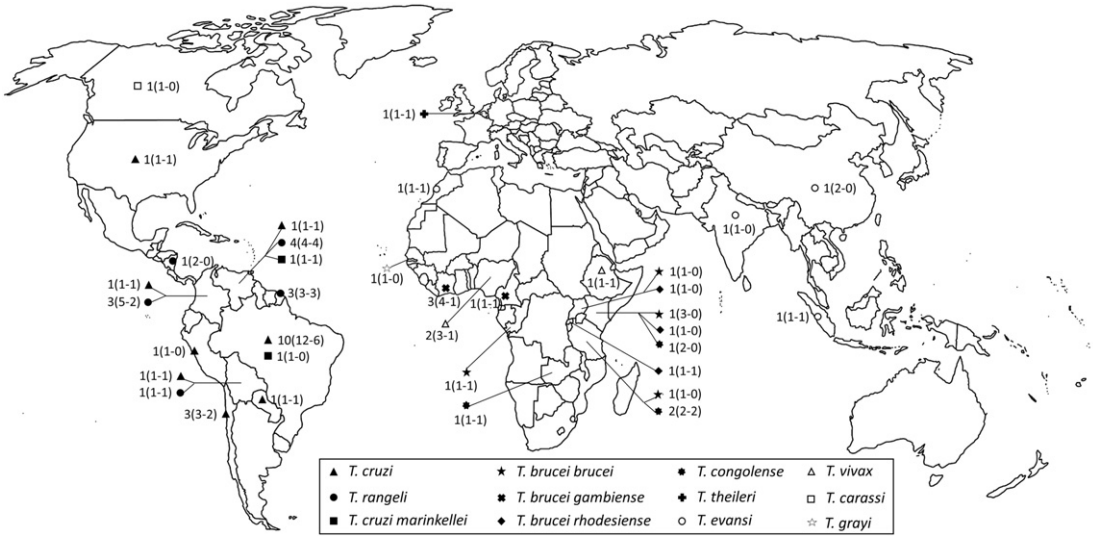

Fig. 1. Geographical origin of the 58 strains and isolates with known origin included in this study. Strains are assigned at country level, the position of the symbols within a country has no meaning. The symbols represent different species, the numbers next to the symbols indicate how many strains were included in our analysis. The first number between parentheses represents the number of sequences used in our alignments, while the second number indicates how many were sequenced and submitted to GenBank for this study.

triphosphate, 0.5 U HotStarTaq Plus DNA polymerase (Qiagen, Hilden, Germany), 0.4 μM of each primer, and 10 ng of genomic DNA isolated from parasite cultures. The thermal cycling parameters of the assay were: initial denaturation at 95 °C for 5 min, followed by 35 cycles consisting of 94 °C for 40 s – 61 °C for 1 min – 72 °C for 2 min, and a final extension step of 8 min at 72 °C. Amplicons were visualized on a 2% agarose gel, and sequenced directly without molecular cloning.

2.3. DNA sequencing

Sequences were generated with the primers listed in Table 2, using the dideoxy nucleotide chemistry from the ABI PRISM BigDye™ Terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, USA). They were analyzed on an ABI 3730 automated sequencer (Perkin Elmer). For phylogenetic analysis only the region between the primers was retained, i.e. 1380 out of the 1422 nucleotides. Additional *hsp*70 sequences (Table S1) where obtained from BLAST searches in the following databases: GeneDB (http://www.genedb.org), TritrypDB (http://www.tritrypdb.org) and GenBank (http://www.ncbi.nlm.nih.gov). Finally, some sequences were taken from unpublished whole genome sequences obtained from the Institute of Tropical Medicine (Antwerp, Belgium); the French Agency for Food, Environmental and Occupational Health & Safety; the University of Glasgow (UK) and the Food and Agriculture Organization (Italy).

2.4. Phylogenetic analysis

The obtained sequences were aligned with previously published sequences as listed in Table S1, using the software package MEGA (Molecular Evolutionary Genetic Analysis Version 5.05, Tamura et al., 2011, www.megasoftware.net). As no length variation is present between the sequences, the alignment was straightforward and no alignment algorithm was needed. The same software was used to build phylogenetic trees with both distance and character-based methods, and to analyze synonymous versus non-synonymous nucleotide substitutions. The number of synonymous differences per synonymous site, and the number of non-synonymous differences per non-synonymous site were averaged over all *Trypanosoma* sequence pairs, using the Nei–Gojobori method (Nei and Gojobori, 1986). If more than one sequence was available for a particular strain (see Table S1), only one sequence was retained for this analysis.

Neighbor-Joining phylogenies were constructed using the Kimura 2-parameter model (Kimura, 1980) for pairwise distance calculations. Character-based phylogenetic analyses were performed as well, with Maximum Parsimony (Eck and Dayhoff, 1966; Fitch, 1971) and Maximum Likelihood (ML) (Felsenstein, 1985) methods. The ML analysis was done under the General Time Reversible and Gamma Distributed model, as selected using the Akaike information criterion in MEGA 5.05. Distances from predicted amino acid sequences were determined with the p-distance model. *Leishmania* spp. and *Paratrypanosoma confusum* were included as outgroups (Table S1). The support of groups was assessed by the bootstrap method (Felsenstein, 1985) with 2000 replicates.

Additionally, a phylogenetic network was inferred from Kimura 2-parameter distances with the Neighbor-Net method in SplitsTree4 (Huson, 1998; Huson and Bryant, 2006). Such networks can depict alternative evolutionary paths supported by the data set, as they do not enforce a single bifurcating dendrogram. Bootstrap was performed with 2000 replicates.

Finally the SSU-rDNA, gGAPDH, and *hsp*70 sequences (Table S2) were concatenated and analyzed with Neighbor-Joining, Maximum Parsimony, and Maximum Likelihood, as described above.

Table 2
Primers used for *hsp*70 gene sequencing and PCR.

Primer	Primer Sequence (5′-3′)	Nucleotide position[a]
HSP70sen[b]	GACGGTGCCTGCCTACTTCAA	435–455
HSP70T-F308[c]	CAAGCGCAAGAACAAGGGCAAGG	741–763
HSP70T-F813[c]	CTGATCAAGCGCAACACGACG	1246–1266
HSP70T-R564[c]	GCACGCTTGTCCATCTTGGC	998–979
HSP70T-R1069[c]	CGCTTACCGGTGCCCTTCTCCTC	1502–1480
HSP70ant[c]	CCGCCCATGCTCTGGTACATC	1856–1836

[a] The annealing position of the primers is given relative to GenBank accession XM_812645 (*T. cruzi* strain CL Brener).
[b] Primers used for PCR amplification (Garcia et al., 2004).
[c] Primer used for sequencing (Fraga et al., 2014).

3. Results

3.1. Sequence characteristics

A fragment of 1380 bp of the *hsp*70 genes from 41 strains of nine *Trypanosoma* species was sequenced (Fig. 1, Table S1). These were aligned with 35 previously available sequences of 8 *Trypanosoma* species, amounting to a total of 76 sequences of 65 strains from 11

Trypanosoma species. For nine strains more than one *hsp*70 sequence was available, originating from different sources. For five strains (CL Brener, Tre, H14, Esm and STIB805) one to six nucleotides differed between sequences of the same strain, while the sequences from each of the remaining four strains (927/4GUTat10.1, ILRAD3000, ABBA and Y486) were identical. There were no sequence ambiguities in 55 out of the 76 sequences, and in total 39 positions in the remaining 21 sequences showed the presence of 2-base ambiguities.

The gene fragments of *Trypanosoma* spp. were GC rich (55.8–65.6%), with a similarity between species ranging from 75 to 100% (overall average 86.8%). The nucleotide sequence variation allowed discriminating all parasite species included in this study, except those of the "*Trypanozoon*" clade that grouped *T. brucei gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *Trypanosoma evansi*, and *Trypanosoma equiperdum*. In total 526 nucleotide positions (38.1%) were polymorphic and 412 positions (29.8%) were parsimony informative. The deduced amino acid sequences (459 amino acids) revealed substitutions at 132 positions (28.8%), of which 83 sites (18.1%) were parsimony informative. The number of non-synonymous substitutions per non-synonymous site *dN* was 3.2%, while the number of synonymous substitutions per synonymous site *dS* was 40.2%, hence $dN/dS < 1$.

3.2. Phylogenetic analysis

The Neighbor-Joining tree constructed from *hsp*70 sequences is presented in Fig. 2, where the sections and subgenera defined by Hoare (1972) and the comparison with two other genes are included. From the selection of species included in this analysis, only two subgenera do not follow the Hoare (1972) classification. First, *Herpetosoma* forms a paraphyletic group, with *Schizotrypanum* branching between *Trypanosoma rangeli* and *Trypanosoma lewisi*. Second, *Megatrypanum* is not supported as a Stercoraria section. Based on *hsp*70, nine groups are recognized: *Trypanosoma carassi*, *Trypanozoon*, *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma theileri*, *Trypanosoma grayi*, *T. lewisi*, *T. rangeli* and *T. cruzi*. The *Trypanozoon* clade includes *T. brucei gambiense*, *T. b. rhodesiense*, *T. b. brucei*, *Trypanosoma evansi*, and *T. equiperdum*. The *T. cruzi* clade includes *T. cruzi* and *T. cruzi marinkellei*.

All these clusters were also observed in Maximum Parsimony (Fig. S1) and Maximum Likelihood phylogenies (Fig. S2). The bootstrap values from these trees are also indicated in Fig. 2, and exceed 95% with all methods. This indicates that the derived groups are robust and not dependent of the choice of evolutionary models underlying the various tree-building algorithms. Trees based upon amino acid sequences (Fig. S3) did not conflict the nucleotide-based phylogenies.

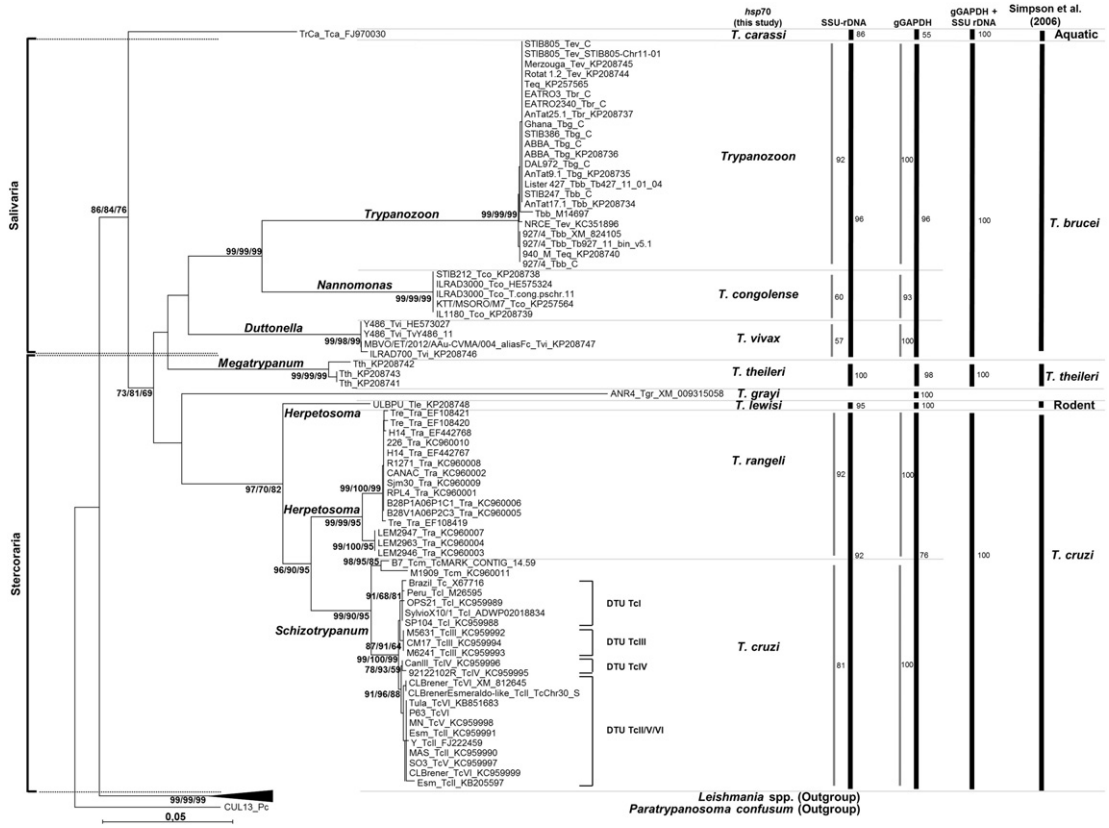

Fig. 2. Neighbor-Joining phylogeny of the *hsp*70 nucleotide sequences belonging to 11 *Trypanosoma* species. Distances were estimated using the Kimura 2-parameter model. Bootstrap support of the branches was inferred from 2000 replicates, and is given in percentages at the internodes when exceeding 70% (first value). For comparison, also the bootstrap values of the Maximum Parsimony (Fig. S1) and Maximum Likelihood (Fig. S2) trees are shown for each respective group (second and third values respectively). The tree is drawn to the scale at the bottom, expressed as distance per nucleotide. The tree was rooted with the sequences of *Paratrypanosoma confusum* and *Leishmania* spp. (*L. aethiopica*, *L. major*, *L. tropica*, *L. donovani*, *L. infantum*, *L. amazonensis*, *L. mexicana*, *L. garnhami*, *L. braziliensis*, *L. peruviana*, *L. lainsoni*, *L. guyanensis*, *L. panamensis*, *L. naiffi*). The names on the branches represent the classical subgenus classification of Hoare (1971). The Stercoraria and Salivaria sections are indicated on the left. Clades proposed in Simpson et al. (2006) and monophyletic groups from other reports are depicted at the right by vertical lines, for SSU-rDNA (Cavazzana et al., 2010; Stevens and Gibson, 1999); gGAPDH (Hamilton et al., 2005); and SSU-rDNA + gGAPDH (Lima et al., 2013). The vertical black and grey lines depict monophyletic clusters from these analyses, with the numbers on their right representing the bootstrap support in percentages. It should be noted that none of these studies used an identical strain and species set, so comparing them is indicative. Strains are identified using the code as in Table S1, followed by the species or subspecies (Tbb: *T. brucei brucei*; Tbg: *T. brucei gambiense*; Tbr: *T. brucei rhodesiense*; Tca: *T. carassi*; Tcm: *T. cruzi marinkellei*; Tco: *T. congolense*; Teq: *T. equiperdum*, Tev: *T. evansi*; Tgr: *T. grayi*, Tle: *T. lewisi*; Tra: *T. rangeli*; Tth: *T. theileri*; Tvi: *T. vivax*; Tc: *T. cruzi* with DTUs I–VI indicated as TcI–TcVI), and the accession number in GenBank, TrypTriDB, or GeneDB (C refers to unpublished confidential data from whole genome sequences).

Fig. 3 displays a phylogenetic network of the *Trypanosoma* sequences, thereby excluding *Leishmania* and *P. confusum*. The groups that could be reliably identified using conventional phylogenetic analysis are also recovered from the network (Fig. 3A). Fig. 3A shows a separation of *T. cruzi* and *T. cruzi marinkellei* as subgroups within the *T. cruzi* clade. In the *T. rangeli* cluster two groups can be distinguished (Fig. 3B). Species or subspecies in the *Trypanozoon* cluster cannot be distinguished (Fig. 3C). Three genetic groups are seen in *T. cruzi* (Fig. 2; Fig. 3D), corresponding to Discrete Typing Units (DTUs, see Zingales et al., 2012) TcI and TcIII, and DTUs TcII, TcV, and TcVI which do not form separate entities, while bootstrap support for TcIV was lacking.

In order to appraise whether a combined analysis of SSU-rDNA, gGAPDH and *hsp*70 sequences would further clarify the phylogenic relationships within the *Trypanosoma* genus, SSU-rDNA, gGAPDH and *hsp*70 sequences from 9 species were concatenated (Fig. 4). The analysis was consistent with single gene analysis, with few exceptions. First, *T. theileri* clustered consistently with the Stercoraria with bootstrap support of nearly 100% in all trees. Second, *T. vivax* clearly grouped with the *Trypanozoon-T. congolense* cluster with 100% bootstrap support in all trees.

4. Discussion

This is the first time that *hsp*70 sequences have been exploited for studying the phylogeny of the *Trypanosoma* genus. The *dN*/*dS* ratio of 0.08 indicates that positive selection did not play a major role in the evolution of the gene, but that instead purifying selection took place to preserve the HSP70 functionality. This includes transport of proteins between cellular compartments, removal of misfolded proteins, folding and refolding of proteins, prevention and dissolution of protein aggregation, and control of regulatory proteins (Requena et al., 2015). Further studies are needed to assess the impact of the observed *hsp*70 diversity on the host-parasite relationship (Folgueira and Requena, 2007).

Within strains, two types of variations between *hsp*70 sequences were observed. On the one hand, sequences from different sources derived from the same strain were occasionally dissimilar. On the other hand, some sequences contained ambiguous nucleotides. Although such ambiguous sites can result from an unclear sequence chromatogram, they can also represent genuine sequence variation. Two phenomena can account for these observed intra-strain sequence differences. One possibility is that the strains contain a mix of parasites, having one or the other observed nucleotide. The other possibility is that all parasites from a particular strain are identical, but that differences exist between the tandemly repeated copies of the *hsp*70 array (Folgueira and Requena, 2007). In order to decide between both options, clones derived from single parasites must be analyzed, and the sequencing strategy must allow revealing intra-parasite variation.

Our *hsp*70 analysis supports the classification in the clades proposed by Simpson et al. (2006), which was previously derived from SSU-rRNA and gGAPDH phylogenies (Simpson et al., 2006; Stevens, 2008; Stevens and Gibson, 1999; Stevens and Rambaut, 2001). We cannot draw conclusions regarding the monophyly of the aquatic (*T. carassi* TrCa), crocodile (*T. grayi* ANR4) and rodent (*T. lewisi* ULBPU) clades because they were represented by a single strain. The separation of aquatic and terrestrial *Trypanosoma* was clearly shown, and confirms data from SSU-rRNA and gGAPDH genes (Cavazzana et al., 2010; Hamilton et al., 2004, 2009; Lima et al., 2013; Stevens, 2008; Stevens and Gibson, 1999; Stevens and Rambaut, 2001). The deep split between aquatic and terrestrial clades provides evidence in support of parasite co-evolution with vertebrate hosts and vectors. The Hoare (1972) classification on the contrary was not supported due to the paraphyletic status of *Herpetosoma* and the lack of support of *Megatrypanum* as part of the Stercoraria except in the concatenated analysis (Fig. 4).

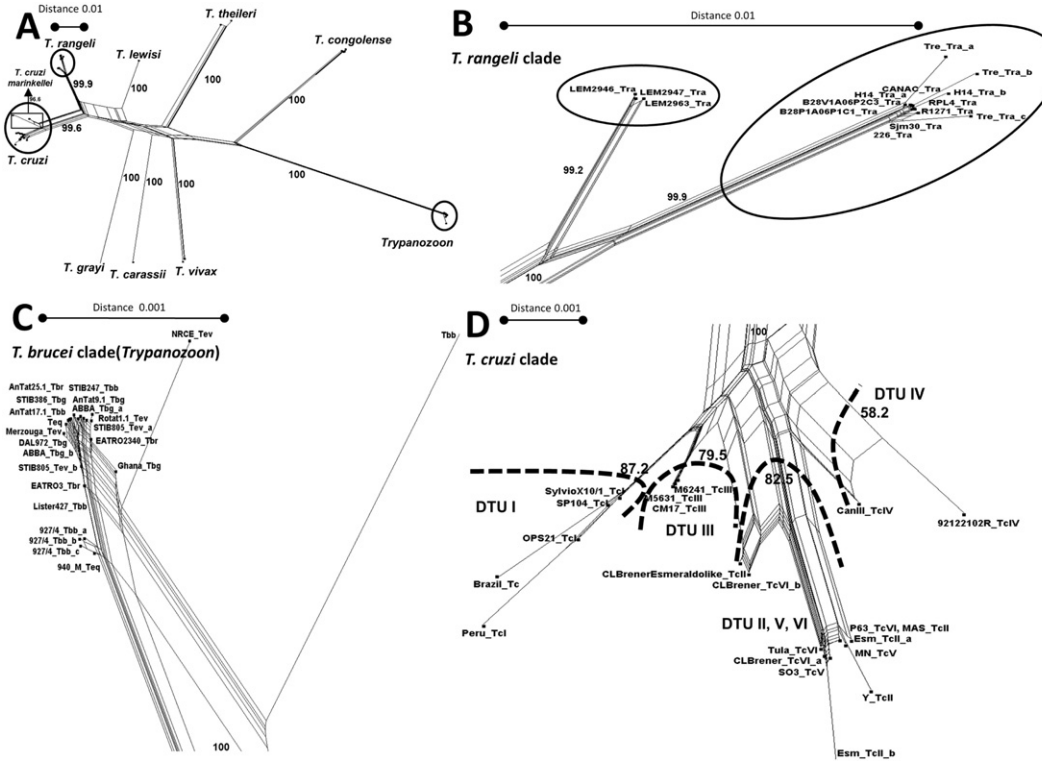

Fig. 3. Phylogenetic network of the *Trypanosoma hsp*70 sequences. The network was constructed using the Neighbor-Net algorithm (Bryant and Moulton, 2004), excluding all conserved sites. The Kimura 2-parameter model for nucleotides was used, calculating the fraction of differences between each pair of sequences. Each of the four panels (A–D) is drawn to the scale indicated, expressed as distance per nucleotide counted over variable sites in the *hsp*70 alignment. The numbers with the splits represent bootstrap values from 2000 replicates in percentages. (A) Complete network with representation of the three groups shown in more detail in the remaining panels; (B) *T. rangeli* clade; (C) *T. brucei* clade (*Trypanozoon*); (D) *T. cruzi* clade. Within the *T. cruzi* clade, splits defining the discrete typing units (DTUs) are indicated by dotted bows, with their bootstrap values. All features and abbreviations are as in Table S1.

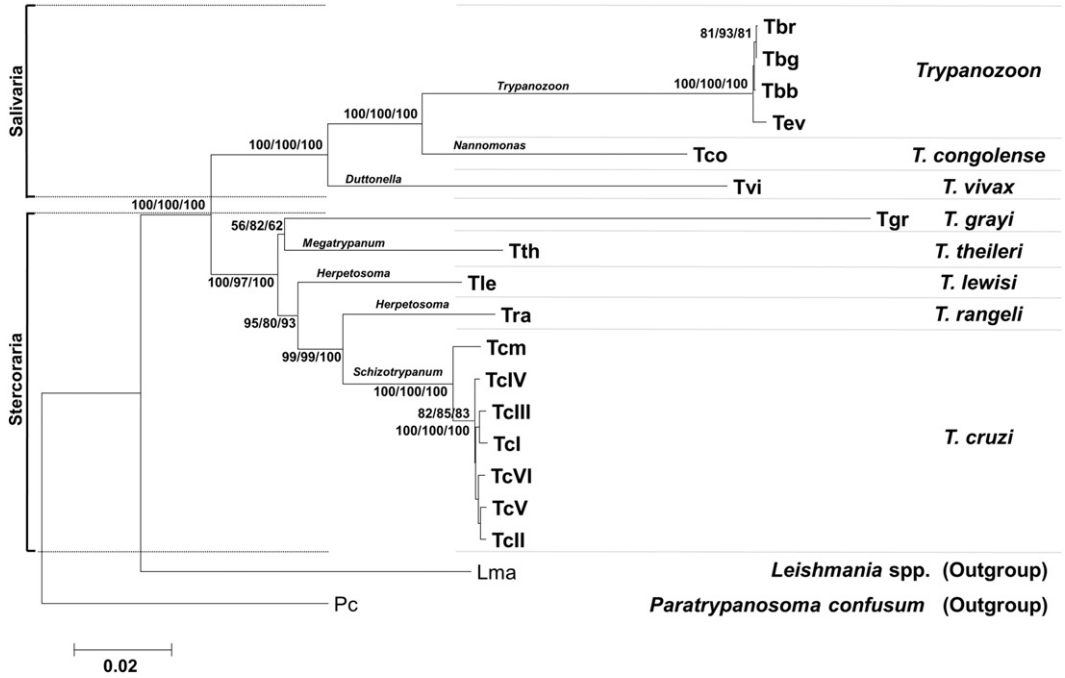

Fig. 4. Neighbor-Joining phylogeny based on concatenated SSU-rDNA + gGAPDH + *hsp*70 sequences belonging to 9 *Trypanosoma* species. Distances were estimated using the Kimura 2-parameter model. Bootstrap support of the branches was inferred from 2000 replicates, and is given in percentages at the internodes when exceeding 70% (first value). For comparison, also the bootstrap values of the Maximum Parsimony and Maximum Likelihood trees are shown for each respective group (second and third values respectively). The tree is drawn to the scale at the bottom, expressed as distances per nucleotide. The tree was rooted with the sequences of *Paratrypanosoma confusum* and *Leishmania major*. The Stercoraria and Salivaria sections are indicated on the left. Only one strain was used for each species. It should be noted that in some cases it was impossible to use identical strains for each gene because sequences were not available (Table S2). Strains are identified using the species or subspecies (abbreviations as in Fig. 2).

Different authors proposed to include in the *T. brucei* clade the species *T. congolense*, *T. vivax* and other tsetse fly transmitted species (*Trypanosoma simiae* and *Trypanosoma godfreyi*), based on the analysis of SSU-rRNA and gGAPDH (Cavazzana et al., 2010; Hamilton et al., 2004; Lima et al., 2013; Simpson et al., 2006; Stevens, 2008; Stevens and Gibson, 1999; Stevens and Rambaut, 2001). Also in our analysis *T. brucei*, *T. congolense*, and *T. vivax* grouped together in support of this clade. Nevertheless, in all analyses *T. congolense* and *T. vivax* formed distinct entities, and thus remained recognizable as separate species (Fig. 2). We should note that for *T. congolense* we only analyzed the Savannah subtype, and not the Forest and Kilifi subtypes. Equally so, our *T. vivax* sample did not include any strains from South America, only from Africa (Table S1).

The *Trypanozoon* subgenus forms a monophyletic cluster, but its constituent (sub-)species (*T. brucei brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. evansi*, and *T. equiperdum*) were not supported as separate groups, neither by *hsp*70 nor by other markers. The monophyly of *Trypanozoon* was also observed from satellite DNA repeats, ITS1-rDNA, and SSU-rDNA sequences (Amer et al., 2011; Cortez et al., 2006; Gibson, 2007; Stevens and Gibson, 1999). *T. evansi* and *T. equiperdum* hence are not monophyletic clades and do not qualify for species status.

Within the *T. brucei* clade reported by Simpson et al. (2006), our analysis identified *T. vivax* as the first diverging species, but it has a controversial status. Several reports agree with *hsp*70 and the combined analysis of SSU-rDNA, gGAPDH and *hsp*70 sequences: SSU-rRNA, LSU-rRNA and gGAPDH genes placed *T. vivax* in the *T. brucei* clade (Hamilton et al., 2004; Lukeš et al., 1997, Stevens and Gibson, 1999). In addition, some authors identified *T. vivax* as a distant member of the Salivaria section as part of the African tsetse fly-transmitted group (Amer et al., 2011; Cortez et al., 2006; Hughes and Piontkivska, 2003). On the contrary, in some analysis it appeared apart from the other African trypanosomes and outside all other Trypanosomatidae and Bodonidae using SSU-rDNA (Hughes and Piontkivska, 2003).

In our analysis based on *hsp*70 sequence alone, *T. theileri* did not convincingly group with either Stercoraria nor with Salivaria species. Its position in the *Trypanosoma* genus has been matter of debate, and no clear relationships could hitherto be established. Some studies demonstrated that *T. theileri* was closer to *T. cruzi* than to the *T. brucei* clade, using SSU-rRNA and gGAPDH genes (Cortez et al., 2006; Hamilton et al., 2004, 2007; Rodrigues et al., 2006). Also the combined analysis of SSU-rDNA, gGAPDH and *hsp*70 sequences grouped *T. theileri* with the Stercoraria. However, other studies showed contradictory results using the same genes (Cavazzana et al., 2010; Lima et al., 2013). Our results support *T. theileri* as an independent clade as proposed by Simpson et al. (2006); Rodrigues et al. (2006) and Hamilton et al. (2007).

Based on *hsp*70 alone, *T. grayi* appeared as an independent taxon, not clearly linked to any other group included in our analysis. However, in the concatenated analysis based on SSU-rDNA + gGAPDH + *hsp*70 sequences, *T. grayi* is clearly more related to the *T. cruzi* clade than to the *T. brucei* clade, and this is supported by bootstrap values between 97 and 100%, which clearly demonstrates the added value of concatenating different genes. Also Kelly et al. (2014) concluded that *T. grayi* relates more to *T. cruzi* than to *T. brucei*, but SSU-rRNA and gGAPDH placed it in a clade separate from both *T. cruzi* and *T. brucei*, often with other reptile or bird trypanosomes (Hamilton et al., 2004, 2007; Stevens and Gibson, 1999; Kelly et al., 2014). Finally, in the concatenated analysis *T. grayi* and *T. theileri* are deriving from the same branch, albeit with weak bootstrap support.

The *hsp*70 phylogeny and the combined analysis based on SSU-rDNA + gGAPDH + *hsp*70 sequences supports the *T. cruzi* clade as described by Simpson et al. (2006) (Fig. 2, Table 1). Indeed, the *hsp*70 gene identified *T. rangeli* as a sister clade of *T. cruzi* and *T. cruzi marinkellei*, as did other molecular markers such as the SSU-rRNA, cathepsin L-like proteases, cytochrome *b* and gGAPDH genes (Maia da Silva et al., 2004, 2007, 2009; Ortiz et al., 2009; Stevens and Gibson, 1999; Stevens and Rambaut, 2001). Traditionally, *T. rangeli* is classified

in the subgenus *Herpetosoma* of Stercoraria together with the subgenus' type species *T. lewisi*, but in our analysis *Herpetosoma* is paraphyletic. Añez (1982) proposed removing *T. rangeli* from the subgenus *Herpetosoma*, creating the subgenus *Tejeraia* within the Salivaria section. Other authors suggested reclassification in the subgenus *Schizotrypanum*, or discontinued the use of subgenus names altogether based on phylogenetic analysis with SSU-rRNA, gGAPDH, cathepsin L-like proteases, cytochrome *b*, and spliced-leader RNA genes (Maia da Silva et al., 2004, 2007, 2009; Ortiz et al., 2009; Stevens and Gibson, 1999; Stevens and Rambaut, 2001).

The *hsp*70 analysis clearly identified two lineages within *T. rangeli* (Fig. 2, Fig. 3B). Previously, two main genetic lineages were defined based on kinetoplast DNA (kDNA): KP1(+) and KP1(−) (Vallejo et al., 1999, 2003, 2009). In addition, ITS-rDNA and spliced leader genes identified at least five lineages (TrA–E) within *T. rangeli* (Maia da Silva et al., 2004, 2007). As we do not dispose of these lineage identifications for the strains included in our study, we could not correlate these subdivisions with the two *hsp*70 clades.

T. cruzi is one of the species of the subgenus *Schizotrypanum* and a heterogeneous monophyletic clade within the *Trypanosoma* genus (Hoare, 1972; Zingales et al., 2012). It is currently accepted that there are at least six defined genetic groups or discrete typing units (DTUs) or near-clades (clades that are blurred by infrequent inter-lineage genetic recombination) (Tibayrenc and Ayala, 2012): TcI-TcVI (Miles et al., 2009; Zingales et al., 2012). Three groups were clearly supported in the *hsp*70 network (Fig. 3D), corresponding to TcI, TcIII, and TcII + V + VI isolates. As TcV and TcVI have arisen by hybridization of TcII and TcIII (Brisse et al., 2000; Ferreira and Briones, 2012; Flores-Lopez and Machado, 2011; Westenberger et al., 2005), it is not surprising that their *hsp*70 sequences are linked to one of both, in particular TcII (Caballero et al., 2015; Lima et al., 2015; Westenberger et al., 2005). Based on *hsp*70 it was hence not possible to discriminate the DTUs TcII, TcV, and TcVI. The bootstrap value of the split defining TcIV was only 58.2, indicating that our data set does not strongly support this DTU, but nor does it support any other affiliation of the two TcIV strains. Two genotypes related to *T. cruzi* are restricted to bats: Tc-bat and *T. cruzi marinkellei* (Lima et al., 2012a; Marcili et al., 2009). Genealogy of *hsp*70 genes inferred in this study confirmed *T. cruzi marinkellei* isolates a sister clade of human *T. cruzi* isolates, both forming monophyletic groups (Fig. 2). This was previously shown by examination of SSU-rRNA, gGAPDH, cytochrome *b* and cruzipain (Cavazzana et al., 2010; Maia da Silva et al., 2009; Lima et al., 2012a, 2012b), and by comparative proteomic analysis (Telleria et al., 2010). More recently, Lima et al. (2015) provided strong evidence for the recognition of Tc-bat as an additional DTU, but unfortunately for our analysis DNA from this lineage was not available.

In conclusion, the *hsp*70-derived *Trypanosoma* phylogeny adds to the existing evidence towards classification into clades. The position of *T. theileri* remained however unclear. The *hsp*70 gene phylogeny could identify three *T. cruzi* groups (TcI, TcIII, and TcII + V + VI), but did not reflect the hybrid DTUs' separate evolution. The *hsp*70 gene was shown adequate for studying *Trypanosoma* evolution, and has been successfully applied for typing in various clinical samples of another kinetoplastid, *Leishmania* (Graça et al., 2012; Fraga et al., 2012; Garcia et al., 2004; Montalvo et al., 2014; Veland et al., 2012). Therefore, it is a promising diagnostic marker for the *Trypanosoma* genus as well, although more variable markers are needed to distinguish the *Trypanozoon* species. In fact, some typing assays have already been developed (Cuervo et al., 2013; Fraga et al., 2014; Rozas et al., 2007, 2008; Westenberger et al., 2005). Such typing assays should comply with a general consensus phylogeny, established by analyzing many genes from all species in parallel, or ultimately the entire genome.

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