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Trypanosoma rangeli is phylogenetically closer to Old World trypanosomes than to *Trypanosoma cruzi* ☆

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ABSTRACT

Trypanosoma rangeli and *Trypanosoma cruzi* are generalist trypanosomes sharing a wide range of mammalian hosts; they are transmitted by triatomine bugs, and are the only trypanosomes infecting humans in the Neotropics. Their origins, phylogenetic relationships, and emergence as human parasites have long been subjects of interest. In the present study, taxon-rich analyses (20 trypanosome species from bats and terrestrial mammals) using ssrRNA, glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), heat shock protein-70 (HSP70) and Spliced Leader RNA sequences, and multilocus phylogenetic analyses using 11 single copy genes from 15 selected trypanosomes, provide increased resolution of relationships between species and clades, strongly supporting two main sister lineages: lineage *Schizotrypanum*, comprising *T. cruzi* and bat-restricted trypanosomes, and Tra[Tve-Tco] formed by *T. rangeli*, *Trypanosoma vespertilionis* and *Trypanosoma conorhini* clades. Tve comprises European *T. vespertilionis* and African *T. vespertilionis*-like of bats and bat cimicids characterised in the present study and *Trypanosoma* sp. Hoch reported in monkeys and herein detected in bats. Tco included the triatomine-transmitted tropicopolitan *T. conorhini* from rats and the African NanDoum1 trypanosome of civet (carnivore). Consistent with their very close relationships, Tra[Tve-Tco] species shared highly similar Spliced Leader RNA structures that were highly divergent from those of *Schizotrypanum*. In a plausible evolutionary scenario, a bat trypanosome transmitted by cimicids gave origin to the deeply rooted Tra[Tve-Tco] and *Schizotrypanum* lineages, and bat trypanosomes of diverse genetic backgrounds jumped to new hosts. A long and independent evolutionary history of *T. rangeli* more related to Old World trypanosomes from bats, rats, monkeys and civets than to *Schizotrypanum* spp., and the adaptation of these distantly related trypanosomes to different niches of shared mammals and vectors, is consistent with the marked differences in transmission routes, life-cycles and host-parasite interactions, resulting in *T. cruzi* (but not *T. rangeli*) being pathogenic to humans.

1. Introduction

Trypanosoma rangeli and *Trypanosoma cruzi* are two generalist trypanosomes of bats and all orders of terrestrial mammals, and are the only trypanosome species infective to humans in the New World. *Trypanosoma cruzi*, the agent of Chagas disease, occurs from the southern United States to southern South America, while the geographical range of *T. rangeli* extends from Central to South America, and both species share diverse ecological niches in a variety of ecosystems where triatomines of the genus *Rhodnius* – the vector of *T. rangeli* – occur. *Trypanosoma rangeli* is prevalent from Central America to Amazonia, where *Rhodnius* spp. are highly abundant in palm trees, and it is also reported in other Brazilian biomes such as the Pantanal, Cerrado, and the Atlantic Forest. *Trypanosoma rangeli* is a common parasite of xenarthrans, marsupials,

☆ Note: Nucleotides sequence data reported in this paper are available in GenBank under accession numbers listed in Supplementary Table S1.

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rodents, carnivores and primates, and recent studies have identified a relevant prevalence of *T. rangeli* in bats (Hoare, 1972; Maia da Silva et al., 2007, 2009; Vallejo et al., 2009; Dario et al., 2017).

Mixed infections with *T. cruzi* and *T. rangeli* are common in triatomines and mammalian hosts including humans (Hoare, 1972; Maia da Silva et al., 2004a,b, 2007, 2009; Ramírez et al., 2014a; Pinto et al., 2015; Dario et al., 2017). However, despite shared mammalian hosts and vectors across Central and South America, the life cycles of *T. cruzi* and *T. rangeli* differ significantly in both vertebrate and invertebrate hosts. *Trypanosoma rangeli* is not pathogenic to its mammalian hosts, in which intracellular forms have not been confirmed, and is transmitted by *Rhodnius* spp. (triatomines) through the inoculation of trypomastigotes present in the salivary glands during feeding on mammalian blood (Hoare, 1972; Vallejo et al., 2009).

Trypanosoma rangeli was originally classified in the subgenus *Herpetosoma*, based exclusively on morphological parameters. On the basis of its route of transmission, *T. rangeli* was considered to be related to African trypanosomes transmitted by tsetse flies of the Salivaria section (Hoare, 1972), and the subgenus *Tejeraia* was proposed to accommodate this species in this section (see Hoare, 1972; Maia Da Silva et al., 2004a; Vallejo et al., 2009). However, based on molecular phylogeny, Stevens et al. (1999) confirmed *T. rangeli* to be closely related to *T. cruzi*, and thus distant from any species of the Salivaria section. In more comprehensive phylogenies, *T. rangeli* and *T. cruzi* clustered with trypanosomes from diverse mammalian hosts from South America, Africa and Europe, forming the *T. cruzi* clade comprising two main subgroups: one headed by *T. rangeli*, and the other by *T. cruzi* and its allied bat-trypanosome species of the subgenus *Schizotrypanum*: *Trypanosoma cruzi marinkellei* (restricted to Central and South America), *Trypanosoma dionisii* (cosmopolitan), and *Trypanosoma erneyi* of African bats (Hamilton et al., 2007, 2012; Lima et al., 2012, 2015a; Pinto et al., 2015). The *T. cruzi* clade also comprises several species of trypanosomes of Neotropical bats (clade Neobats that includes *Trypanosoma wauwau*) that clustered with *Trypanosoma noyesi* from Australian marsupials, unnamed trypanosomes from Australian rodents (Cottontail et al., 2014; Lima et al., 2015a; Pinto et al., 2015; Botero et al., 2016; Barbosa et al., 2017), and with one trypanosome reported from lemurs in Madagascar (Larsen et al., 2016); all placed basal to the assemblage including *T. rangeli* and *T. cruzi* clades. *Trypanosoma livingstonei* from African bats is currently positioned at the edge of the *T. cruzi* clade (Lima et al., 2013, 2015a; Dario et al., 2017).

Trypanosoma cruzi is widespread in virtually all terrestrial mammalian orders, and is transmitted by triatomines of both the Triatomini and Rhodniini tribes (Reduviidae: Triatominae); its development is restricted to the digestive tract of the vectors. *Trypanosoma cruzi* is a genetic complex, comprising at least six discrete taxonomic units (DTUs, TcI-TcVI), plus the Tcbat DTU tightly linked to bats, and bats are hosts of all DTUs (Marcili et al., 2009; Pinto et al., 2012; Ramírez et al., 2014b; Lima et al., 2015b; Dario et al., 2016, 2017). All species of *Schizotrypanum* exhibit intracellular (cytoplasm) multiplication of amastigote forms and differentiation to infective trypomastigote forms that are released by the cells, a trait unique to the subgenus (Molyneux, 1991; Cavazzana et al., 2010; Lima et al., 2012). To date, only *Cavernicola pilosa* of the rare Cavernicolini tribe of Triatominae has been proven to be a vector of *T. c. marinkellei.* Cimicidae bat bugs are known vectors of *T. dionisii* and *T. vespertilionis* to Old World bats and *Trypanosoma hedricki* in North America; these ectoparasites are common in bat shelters (Bower and Woo, 1981; Gardner and Molyneux, 1988; Molyneux, 1991). The high prevalence of *T. c. marinkellei* and *T. dionisii* in regions where neither *Cavernicola* spp. nor bat bugs are reported suggests that alternative vectors (triatomines of different species) transmit (cyclically or mechanically) *Schizotrypanum* trypanosomes to bats (Cavazzana et al., 2010; Lima et al., 2015a,b).

Trypanosoma vespertilionis appears to be restricted to bats and transmitted by cimicids (development being restricted to the digestive tract) in Europe and Africa (Hoare, 1972; Gardner and Molyneux, 1988; Molyneux, 1991). *Trypanosoma conorhini* is a parasite of *Rattus* spp. (restricted to the bloodstream), and is thought to be transmitted exclusively by *Triatoma rubrofasciata* (with development restricted to the digestive tract); *Tr. rubrofasciata* is known to transmit at least two trypanosomes: *T. cruzi* in Latin America and *T. conorhini* worldwide. *Trypanosoma conorhini* was first reported in *Tr. rubrofasciata* in India, and has since been reported throughout the tropical world, especially in Asian-Pacific, African and Latin American seaports (Hoare, 1972; Dujardin et al., 2015). *Trypanosoma conorhini* and *Tr. rubrofasciata* likely dispersed together with domestic rats (Patterson et al., 2001; Hypsa et al., 2002; Dujardin et al., 2015). The presence of *T. conorhini* in field monkeys and *Tr. rubrofasciata* has never been confirmed by molecular methods, and only a single isolate derived from *Rattus rattus* from Brazil has been included in phylogenetic trees (Stevens et al., 2001; Rodrigues et al., 2006; Hamilton et al., 2009).

Phylogenetic analyses of isolates of *T. rangeli* from different vertebrates and vectors have to date identified five phylogenetic lineages: TrA-TrE (Maia da Silva et al., 2004a,b, 2007, 2009; Ortiz et al., 2009; Caballero et al., 2015). In contrast to *T. rangeli* and *T. cruzi*, both constituted by an increasing number of genotypes supported by different molecular markers, there are no studies on the genetic diversity of *T. conorhini* and *T. vespertilionis*, whose ranges of host species, geographical distribution, and relationships with other species of trypanosomes remain unclear. Recent phylogenies based on ssrRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) have left uncertainty regarding the relationships of *T. rangeli* with *T. cruzi*, *T. conorhini*, European *T. vespertilionis* and African trypanosomes from bats, monkeys and civets (Stevens et al., 1999, 2001; Hamilton et al., 2007, 2009; Lima et al., 2013, 2015a; Barbosa et al., 2016).

The unresolved relationships of *T. rangeli* with Old World trypanosomes of bats and non-volant hosts, and *Trypanosoma teixeirae* of Australian bats have challenged the earlier hypotheses about the origin and hosts of recent ancestors of *T. rangeli*, and the usually assumed close relationships with *T. cruzi*. The addition of new taxa into the weakly supported lineage comprising *T. rangeli* and the use of multilocus analyses appear critical to resolution of these relationships. With this aim, we characterised eight new African (Guinea Bissau (GW)) trypanosomes from bats and one from a bat cimicid related to *T. vespertilionis*, plus nine trypanosomes morphologically resembling *T. conorhini* isolated from *Rattus* spp., *Tr. rubrofasciata*, and Asian (Malaysia) monkeys. To clarify the tangled phylogenetic relationships of *T. rangeli* with Old World trypanosomes related to *T. conorhini* and *T. vespertilionis*, and with *T. cruzi* and other species of the subgenus *Schizotrypanum*, we performed taxon-rich phylogenetic analyses using ssrRNA, gGAPDH and heat shock protein-70 (HSP70) sequences. Thereafter, trypanosomes from bats and non-volant mammals representative of the genetic diversity of the whole data set of trypanosomes transmitted by triatomines and cimicids, from the New and Old Worlds, were selected for Spliced Leader (SL) RNA and multilocus phylogenetic analyses.

2. Materials and methods

2.1. Study area in Guinea Bissau, bats, and blood samples

Bats examined in this study were captured using mist nets in the National Park of "Lagoas de Cufada" (S11°60′ E15°04′), Guinea

Bissau (GW) in West Africa (WA), in 2010 (Fig. 1). Surveys of trypanosomes in 54 bats from GW using DNA from blood samples preserved in ethanol were performed as described previously (Lima et al., 2012b). We also analysed blood samples from monkeys and civets captured in Cameroon, Central Africa (Fig. 1) (Njiokou et al., 2006), that were previously shown to be infected with the HochNdi1 and NanDoum1 trypanosomes (Hamilton et al., 2009), and nine trypanosomes morphologically resembling *T. conorhini* isolated from *Rattus* spp., *Tr. rubrofasciata*, and Asian (Malaysia) monkeys. All trypanosomes included in the phylogenetic analyses, and their respective hosts and geographical origins, are detailed in Supplementary Table S1.

Bats were identified by morphological keys, and as done previously, specimens of each putative species of African bat were confirmed by cytochrome c oxidase subunit I (*CoxI*) barcoding (Lima et al., 2013, 2015a). A cimicid bug taken from a bat was identified by 16S rDNA barcoding (Maia da Silva et al., 2009).

Ethical approval for animal handling was obtained from University of São Paulo (Brazil) and Biodiversity Institute of Guinea Bissau. All procedures undertaken in Brazil were in accord with the Committee on the Ethics of Animal Experimentation of the Institute of Biomedical Sciences and Biosciences, University of São Paulo, Brazil (Approved protocols: no17/page 3/book2 and no109/03).

2.2. Culture, in vitro tests of cell invasion, and cryopreservation of trypanosomes from Guinea Bissau

For surveys of trypanosomes, 54 bats captured in GW were examined by haemoculture (HE) as previously described (Lima et al., 2012, 2013). Blood samples (~200 µl) were inoculated into culture tubes with a blood agar base containing 15% sheep blood as a solid phase with an overlay of TC100 medium (= Grace's medium) containing 10% FBS, and incubated at 25 °C. Five cimicid bugs taken from bats were dissected, examined microscopically for trypanosomes, and positive guts were inoculated into culture tubes as described for HEs. Cultures were cryopreserved in the Trypanosomatid Culture Collection (TCC) of the University of São Paulo (Supplementary Table S1). To verify whether trypanosomes invade and develop within mammalian cells, cultures showing many trypomastigotes were transferred to monolayers of monkey LLC-MK2 cells at 37 °C (Lima et al., 2012, 2013).

2.3. Trypanosome cultures and PCR amplifications

Cultures of trypanosomes were grown in TC100 medium as described in Section 2.2, and DNA was extracted using the phenol–chloroform method (Sambrook et al., 1989). DNA samples were used for PCR amplification of the variable V7-V8 region of ssrRNA (~800 bp) as described previously (Borghesan et al., 2013). To detect trypanosomes in blood samples, we used a nested PCR for amplification of partial (~560 bp) V7-V8 ssrRNA sequences (Noyes et al., 1999). Sequences of gGAPDH (~800 bp) were amplified as described previously (Borghesan et al., 2013). Sequences of HSP70 were amplified using the primers HSP70F (5′-TGATG CAGCTGGTGTCGGACTT-3′) and HSP70R (5′-CTGGTACATCTTCGT CATGATG-3′). PCR was performed using 100 ng of each primer, 200 µM of each dNTP, 1.5 mM of $MgCl_2$, 2.5 U of Taq DNA polymerase and ~100 ng of DNA template. PCR amplifications of HSP70 consisted of 34 cycles as follows: 1 min at 94 °C, 2 min at 58 °C and 2 min at 72 °C, with a first cycle of 3 min at 94 °C and a final cycle of 10 min at 72 °C. Host and geographical origins of the trypanosomes included in our phylogenetic analyses are presented in Supplementary Table S1.

2.4. Phylogenetic analyses of ssrRNA, gGAPDH and HSP70 gene sequences

To infer broad phylogenies based on conventional ssrRNA and gGAPDH genes of a large set of samples from the *T. cruzi* clade, the following alignments of DNA sequences were created: (i) V7–V8 ssrRNA (73 sequences from 20 trypanosome species) including 11 sequences determined in the present study of the novel bat isolates from GW aligned with sequences from other trypanosomes of bats and other hosts; sequences from two recently reported trypanosomes of the *T. cruzi* clade – *T. teixeirae* from an Australian bat (Barbosa et al., 2016), and *Trypanosoma* sp. TVY from the blood of lemurs (*Indri indri*) captured in Madagascar (Larsen et al., 2016) were included in the alignment (Supplementary Table S1); (ii) gGAPDH sequences of 11 new trypanosomes (from bats, rats, monkeys, and cimicid and triatomine bugs) aligned with 49 published *T. cruzi* clade sequences (Supplementary Table S1); (iii) concatenated sequences of V7–V8 ssrRNA and gGAPDH genes from trypanosomes of the *T. cruzi* clade, using *Trypanosoma lewisi* and *Trypanosoma microti* as outgroup taxa; (iv) HSP70 sequences (n = 37) obtained in the present study by PCR sequencing and genome surveys from trypanosomes of the *T. cruzi* clade in isolation, or combined with V7-V8 ssrRNA and gGAPDH sequences. All sequences determined in this study were submitted to GenBank (Accession numbers are given in Supplementary Table S1).

Phylogenies were inferred using maximum likelihood (ML), parsimony (P) and Bayesian inferences (BI) analyses. Parsimony and bootstrap analyses were performed using PAUP (Swofford, 2002) with 500 replicates of random addition sequences followed by branch swapping (RAS-TBR). The ML analyses were performed using RAxML (Stamatakis, 2006) with tree searches performed using a GTR model with gamma-distributed rate variation across sites and proportion of invariable sites (GTRGAMMA model), and 500 maximum parsimony-starting trees; model parameters were estimated in RAxML for the duration of the tree search. Nodal supports were estimated with 500 bootstrap replicates in RAxML using GTRGAMMA and maximum parsimony starting trees. MrBayes (Huelsenbeck and Ronquist, 2001) was employed for the BI analyses (GTRGAMMA); the first 25% of trees from 1 million generations were discarded as burn-in.

2.5. Genes retrieved from trypanosome genomes for multilocus phylogenetic analysis

Searches of 11 single copy genes previously employed for multilocus analysis of *T. cruzi* DTUs (Flores-López and Machado, 2011; Lima et al., 2015b) were performed using BLAST against genomes freely available in TriTrypDB and/or NCBI databanks for *T. cruzi* (Esmeraldo and CL Brener), *T. c. marinkellei* B7, *T. rangeli* SC58 and *Trypanosoma brucei brucei* 427, and against unpublished draft genomes generated by our group, to facilitate multilocus phylogenetic analyses, and analyses of particular genes and gene families (Lima et al., 2012b; Caballero et al., 2015). Draft genomes of *T. cruzi* clade trypanosomes (cryopreserved at the TCC collection) were used for gene surveys: *T. cruzi* G, *T. c. marinkellei* 344, *T. dionisii*, *T. erneyi*, *T. rangeli* AM80, *T. conorhini*, *T. noyesi* H25, *T. wauwau*, *T. livingstonei*, *T. vespertilionis*-like G1, and *Trypanosoma* sp. Hoch G3 (Supplementary Table S2). The selected genes were also retrieved from draft genomes of *Trypanosoma lewisi*, *Trypanosoma theileri*, *Trypanosoma cyclops* and *Trypanosoma b. brucei*, and all these species were used as outgroup taxa of the *T. cruzi* clade. The 11 genes selected for this study were: Glucose-6-phosphate isomerase (GPI), Glutathione peroxidase (GPX), 3-Hidroxy-3-metylglutaryl-CoA reductase (HMCOAR), Leucine aminopeptidase (LAP), Mitochon-

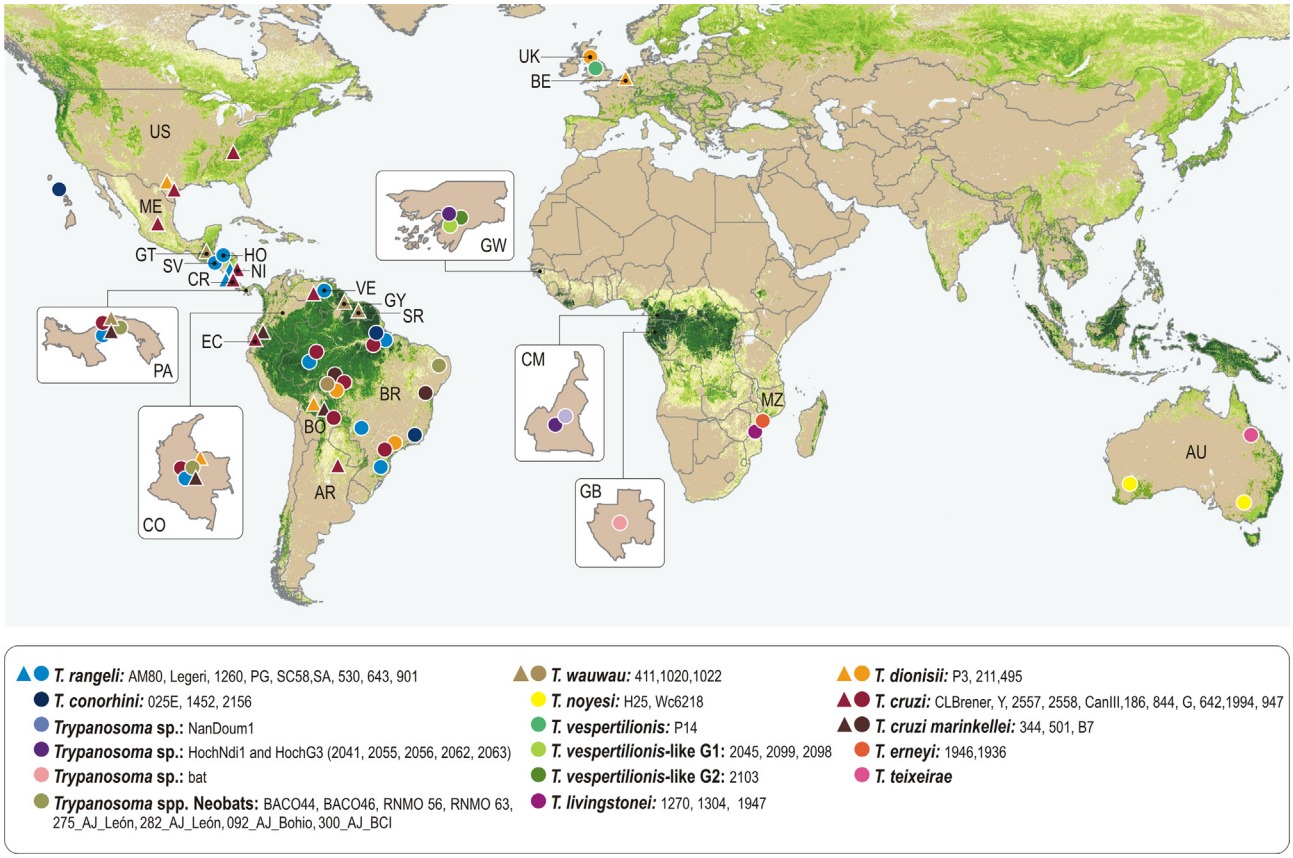

Fig. 1. Geographical distribution of the *Trypanosoma cruzi* clade trypanosomes. Isolates characterised in this (●) or in previous studies (▲), most included in our analyses, were plotted on the map to illustrate the geographical range of each trypanosome species. AU, Australia; AR, Argentina; BE, Belgium; BO, Bolivia, BR, Brazil, CM, Cameroon; CO, Colombia; CR, Costa Rica; EC, Ecuador; GT, Guatemala, GY, French Guyana; GB, Gabon; GW, Guinea Bissau; HO, Honduras; ME, Mexico; MZ, Mozambique; NI, Nicaragua; PA, Panamá; SV, El Salvador; SR, Surinam; VE, Venezuela; UK, United Kingdom; US, United States.

drial peroxidase (TcMPX), Pyruvate dehydrogenase E1 component alfa subunit (PDH), RNA-binding protein-19 (RB19), Rho-like GTP binding protein (RHO1), Superoxide dismutase A (sodA), Superoxide dismutase B (sodB) and Serine/threonine-protein phosphatase PP1 (STTP2) (Flores-López and Machado, 2011). Phylogenetic inferences were performed using individual and combined genes as above. Access to the unpublished draft genomes analysed in this paper can be obtained by contacting the corresponding author.

2.6. SL RNA sequences: amplification, sequencing, and data analysis

Entire SL RNA gene repeats were amplified and sequenced at both strands of three to five clones of each trypanosome, and the secondary structures of SL transcripts were obtained using the RNAdraw programme with default settings (Maia da Silva et al., 2007; Lima et al., 2013, 2015a). The alignment of exon and intron sequences was manually refined. Network analysis of SL RNA genes was inferred by SplitsTree v4.11.3 using the neighbour-net method (Huson and Bryant, 2006). Internode supports were estimated by performing 100 bootstrap replicates using the same parameters optimised for network inferences.

2.7. DNA sequences and alignments employed in this study

DNA sequences obtained in the present study were deposited in GenBank, and accession numbers for sequences used in all analyses are provided in Supplementary Table S1. All nucleotide sequence alignments employed in the present study are available at Mendeley Data (https://doi.org/10.17632/dj6w6z69ch.2).

3. Results

3.1. African trypanosomes related to T. vespertilionis selected by V7-V8 ssrRNA barcoding

New trypanosomes were selected for this study using V7-V8 ssrRNA barcoding of trypanosomes from bats captured in GW, West Africa, and compared with bat trypanosomes from Mozambique, East Africa, and Brazil, Colombia and Venezuela in South America (Fig. 1). Eight cultures of trypanosomes obtained from bats (*Scotophilus* sp.), captured in GW, were characterised for the first time in this study, as well as one culture obtained from the gut of a bat cimicid (*Cacodmus* sp.) taken from one GW bat (*Pipistrellus* sp.) (Table 1).

Phylogenetic analysis of V7-V8 ssrRNA revealed that the trypanosomes from GW bats are closely related – but not identical – to *T. vespertilionis* P14 from European bats. Three trypanosomes from GW bats (TCC2045, 2098, 2099) clustered with *T. vespertilionis* P14 and are hereafter referred as *T. vespertilionis*-like G1 (a genotype different from P14). One trypanosome (TCC2103) from a bat cimicid clustered with *T. vespertilionis* P14, but diverged sufficiently to be considered a new species, and was provisionally referred to as *T. vespertilionis*-like G2. Five cultures of trypanosomes from bats (TCC2041, 2055, 2056, 2062, 2063) shared almost identical sequences with HochNdi1 of a monkey from Cameroon, forming a clade separated by relevant sequence divergence from all species of the lineage [Tve-Tco], and they represent a new trypanosome species that are hereafter referred to as *Trypanosoma* sp. HochG3 (Table 1, Fig. 2). PCR screening of GW bat

Table 1
Host, geographical origin, behaviour and vectors of trypanosomes of the *Trypanosoma cruzi* clade.

Trypanosoma spp.	Geographic distribution	Mammal hosts	Infectivity/ pathogenicity[a]		Intracellular development	Vector
			Human	Mouse		
Clade T. rangeli						
T. rangeli	Central/South America	Generalist[b]	Yes/No	Yes/No	No	Triatominae (*Rhodnius* spp.) Cimicidae [c]
Clade T. vespertilionis						
T. vespertilionis	Europe/UK	Chiroptera	No	No	No	Cimicidae
T. vespertilionis-like G1	Africa: Guinea Bissau	Chiroptera	?	No	No	Cimicidae
T. vespertilionis-like G2	Africa: Guinea Bissau	Chiroptera	?	No	No	Cimicidae
Trypanosoma sp. HochNdi1	Africa: Cameroon	Primata (monkey)	?	?	?	?
Trypanosoma sp. HochG3	Africa: Guinea Bissau	Chiroptera	?	No	No	?
Clade T. conorhini						
T. conorhini	Cosmopolitan	Rodentia (*Rattus* spp.) Primata (monkey)	?	Yes/No	No	Triatominae (*T. rubrofasciata*) Cimicidae[c]
Trypanosoma sp. NanDoum1	Africa: Cameroon	Carnivora (civet)	?	?	?	?
Clade Schizotrypanum						
T. cruzi DTU: TcI-TcVI	North/Central/South America	Generalist[b]	Yes/Yes	Yes/Yes	Yes	Triatominae Cimicidae[c]
T. cruzi Tcbat	Central/South America	Chiroptera	No[d]	Yes/No	Yes	Cimicidae [c]
T. cruzi marinkellei	Central/South America	Chiroptera	No	No	Yes	Triatominae (Cavernicola), other?
T. erneyi	Africa: Mozambique	Chiroptera	No	No	Yes	Cimicidae?
T. dionisii	Cosmopolitan	Chiroptera	No[e]	No	Yes	Cimicidae, other?
Australian clade						
T. noyesi[f] H25/W6218/G8/D15/D17/ D68/BDA1/OTU41/OTU140	Australia	Marsupialia (kangaroo, woylie, possum, wallaby and koala)	?	ND	No	Tabanidae? Ceratopogonidae?
Trypanosoma sp. BRA2	Australia	Rodentia	?	?	?	?
Clade T. wauwau						
T. wauwau	Central/South America	Chiroptera	?	?	No	?
Trypanosoma spp. Neotropical	Central/South America	Chiroptera	?	?	?	?
Species of other clades						
Trypanosoma sp. bat	Africa: Gabon	Chiroptera	?	No	No	?
T. teixeirae[f]	Australia	Chiroptera	?	?	?	?
Trypanosoma sp. TVY	Africa: Madagascar	Primata (lemur)	?	No	?	?
Basal species of the clade T. cruzi						
T. livingstonei	Africa: Mozambique	Chiroptera	?	No	No	?

[a] Experimental infection in BALB/c mice.
[b] Bats and terrestrial mammals.
[c] Experimental infection of cimicids (Hoare, 1972).
[d] One Colombian child positive by PCR (Ramírez et al., 2014a).
[e] One human tissue sample positive by PCR (Dario et al., 2016).
[f] Potentially pathogenic in bats (Barbosa et al., 2016).
[?] Unknown.

blood samples revealed *T. vespertilionis*-like G1, *Trypanosoma* sp. HochG3, and mixed infections with these two trypanosome species. Other trypanosomes detected in GW bat blood samples using this method have been barcoded, and preliminary results showed that they are related to *T. dionisii* or *T. livingstonei* (data not shown).

In addition to the phylogenetic positioning and the level of sequence divergence separating *T. vespertilionis*-like G2 and *Trypanosoma* sp. HochG3 from their closest relatives, formal descriptions of these trypanosomes as new species will be done using a combined taxonomic approach based on phylogenetic positioning, morphological features, development in culture, plus behavioural and biogeographical data. Currently, we are carrying out surveys of trypanosomes in bats and bat cimicids from other African regions aiming to assess the geographical distribution and any association of *T. vespertilionis*-like and *Trypanosoma* sp. HochG3 with bat and cimicid species. Similar to *T. rangeli* and *T. conorhini*, the newly characterised *T. vespertilionis*-like and *Trypanosoma* sp. HochG3 did not develop intracellularly (as herein demonstrated by in vitro cultures), thereby they are more similar to *T. rangeli*, and differ from *Schizotrypanum* spp. from the New and Old Worlds, all of which develop as amastigotes and trypomastigotes within mammalian cells (Table 1) (Molyneux, 1991; Cavazzana et al., 2010; Lima et al., 2012).

The phylogenetic analysis presented in this study using V7-V8 ssrRNA sequences is the most taxon-rich analysis of the *T. cruzi* clade performed to date, including 73 sequences from 20 different trypanosome species of several mammalian orders (formally named or not) from Central and South America, West, Central and East Africa plus Madagascar, and some samples from Europe and Australia. In the ssrRNA analysis, trypanosomes from blood samples of lemurs from Madagascar (Larsen et al., 2016) clustered in the Australian clade, which comprises trypanosomes from marsupial and rodents that is closely related to the Neobats clade formed by a diversity of trypanosome species from Neotropical bats (Cottontail et al., 2014; Lima et al., 2015a; Pinto et al., 2015). The lineage composition within the *T. cruzi* clade revealed by the V7-V8 ssrRNA phylogram (Fig. 2) was concordant with results from previous phylogenies (Lima et al., 2012, 2013, 2015a), and with phylogenetic relationships inferred in this study using other genes (Figs. 3, 4).

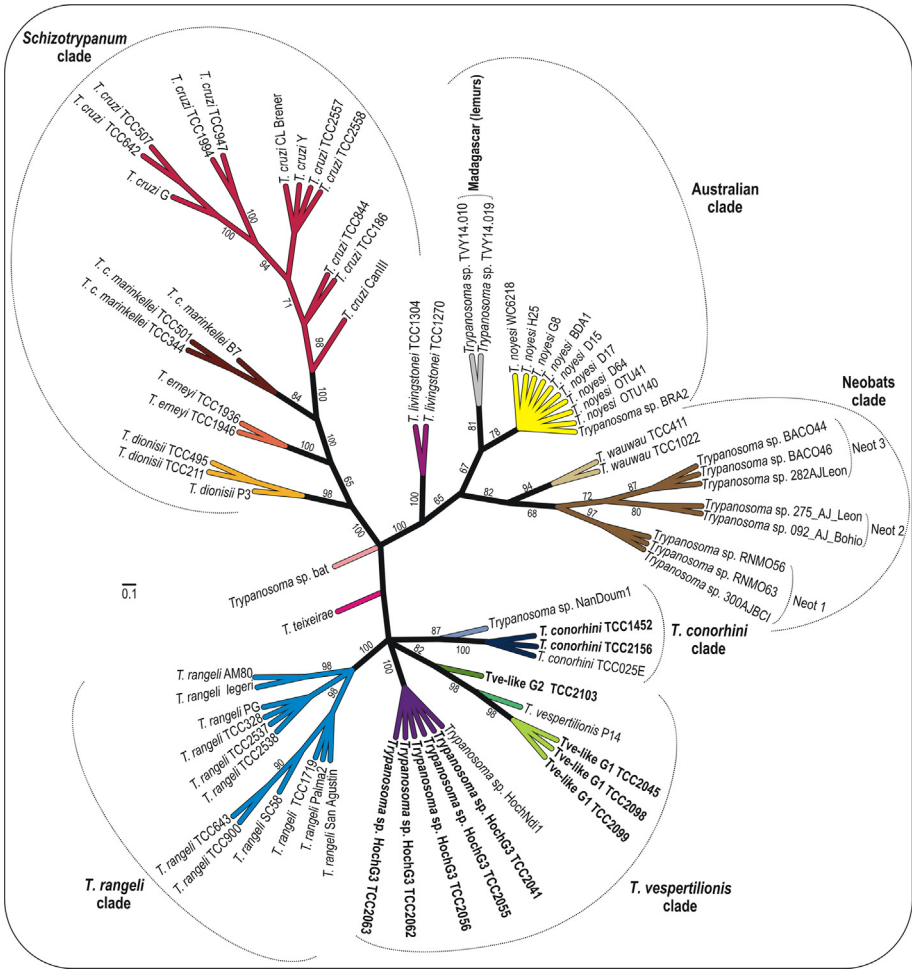

Fig. 2. Unrooted phylogram of the *Trypanosoma cruzi* clade based on variable V7–V8 region of ssrRNA barcode sequences. The dendrogram (P) was inferred using 73 sequences (~800 bp) from 20 trypanosomes (most from bats) from the Neotropics, Afrotropics and Australia, showing the phylogenetic positioning of eight new trypanosomes from bats and one from a bat cimicid from Guinea Bissau, West Africa, and new isolates of *Trypanosoma conorhini* in the clade composed of *Trypanosoma vespertilionis* and *T. conorhini* lineages, respectively. Barcodes determined in the present study are in bold. Numbers at the nodes are support values from 500 replicates.

3.2. Molecular characterisation, host and geographical ranges of T. conorhini

We compared one reference isolate of *T. conorhini* (BR1) from *Rattus rattus* captured in Brazil and infective to *Tr. rubrofasciata*, with two additional isolates: *T. conorhini* BR2 of *R. rattus* from Belém, Brazilian Amazonia, and *T. conorhini* Rub1 of *Tr. rubrofasciata* from Hawaii (Supplementary Table S1). These isolates of *T. conorhini* showed very similar but not identical ssrRNA barcodes, and were separated by a maximum of 0.7% gGAPDH sequence divergence. The *T. conorhini* isolates analysed were collected from rats or *Tr. rubrofasciata* captured near ports, and the small degree of polymorphism between these isolates reinforces the hypothesis of relatively recent dispersal of *T. conorhini* in *Rattus* spp. and *Tr. rubrofasciata*.

Here, we analysed three isolates from Asian monkeys deposited in the ATCC (American Type Culture Collection) reported previously as showing morphological resemblance to *T. conorhini* in the blood of rodents, and able to develop in the gut of experimentally infected *Tr. rubrofasciata* (Weinman, 1977). Nevertheless, all isolates were identified as *T. cyclops*, a trypanosome of southeast Asian monkeys that did not cluster in the *T. cruzi* clade, but were shown to be closely related to *T. theileri* (Stevens et al., 2001; Rodrigues et al., 2006; Hamilton et al., 2007). This species is thought to be transmitted by triatomines in southeastern Asia, the only place outside Latin America where triatomines occur (Patterson et al., 2001; Hypsa et al., 2002).

3.3. Phylogenies based on ssrRNA, gGAPDH and HSP70 sequences strongly support the clustering of T. conorhini and T. vespertilionis forming the sister lineage of T. rangeli

In recent phylogenies, *T. rangeli* was nested in an unresolved and poorly understood assemblage comprising four trypanosomes: *T. conorhini*, European *T. vespertilionis* (P14), and African trypanosomes from monkey (HochNdi1) and civet (NanDoum1) (Hamilton et al., 2009; Lima et al., 2012, 2013, 2015a). Here, the inclusion of eight new trypanosomes from African (GW) bats and one from a bat cimicid plus two additional isolates of *T. conorhini* in combined (ssrRNA, gGAPDH and HSP70 genes) phylogenetic analyses strongly supported a monophyletic assemblage comprising the sister lineages Tra and [Tve-Tco].

Here, our analyses allowed better resolution of phylogenetic relationships among species within each lineage, and their sister relationships with *Schizotrypanum* spp. Congruent phylogenies were inferred using concatenated gGAPDH and V7-V8 ssrRNA (Fig. 3), HSP70 (Fig. 4A) or concatenated HSP70, gGAPDH, and V7-V8 ssrRNA (Fig. 4B) gene sequences. The analyses strongly supported Tve as a lineage formed by the European *T. vespertilionis*

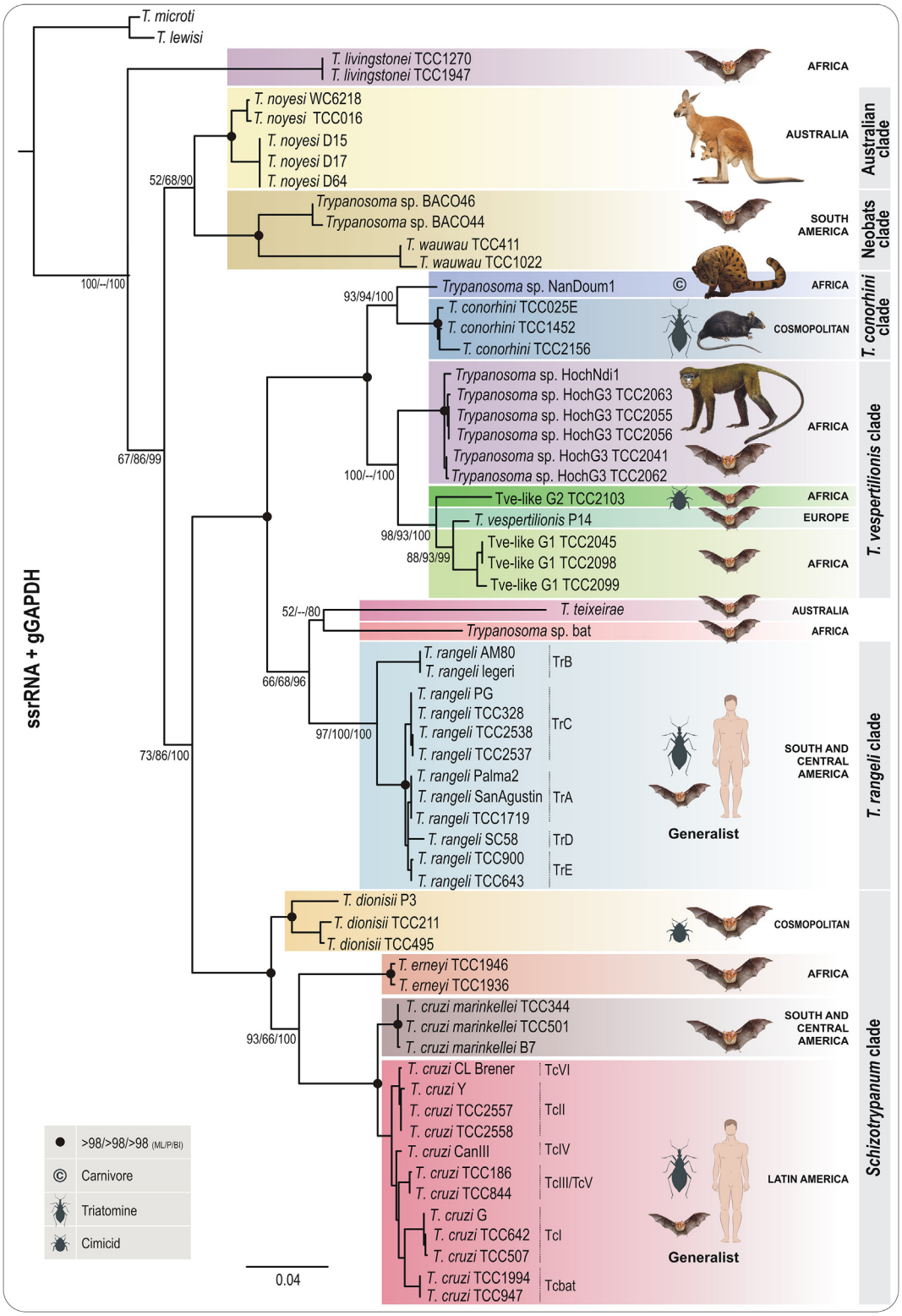

Fig. 3. Phylogeny of *Trypanosoma* spp. with a focus on the *Trypanosoma cruzi* clade inferred using combined ssrRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) sequences. The inferred maximum likelihood phylogenetic tree (1.548 characters, –Ln = 8659.072660) supports a major assemblage comprising the lineages Tra, Tve and Tco, all clustering together forming the major clade Tra[Tve-Tco] and its sister *Schizotrypanum* (type-species = *T. cruzi*) clade. New trypanosomes from African bats and a bat cimicid (Tve-like G1 and G2, and *Trypanosoma* sp. HochG3) were placed in the [Tve-Tco] clade. *Trypanosoma lewisi* and *Trypanosoma microti* were used as outgroups of the *T. cruzi* clade. Numbers at the nodes are, respectively, maximum likelihood/parsimony (P) (500 replicates) and Bayesian inference support values.

P14, three new isolates of *T. vespertilionis*-like G1 from GW bats, and *T. vespertilionis*-like G2 from GW bat cimicid; with gGAPDH sequence divergence of 2.0% separating European *T. vespertilionis* P14 and African *T. vespertilionis*-like G1. These are the first African trypanosome isolates confirmed as *T. vespertilionis* by molecular phylogenetic analysis. This finding suggests that *T. vespertilionis*

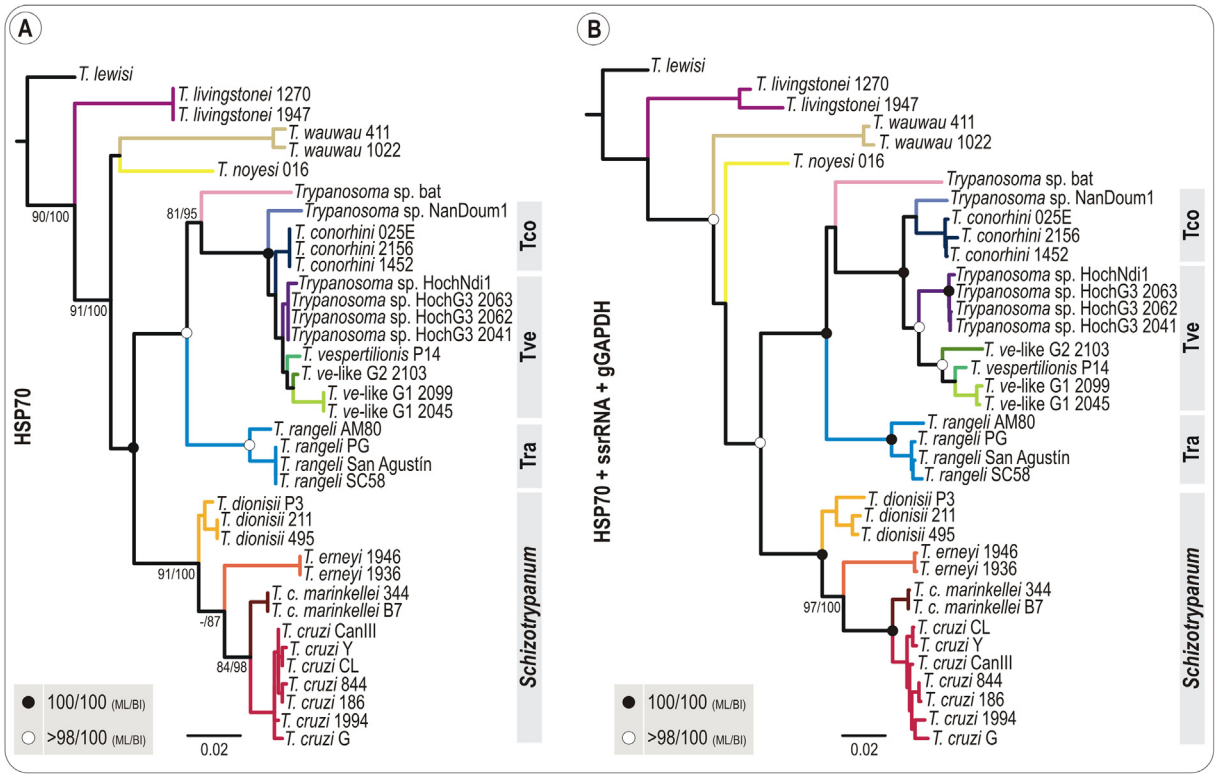

Fig. 4. Phylogeny of Tra[Tve-Tco] and *Schizotrypanum* lineages, and *Trypanosoma wauwau*, *Trypanosoma noyesi* and *Trypanosoma livingstonei*, all species of the *Trypanosoma cruzi* clade inferred using heat shock protein-70 (HSP70) sequences. Maximum likelihood analysis of (A) HSP70 gene sequences (~800 characters, −Ln = 2442.385253), (B) concatenated HSP70, ssrRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) sequences (2.246 characters, −Ln = 10446.636791). *Trypanosoma wauwau*, *T. noyesi* and *T. livingstonei* are the basal species of the major Tra[Tve-Tco]-*Schizotrypanum* lineage, and *Trypanosoma lewisi* was used as an outgroup of the clade *T. cruzi*. Numbers at the nodes correspond respectively to maximum likelihood (500 replicates) and Bayesian inference support values.

may have dispersed in bats through the Mediterranean, although whether its ancestral form originated in Africa or Europe remains unclear at this time. *Trypanosoma vespertilionis*-like G2 from a cimicid bat bug clustered with *T. vespertilionis* P14 and *T. vespertilionis*-like G1, but was separated from these trypanosomes by 3.0% gGAPDH sequence divergence (Fig. 3). *Trypanosoma* sp. HochNdi1 from African monkeys was the only trypanosome from non-bat hosts that nested in the Tve lineage. In the present study, this unnamed species of trypanosome was found for the first time in bats from Africa; five isolates from GW bats sharing almost identical sequences with *Trypanosoma* sp. HochNdi1 are hereafter referred to as *Trypanosoma* sp. HochG3 (Figs. 2–4; Table 1). The clade formed by three very similar isolates of *T. conorhini* included *Trypanosoma* sp. NanDoum1 of a civet from Cameroon, Central Africa (Figs. 2, 3; Table 1); these two trypanosomes exhibited a relatively small (2.4%) gGAPDH sequence divergence.

The phylogenetic trees including new bat and cimicid trypanosomes, and three isolates of *T. conorhini*, enabled better resolution within the [Tve-Tco] lineage, and produced relatively well resolved phylogenetic relationships among the six distinct taxa characterised within this clade. All phylogenetic analyses strongly supported the sister relationships of the lineages Tra and [Tve-Tco] (Figs. 3, 4). Despite including trypanosomes from bats, monkeys, civets and rodents, the average gGAPDH sequence divergence within the [Tve-Tco] clade was only 2.3%, with a maximum divergence of 5.3% between *T. vespertilionis*-like G2 (Tve clade) and NanDoum1 (Tco clade). *Trypanosoma rangeli* was slightly more similar to *T. conorhini* and *Trypanosoma* sp. NanDoum1 (9.0% divergence) than to *Trypanosoma* sp. HochNdi1 (9.4%) and *T. vespertilionis* (10%), while it diverged by 13% from *Schizotrypanum* spp.

In addition to the clades Tra[Tve-Tco] and *Schizotrypanum*, the composition and relationships of other clades within the main *T. cruzi* clade were concordant with results from previous phylogenies (Hamilton et al., 2007, 2012; Lima et al., 2013, 2015a). However, the relationships of Tra[Tve-Tco] with two trypanosomes from megabats, *Trypanosoma* sp. bat from Gabon and *T. teixeirae* from Australia, remained unresolved. With a large gGAPDH sequence divergence (14%), these two trypanosomes grouped together with weak support, forming a long branch equally distant from the Tra (~12%) and Tco-Tve (~13%) clades. Exclusion of these two species or inclusion of only *Trypanosoma* sp. bat produced more robustly supported phylogenies (Figs. 3, 4).

Trypanosoma wauwau and widespread trypanosomes from Neotropical bats (*Trypanosoma* sp. 1, 2 and 3) (Cottontail et al., 2014) clustered together forming a clade labelled as Neobats (Figs. 2, 3) (Lima et al., 2015b), which was placed basal to the major lineage comprising Tra[Tve-Tco] and its sister *Shizotrypanum* clade. The clade Neobats was closely related to the Australian clade (Figs. 2, 3; Table 1), which included *T. noyesi* from marsupials such as woylie (G8 and BDA1), kangaroo (H25), possum (D15, D17 and D64), and koala (OTUs 41 and 140), plus one trypanosome from a rodent (BRA2) (Botero et al., 2016; Barbosa et al., 2017). One trypanosome (*Trypanosoma* sp. TVY) of lemurs from Madagascar (Larsen et al., 2016) nested in the Australian clade (Fig. 2). The phylogenetic analyses based on HSP70 sequences (Fig. 4A) or HSP70 combined with ssrRNA and gGAPDH sequences (Fig. 4B), generate trees showing topologies largely congruent with those inferred using concatenated ssrRNA and gGAPDH genes (Fig. 3), as observed in a phylogenetic study including exclusively *T. cruzi*, *T. c. marinkellei*, and *T. rangeli* from the broader *T. cruzi* clade (Fraga et al., 2016).

3.4. Multilocus phylogenetic analyses of the T. cruzi clade trypanosomes

Aiming for a more robust assessment of the phylogenetic relationships between the Tra[Tve-Tco] and *Schizotrypanum* clades, we undertook multilocus analysis using 11 single copy protein-coding genes (Fig. 5A, Supplementary Table S2) retrieved from published and unpublished genomes of the following trypanosomes: *T. rangeli* (AM80, SC58), *T. vespertilionis*-like G1, *T. conorhini* BR1, *Trypanosoma* sp. HochG3, *T. erneyi*, *T. dionisii*, *T. c. marinkellei* (B7, 344), *T. cruzi* (Esmeraldo, CL Brener, G), *T. wauwau*, *T. noyesi* H25, and *T. livingstonei*. The genes selected for multilocus analysis were first analysed independently; these individual analyses consistently recovered two lineages: Tra[Tve-Tco] and *Schizotrypanum*. Topologies clustering Tra[Tve-Tco] taxa together in a clade sister to *Schizotrypanum* – a finding highly congruent with those generated by conventional ssrRNA and gGAPDH sequences – were obtained using TcMPX, GPX and HMCOAR gene sequences. Nevertheless, in general, the topologies recovered using independent genes were only weakly supported, and the positioning of *T. livingstonei*, *T. wauwau*, and *T. noyesi* remained unresolved (Supplementary Fig. S1). Thus, we advise caution when attempting to infer phylogenies of trypanosomes using sequence data from single genes. Nonetheless, analysis including 11 single copy genes (Fig. 5A), together with sequences from gGAPDH and HSP70 genes (Fig. 5B), resulted in well resolved phylogenies, corroborating the topology generated by analysis of ssrRNA + gGAPDH genes using much larger taxon coverage (Figs. 3, 4). The multilocus phylogenetic analyses inferred in the present study are the first including many species of the *T. cruzi* clade. The inferred trees strongly supported the placement of Tra sister to [Tve-Tco] clades, and the whole Tra[Tve-Tco] lineage as sister to the *Schizotrypanum* lineage (Fig. 5A, B).

Our previously inferred multilocus phylogeny of *T. cruzi*, which included all reported DTUs, strongly supported the placement of *T. c. marinkellei* at the edge of the clade comprising all DTUs while Tcbat was closest to TcI (Lima et al., 2015b). Here, our multilocus analyses included *T. cruzi* of DTUs TcVI (CL Brener), TcII (Esmeraldo), and TcI (G), and isolates of TrB (AM80) and TrD (SC58), the two main evolutionary lineages of *T. rangeli* (Maia da Silva et al., 2007; Caballero et al., 2015). Previously, another study compared the genomes of *T. rangeli* strains assigned to TrA (Choachi strain, genome data not available) and the closely related TrD (SC58 strain, draft genome) (Stoco et al., 2014). Isolates of all Tra lineages examined using ssrRNA, gGAPDH and HSP70 sequences clustered tightly together, forming a clade exclusive of *T. rangeli* isolates (Figs. 2–4).

3.5. Trypanosoma rangeli shares highly similar SL RNA primary and secondary structures with Old World trypanosomes from bats, rats, monkeys and civets

Sequences of whole repeats of SL RNA were obtained in the present study for *T. vespertilionis* P14, *T. vespertilionis*-like G1 and G2, *Trypanosoma* sp. HochNdi1 and HochG3, *Trypanosoma* sp. NanDoum1, and *T. conorhini* BR1, BR2 and Rub1. These sequences were compared with those available for other trypanosomes of the *T. cruzi* clade previously characterised by our group (Lima et al., 2013, 2015a). The analyses of SL RNA primary (Fig. 6A, B) and secondary (Fig. 7) structures of trypanosomes in the [Tve-Tco] clade corroborated *T. rangeli* as being their closest relative. In addition, results supported *Trypanosoma* sp. bat from African megabat as being closely related to Tra[Tve-Tco] (Figs. 6A, 7). Here, the SL transcript sequence of *Trypanosoma* sp. bat, for which positioning remained uncertain in phylogenetic analyses, could be confidently aligned and shared highly similar secondary structures (Fig. 7) with SL RNA from the Tra[Tve-Tco] lineage (Fig. 6A), corroborating the close relationships between this African bat trypanosome and this lineage. Regarding *Schizotrypanum* trypanosomes, although the alignment of SL RNA sequences from the Tra[Tve-Tco] lineage was reasonable with sequences of *T. dionisii* (Fig. 6A), very large polymorphisms precluded reliable alignments of *T. cruzi* sequences (data not shown). In addition, SL RNA sequences of *T. wauwau* and *T. noyesi*, which are basal species of the major clade formed by Tra[Tve-Tco] and *Schizotrypanum* lineages, could not be reliably included in the alignment despite their secondary structure being quite comparable with those of Tra[Tve-Tco] trypanosomes (Lima et al., 2015a).

We previously employed the small SL RNA transcript sequences as markers for genotyping of *T. rangeli* (Maia da Silva et al., 2007, 2009). Here, we compared whole repeats (833–975 bp) of isolates representing all Tra lineages (Fig. 6C). In agreement with its basal phylogenetic positioning, TrB exhibited the most divergent sequences compared with the other lineages (Fig. 6A, B). Similar to *T. rangeli* of all lineages, all trypanosomes of the [Tve-Tco] clade had a copy of the 5S RNA inserted into the intergenic region (Fig. 6C). Although SL gene repeats of [Tve-Tco] varied in length from 631 to 1180 bp (Fig. 6C), they shared highly conserved transcript sequences, comprising an almost identical exon (39 nucleotides (nt)), very conserved introns (110 nt), and variable intergenic sequences containing blocks of conserved sequences unique to each species (Fig. 6A, B). Both SL transcripts and blocks of intergenic sequences of Tra[Tve-Tco] species could be aligned with high confidence (Fig. 6A, B).

Results from this study corroborated SL RNA sequences as valuable markers for the differentiation of trypanosomes, with intron sequences (Fig. 6A) and intergenic sequences (Fig. 6B) being sufficiently polymorphic to distinguish species and genotypes. However, the SL gene sequences are not suitable for broader phylogenetic analyses (Gibson et al., 2000; Lima et al., 2013, 2015a). According to available data, SL sequences evolve at very different mutation rates dependent on the species and phylogenetic lineages under consideration, being faster in *T. cruzi* and all other *Schizotrypanum* spp. compared with the apparently more slowly evolving trypanosomes of Tra[Tve-Tco], Neobats and Australian clades. Therefore, their use as evolutionary and taxonomic markers requires comparison with data obtained using conventional ssrRNA and gGAPDH genes.

4. Discussion

The finding, in the present study, of an African bat cimicid infected with *T. vespertilionis*-like is the first known molecular evidence of transmission of an African *T. cruzi* clade trypanosome by cimicid bugs. Data from this study provides relevant support for an important association of trypanosomes nested in the [Tve-Tco] clade with cimicid bugs (Cimicidae), whereas Tra and *Schizotrypanum* are linked to triatomines. Therefore, trypanosomes of both Tra[Tve-Tco] and *Schizotrypanum* lineages are all associated to haematophagous hemipterans of Cimicidae and Triatominae (Reduviidae). To date, no other vectors able to cyclically transmit these trypanosomes have been reported. An evolutionary hypothesis of the relatively younger age of triatomines (~32 Million years ago) for Triatomini, and ~27.5 Ma for Rhodniini + Cavernicolini) compared with ancient cimicids (~100 Mya) (Schuh et al., 2009; Hwang and Weirauch, 2012) is consistent with a bat trypanosome transmitted by cimicids as the last common ancestor of the major lineage comprising Tra[Tve-Tco] and *Schizotrypanum*. Most likely, bat cimicids and triatomine vectors play important roles in the evolution of these trypanosomes, and have shaped their geographical distribution, as well as their vertebrate host species ranges.

Trypanosoma cruzi of different genotypes (DTUs) colonise and undergo metacyclogenesis in the digestive tract of diverse species

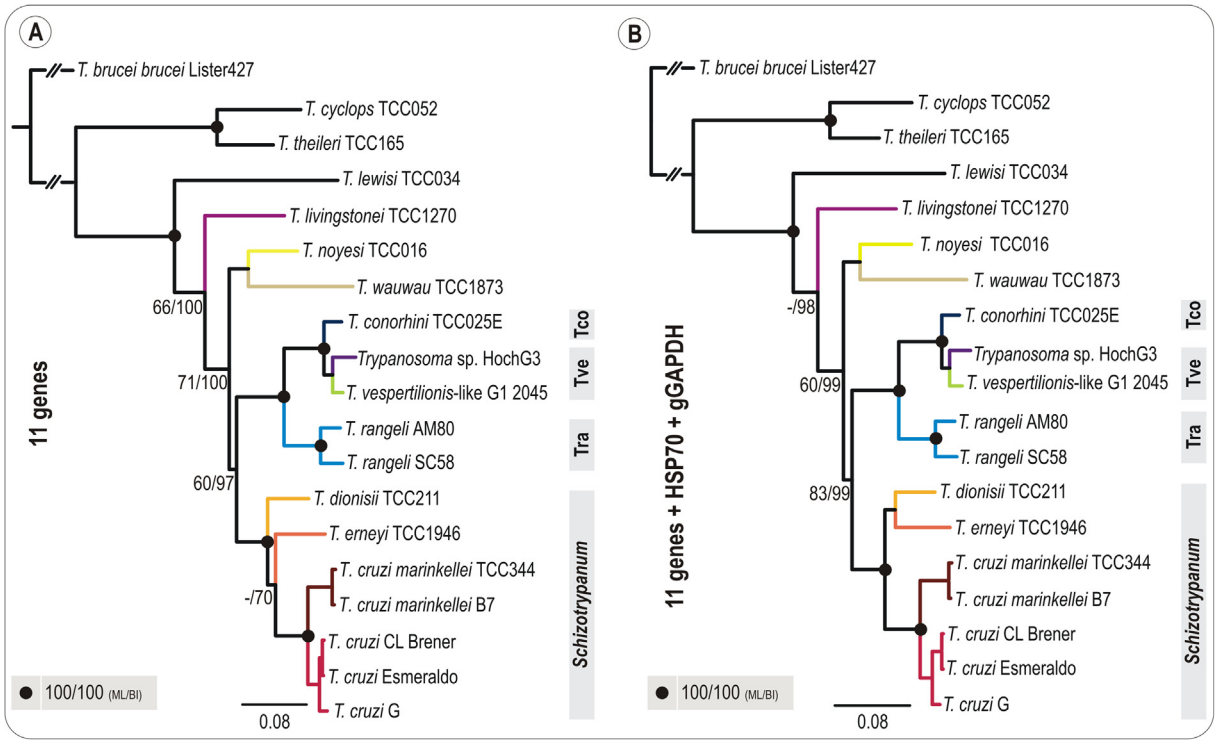

Fig. 5. Multilocus phylogenetics of Tra[Tve-Tco] and *Schizotrypanum* lineages, and *Trypanosoma wauwau*, *Trypanosoma noyesi* and *Trypanosoma livingstonei*. Maximum likelihood analyses of concatenated sequences from (A) 11 single copy protein-coding genes: GPI, Glucose-6-phosphate isomerase; GPX, Glutathione peroxidase; HMCOAR, 3-Hidroxy-3- metylglutaryl-CoA reductase; LAP, Leucine aminopeptidase; TcMPX, Mitochondrial peroxidase; PDH, Pyruvate dehydrogenase E1 component alfa subunit; RB19, RNA-binding protein-19; RHO1, Rho-like GTP binding protein; sodA, Superoxide dismutase A; sodB, Superoxide dismutase B; STTP2, Serine/threonine-protein phosphatase PP1 (4.396 characters, −Ln = 31062.587854). (B) Sequences from the 11 genes included in the analysis shown in (A) plus glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH), and Heat Shock Protein-70 (HSP70) (5.782 characters, −Ln = 37115.145486). *Trypanosoma wauwau*, *T. noyesi* and *T. livingstonei* are the basal species of the major Tra[Tve-Tco]-*Schizotrypanum* lineage. *Trypanosoma lewisi*, *Trypanosoma theileri*, *Trypanosoma cyclops* and *Trypanosoma brucei brucei* were used as outgroups of the clade *Trypanosoma cruzi*. Numbers at the nodes are maximum likelihood (500 replicates) and Bayesian inference support values, respectively.

of triatomine genera. A notable exception appears to be Tcbat, for which experimental infection failed in many triatomines from laboratory colonies, although the parasites can survive for more than two weeks, similarly to we previously observed for *T. c. marinkellei* and *T. dionisii* (Marcili et al., 2009; Cavazzana et al., 2010). However, infectivity of Tcbat to triatomines that share shelters with bats, and development of infective trypomastigotes, remains to be investigated. In contrast, metacyclogenesis in *T. rangeli* occurs exclusively in the salivary glands of particular *Rhodnius* complex species depending on the parasite lineage, whereas development restricted to the digestive tract occurs in triatomines of other genera. *Trypanosoma rangeli* is composed of two main lineages: one containing TrA, TrC, TrD and TrE, and the other formed by the phylogenetically basal lineage TrB comprising the most divergent isolates (Maia da Silva et al., 2004b, 2007, 2009; Ortiz et al., 2009; Caballero et al., 2015). Differential behaviour in *Rhodnius* spp. of TrA and TrC has been linked to the complexes *prolixus* and *pallescens*, respectively. Nevertheless, a much more tangled vector-lineage association has been suggested for TrB, which although earlier associated with the Amazonian *Rhodnius brethesi* (*pictipes* complex), is common in *Rhodnius robustus* (*prolixus* complex), many times mixed with TrA. Consistent with wide-range TrB transmission by different vectors, experimental evidence has demonstrated that isolates of TrB develop in salivary glands of both cis (Maia da Silva et al., 2004b, 2007, 2009) and trans-Andean species of *Rhodnius* (unpublished data). The lineage TrE has been associated with *Rhodnius stali* and *Rhodnius pictipes* (*pictipes*). Field isolates of TrD were so far restricted to the guts of *Panstrongylus megistus*, a widespread species of triatomine (Maia da Silva et al., 2004a,b, 2007, 2009, Ortiz et al., 2009; Vallejo et al., 2009; Urrea et al., 2011; Caballero et al., 2015; Sincero et al., 2015). Although consistently supporting three complexes, *pictipes*, *prolixus* and *pallescens*, different phylogenetic relationships have been suggested for the evolutionary history of the *Rhodniini*. Consistent with relationships among Tra lineages, one hypothesis suggests that the complex *pictipes* evolved in the Amazon-Orinoco region, and gave origin to both *prolixus* and *pallescens* complexes (Abad-Franch et al., 2009). A different relationship supports a single cis-Andean (*pictipes* + *prolixus*) lineage sister to the trans-Andean (*pallescens*) lineage (Justi et al., 2014, 2016). This alternative phylogeny agrees with all lineages of *T. rangeli* developing in *cis*-Andean *Rhodnius* spp. except TrC, for which salivary gland invasion appears to be restricted to trans-Andean *Rhodnius* spp. (Vallejo et al., 2009). *Trypanosoma rangeli* of different lineages infect mammals of diverse orders. Humans have been reported to be infected with TrA, TrB and TrC, monkeys with TrA and TrB, and bats with TrA, TrB, TrC, TrD and TrE. The basal TrB lineage has been found in animals of many orders, from the Amazon to the Atlantic Forests (Maia da Silva et al., 2004a,b, 2007, 2009; Ortiz et al., 2009; Pinto et al., 2012, 2015; Sincero et al., 2015; Dario et al., 2017).

Molecular data obtained in the present study demonstrated, to our knowledge for the first time, the presence of *T. vespertilionis* in African bats and bat cimicid. African *T. vespertilionis*-like G1 (from bats) and G2 (from bat cimicids) are closely related to European *T. vespertilionis* from bats (Figs. 1–3, Table 1). Molecular surveys in bats from Brazil, Bolivia, Colombia, Panama and Ecuador did not reveal any trypanosome closely related to *T. vespertilionis*. Thus, "*T.* (*Schizotrypanum*) *vespertilionis*" reported in Neotropical bats may correspond to *T. dionisii*, a species highly prevalent in recent surveys, but unknown in the Neotropics before the advent of

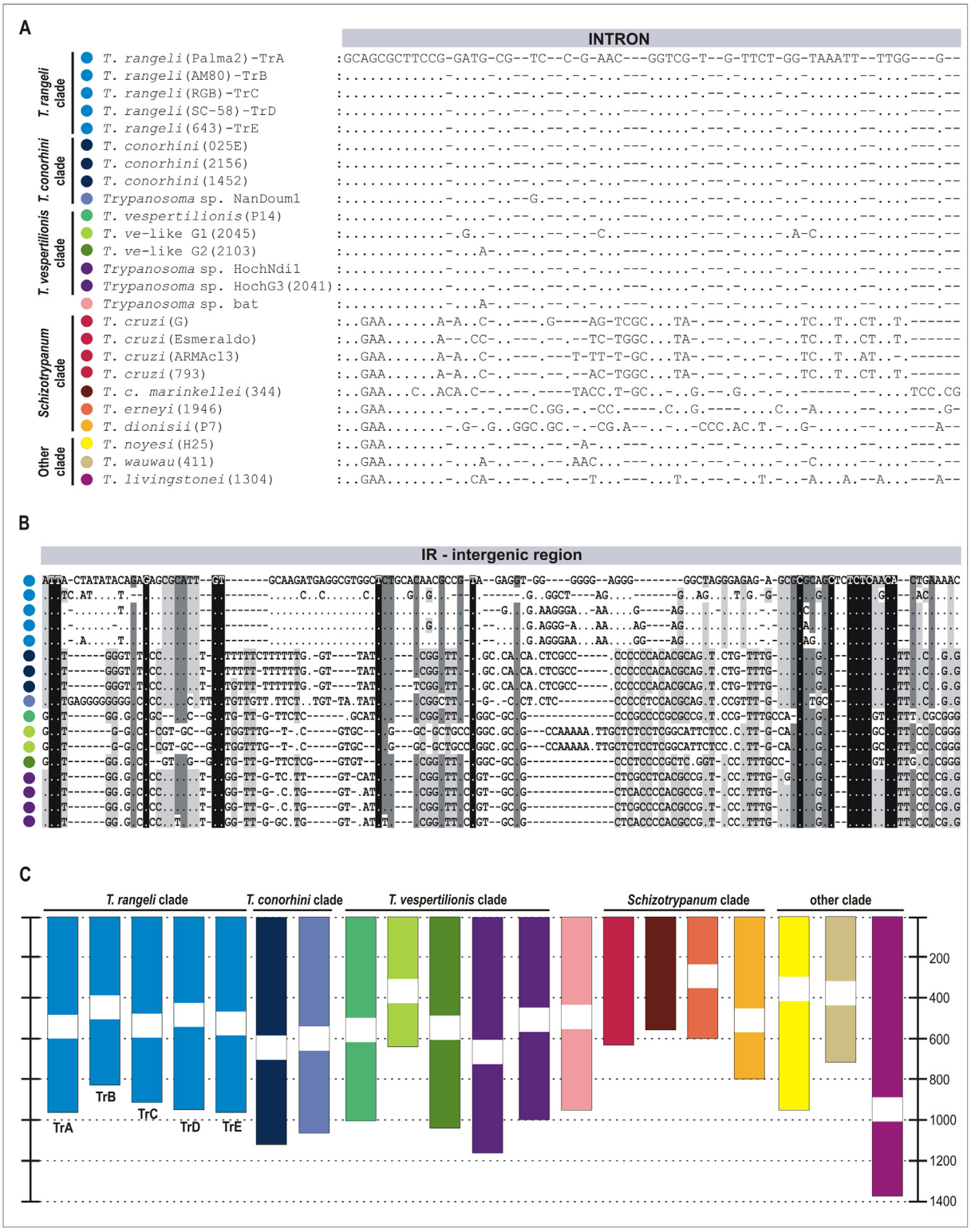

Fig. 6. Length and sequence polymorphisms of Spliced Leader RNA sequences of trypanosome species of the Tra[Tve-Tco]. (A) Alignment of Spliced Leader intron sequences from isolates selected to represent the genetic diversity of the species nested into the Tra, Tve and Tco clades, the closely related *Trypanosoma* sp. bat, species of the *Schizotrypanum* clade, and *Trypanosoma noyesi*, *Trypanosoma wauwau* and *Trypanosoma livingstonei* basal species. (B) Conserved block of the intergenic region from species of Tra[Tve-Tco] lineage selected to illustrate their very close relationships. (C) Intra-specific and intra-lineage length polymorphisms of Spliced Leader repeat units of Tra[Tve-Tco]-*Schizotrypaum* major lineage and its basal species. Each species is represented by dots and bars of the specific colour shown in (A).

molecular surveys (Molyneux, 1991; Cavazzana et al., 2010; Pinto et al., 2012; Cottontail et al., 2014; Ramírez et al., 2014a,b; Dario et al., 2017). Our findings suggest that *T. vespertilionis* may have dispersed in bats through the Mediterranean; however, whether its ancestral species originated in Africa or Europe is unclear at this time.

Data from this and previous studies demonstrated that *T. vespertilionis*, apparently found only in the Old World, is

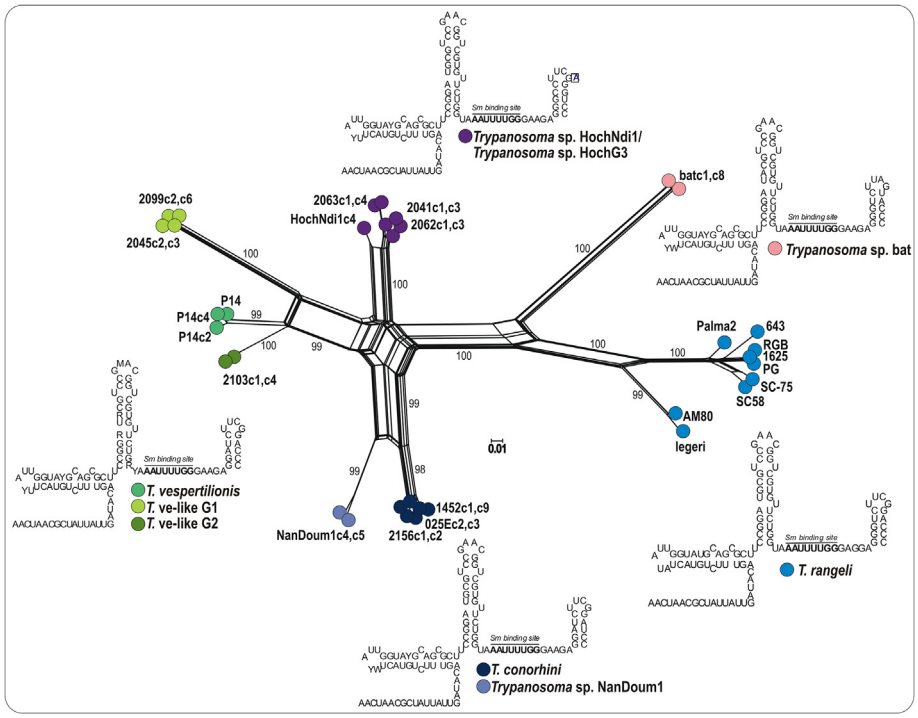

Fig. 7. Network and predicted secondary structure of Spliced Leader RNA sequences. Highly similar primary and secondary structures of Spliced Leader RNA transcripts (~200 bp) are shared by trypanosomes of the Tra[Tve-Tco] lineage, and their closely related *Trypanosoma* sp. bat from African megabat. The network included sequences from different isolates/clones of each species. Numbers at nodes correspond to bootstrap values estimated with 500 replicates using the same parameters optimised for network inference.

transmitted by cimicids (Gardner and Molyneux, 1988; Molyneux, 1991). Different genera and species of cimicids are associated with bats across the Old World while in the New World bat cimicids occur mainly in temperate zones. Cimicids are common temporary ectoparasites of bats (and birds), surviving off-host in the nest between blood meals. Most species are host-specific, but a few species (*Cimex lectularius*, *Cimex hemipterus*, and *Leptocimex boueti*) feed on a range of hosts, although populations of *C. lectularius* feeding on humans and bats have undergone genetic differentiation (Balvín et al., 2014; Booth et al., 2015). The invasion of the New World by cimicids appears to have occurred via both the Bering Land Bridge and overwater dispersal (Schuh et al., 2009), their spread likely being facilitated by highly mobile winged hosts. To our knowledge, *T. vespertilionis*-like G2 found in this study in the bat bug *Cacodmus* sp. is the first trypanosome isolated in culture and characterised by molecular methods from a bat cimicid, and the first report of a trypanosome in a cimicid species of the Cacodminae, a family exclusive to the Old World.

The newly characterised *Trypanosoma* sp. HochG3 from GW bats is very closely related to *Trypanosoma* sp. HochNdi1 of one monkey (*Cercopithecus nictitans*) from Cameroon, both being genotypes of the first African trypanosome species placed in the *T. cruzi* clade parasitizing both bats and primates (Figs. 2, 3; Table 1). *Cercopithecus* spp. from Cameroon, Tanzania and Congo were previously reported as being infected with *Trypanosoma primatum* (Hoare, 1972), a species not available for molecular studies. Vectors of *T. primatum* and *Trypanosoma* sp. HochNdi1, which may correspond to a single species, are unknown. Interestingly, bats share palms used as nests by monkeys (*Cercopithecus* spp. and chimpanzees) in the area where the bats harbouring *Trypanosoma* sp. HochG3 were captured in GW; thus, several haematophagous arthropods may feed on both bats and monkeys. In addition, predation of bats by monkeys (including *Cercopithecus* spp.) occurs throughout the Afrotropical and Neotropical regions (Boinski and Timm, 1985; Tapanes et al., 2016), favouring trypanosome host-switching by oral infection.

Results from the present study provide the first molecular evidence that isolates of *T. conorhini* from rats and *Tr. rubrofasciata*, from distant locations such as Brazil and Hawaii, are indeed closely related. These findings strongly supported the tight association of *T. conorhini* with rats, and *Tr. rubrofasciata* are critical factors in their joint dispersal throughout the world (Dujardin et al., 2015). Because wild foci of *Tr. rubrofasciata* that may represent its original populations have never been reported, longstanding hypotheses support either an Asian or Neotropical origin for this species. Phylogenetic studies of triatomines support a New World origin for *Tr. rubrofasciata* (Patterson et al., 2001; Hypsa et al., 2002). In a broad phylogeny of reduviids, *Tr. rubrofasciata* was placed basally in the single Asiatic clade of triatomines. The time of arrival of triatomines in the Old World was estimated at between 25 and 10 ma, with probable dispersal via the Bering Land Bridge (Hwang and Weirauch, 2012; Justi et al., 2016).

The origin of *T. conorhini* remains far from clear, but its closer relationships with both the African *Trypanosoma* sp. NanDoum1 from a civet and *T. vespertilionis* (Europe and Africa) than with any Neotropical trypanosome examined to date is more compatible with an Old World origin and its dispersal carried by *Rattus/Tr. rubrofasciata.* Consistent with this hypothesis, *Tr. rubrofasciata* has never been reported infected with *T. cruzi* in the Old World, and in the Neotropics, *T. conorhini* has been reported in rats and *Tr. rubrofasciata* captured near ports (Hoare, 1972; Weinman, 1977). Due to the presence of trypanosomes resembling *T. conorhini* in monkeys and *Tr. rubrofasciata* from Malaysia, The Philippines and Indonesia (Weinman, 1977), it was previously hypothesised that original hosts of *T. conorhini* were Asian monkeys (Deane et al., 1986). This hypothesis was reinforced by the demonstration that *T. conorhini* can experimentally infect monkeys of both the New and Old Worlds (Deane et al., 1986). Our study did

not confirm the existence of *T. conorhini* in Asian monkeys, and to our knowledge this trypanosome has not yet been confirmed by molecular analysis in *Tr. rubrofasciata* or *Rattus* spp. captured in Asia. Molecular surveys of trypanosomes in Asian mammals, as well as in rats and *Tr. rubrofasciata* across their wide geographic distributions, will be valuable to clarify this history.

The vector of *Trypanosoma* sp. NanDoum1 from African civet is unknown. Interestingly, another trypanosome of African carnivores, *Trypanosoma helogalei* from mongoose, shares morphological features of culture and blood forms with *T. conorhini*, as well as infectivity to rats and mice, and the ability to experimentally develop in *C. lecturalis* and *R. prolixus* (Hoare, 1972). In addition to natural/experimental infection of cimicids with *T. vespertilionis*, *T. vespertilionis*-like, *T. dionisii*, *T. hedricki* and *T. helogalei* (Hoare, 1972; Bower and Woo, 1981; Gardner and Molyneux, 1988; Molyneux, 1991), other species including *T. rangeli*, *T. conorhini* and *T. cruzi* have been shown experimentally to develop in *C. lecturalius* (revised in Hoare, 1972; Salazar et al., 2015). Similarly, the vectors of the African *T. erneyi* and *T. livingstonei*, and the Neotropical *T. wauwau* and *Trypanosoma* spp. of the clade Neobats are unknown. However, these trypanosomes appear to be unable to develop in triatomines (inoculated with culture forms) in laboratory conditions, although neither triatomines nor cimicids that usually feed on bats were investigated (Cavazzana et al., 2010; Lima et al., 2012, 2013, 2015a). Nevertheless, while the African trypanosomes can be transmitted by bat cimicids, the high prevalence and wide distribution of bats infected with *T. cruzi* clade trypanosomes in South America strongly suggest the existence of additional vectors other than triatomines and cimicids. In addition to the paucity of bat cimicids, the species repertoires of triatomines are very different in the distant regions, ecosystems and ecological niches where bats have been found infected with *T. dionisii*, *T. c. marinkellei*, and *Trypanosoma* spp. of the Neobats clade (Cavazzana et al., 2010; Pinto et al., 2015; Ramírez et al., 2014b; Lima et al., 2015a; Hodo et al., 2016; Dario et al., 2017). Although the vectors of the Australian *T. noyesi* are also unknown, molecular surveys have suggested that tabanid flies and biting midges can be its vectors (Botero et al., 2016). A large diversity of haematophagous hemipterans (triatomines and cimicids) and dipterans (including cave-dwelling sand flies and culicids) in addition to a range of ectoparasites (flies, ticks, mites, and fleas) feed on bats (Obame-Nkoghe et al., 2017), and all these arthropods should allow for both cyclical and mechanical trypanosome transmission. The gregarious behaviour and cooperative relationships of bats favour host switching of ectoparasites. Additionally, grooming (with the ingestion of ectoparasites that may carry trypanosomes) and regurgitated blood sharing behaviour (Carter and Leffer, 2015), may all facilitate mechanical transmission of bat trypanosomes.

The characterisation of nine new African trypanosomes from bats and bat cimicids by multilocus phylogenetic analysis has provided additional support for the bat seeding hypothesis (Hamilton et al., 2012). The evolutionary scenarios hypothesised in this study reaffirm that the majority of bat trypanosomes identified to date throughout the world cluster in the *T. cruzi* clade. *Trypanosoma evansi*, a generalist pathogen of mammals that sporadically infect humans (Truc et al., 2013), and *Trypanosoma vegrandis*, found infecting bats and a range of marsupials in Australia (Austen et al., 2015), did not cluster in the *T. cruzi* clade. Our phylogenetic analyses greatly increased the resolution of the relationships within the [Tve-Tco] clade, and strongly supported the clade Tra sister to the [Tve-Tco] clade forming a monophyletic lineage sister to the *Schizotrypanum* lineage. In a plausible (most parsimonious) evolutionary scenario, the trypanosomes of the Tra[Tve-Tco] lineage evolved from within a lineage of bat-trypanosomes. Most probably, an Old World ancestral bat trypanosome transmitted by cimicids diversified and dispersed in bats, giving origin to deeply rooted phylogenetic lineages of trypanosomes, including those that evolved by successive host switching to give origin to *T. rangeli* and *T. cruzi* in the Neotropics. The fact that all these trypanosomes develop (naturally and or experimentally) in cimicids is consistent with a common ancestor transmitted by bat cimicids. In addition to contaminative transmission by trypomastigotes present in the faeces of cimicid vectors, oral transmission by eating insects or by carnivory may also have played important roles in the evolution of the Tra[Tve-Tco] trypanosomes. It is unknown whether *T. vespertilionis*, *T. conorhini* and their allied trypanosomes able to infect Old World monkeys can also infect humans.

Our multilocus phylogenetic analysis provides relevant additional support for the hypothesis that the deepest split separating the most basal species of the *T. cruzi* clade from all other trypanosomes, at present represented by *T. livingstonei* from Africa, is compatible with an ancient Old World origin of this clade (Lima et al., 2013, 2015a; Botero et al., 2016). An alternative hypothesis, with a common ancestor of the *T. cruzi* clade being a New World trypanosome, has gained support following the discovery of an increasing diversity of Neotropical bat trypanosomes closely related to trypanosomes from marsupials and rodents, all placed at a basal position of the assemblage formed by the lineages Tra[Tve-Tco] and *Schizotrypanum* (Lima et al., 2013, 2015a; Pinto et al., 2015; Botero et al., 2016; Barbosa et al., 2017; Dario et al., 2017).

Corroborating the previous bat-seeding hypothesis (Hamilton et al., 2012), multilocus phylogenetic analyses performed in the present study strongly support a bat-restricted trypanosome as the last ancestor of the *Schizotrypanum* lineage in which all currently recognised species are restricted to bats, except the generalist *T. cruzi*. It is likely that a bat trypanosome adapted to triatomines gave origin to two lineages, one that continued evolving in bats represented by *T. c. marinkellei*, and another that gave origin to all *T. cruzi* DTUs. Accordingly, *T. cruzi* appears to have arisen recently from a bat trypanosome and diversified, giving origin to many infraspecific genotypes including Tcbat. The ancestor of all *T. cruzi* DTUs adapted to different mammalian hosts and triatomine species, evolving by successive host-switching to become a generalist parasite. In addition to Tcbat, bats have been found infected by almost all DTUs (Marcili et al., 2009; Lima et al., 2015b). The relationships among the genotypes of *T. cruzi* do not support Tcbat, which is closest to TcI, as the common ancestor of all *T. cruzi* DTUs as previously hypothesised (Ramírez et al., 2014a). Interestingly, *T. erneyi* from African bats appears to be more closely related to *T. cruzi* than to the cosmopolitan *T. dionisii*, which is currently the most widespread bat trypanosome, likely the basal species of the whole *Schizotrypanum* clade (Lima et al., 2012, 2013, 2015a; Hamilton et al., 2012).

Despite recent findings of bats infected with different lineages of *T. rangeli*, available data do not permit one reliable hypothesis about the origin (New or Old World) and the vertebrate species that harboured the last common ancestor of the *T. rangeli* lineage. The answer to these questions may rely on trypanosomes of non-bat hosts such as marsupials, xenarthrans, non-human primates and rodents, all natural hosts of *T. rangeli*, and known to have played important roles in the evolution of *T. cruzi* clade trypanosomes in the Neotropics. Recent studies have suggested a link between Neotropical and Australian trypanosomes from bats and marsupials, supporting an evolutionary scenario whereby a *T. cruzi* clade lineage (of unknown geographic and host origin) dispersed through a southern supercontinent route (Lima et al., 2015a; Barbosa et al., 2016; Botero et al., 2016).

Our findings strongly support the hypothesis that divergent bat trypanosomes of long-established lineages adapted independently and at different times to different niches of triatomines and mammals. In this scenario, successive jumping of bat trypanosomes into

new hosts has occurred repeatedly, and the different behaviours in both mammalian and insect hosts may have led to separate *T. cruzi* and *T. rangeli* evolutionary lineages. *Trypanosoma cruzi* and *T. rangeli* differ greatly in life cycles either in vertebrate and insect hosts as well as in host infection and immune evasion strategies. A long, independent evolutionary history of *T. rangeli*, more related to Old World trypanosomes of the [Tve-Tco] lineage than to *Schizotrypanum* spp. is consistent with the marked differences in transmission routes and host-parasite interactions, prompting human infection with (*T. cruzi*) or without pathogenicity (*T. rangeli*).

At this time, little or nothing is known about the mechanisms by which bat trypanosomes can cross host species barriers and emerge as human parasites. In the hypothesised evolutionary scenario, bat trypanosomes of very different genetic backgrounds lacked host specificity, and successively adapted to a range of mammalian hosts.

To our knowledge, *T. cruzi*, *T. rangeli*, *T. conorhini* and *Trypanosoma* sp. Hoch (Ndi1 and G3 isolates) are the only species of the *T. cruzi* clade adapted to primates, and despite the fact that all these species are able to infect Old Word monkeys, only *T. cruzi* and *T. rangeli* were found infecting humans. The ability of these distantly related trypanosomes to infect Old Word primates suggests that other species of the *T. cruzi* clade, besides *T. cruzi* and *T. rangeli*, may adapt to a range of hosts including humans. Bat-borne parasites (mainly viruses) are well known for their facility to jump to many unrelated host species. Spillover of viruses from bats to intermediate hosts such as civets and non-human primates are thought to be the most likely route to their emergence as human pathogens, although direct spillover from bats to humans can also occur (Han et al., 2015). Infections with *T. lewisi*, a rat-borne zoonosis transmitted by fleas, have been diagnosed in children, immune-depressed adults, and non-human-primates, and this species is currently considered an emergent human pathogen. This trypanosome is commonly transmitted from Old World rats to native rodents (Ortiz et al., 2017), and belongs to the subgenus *Herpetosoma*, a clade of flea-transmitted trypanosomes (Maia da Silva et al., 2010; Truc et al., 2013). In addition, children harbouring *T. cruzi* Tcbat and *T. dionisii* were recently reported by PCR blood screening (Ramírez et al., 2014a; Dario et al., 2016). However, active (even transient) infection in humans with these and other trypanosomes tightly linked to bats still needs to be confirmed, as indubitably demonstrated for human infections caused by *T. lewisi* and *T. evans*i (Truc et al., 2013).

In conclusion, taxon-rich and multilocus phylogenetic analysis of *T. cruzi* clade trypanosomes reconstructed in the present study, together with biogeographical data about mammalian hosts and vectors, allowed us to hypothesise one plausible evolutionary scenario for *T. rangeli*. All analyses suggested a long-established divergence, at different times, from Old World trypanosomes giving origin to Tra[Tve-Tco] and *Schizotrypanum* lineages. In addition, we discussed how the deep rooting of these two main lineages relates to their independent evolution over a time horizon that begins well before the dispersal from the Old World of the ancestors of both *T. cruzi* and *T. rangeli*. The knowledge of deep-rooted lineages is particularly informative in the reconstruction of any evolutionary scenario. However, this study focused only on the Tra[Tve-Tco] and *Schizotrypanum* lineages, and the deepest branches in the whole *T. cruzi* clade still remain not very well resolved. Our analyses showed that the placement of the basal clades depended on the genes and especially on the taxa employed for phylogenetic inferences. Therefore, the phylogenetic relationships between lineages could be reordered, including the placement of the root, with the inclusion of additional taxa.

The evolutionary history of the whole *T. cruzi* clade is still very fragmented. Available data are concentrated on bat trypanosomes from the Neotropics, with very few data from African, European and Australian bats. Here, we provide stronger support for a close relationship between Neotropical, African and European trypanosomes of bats and other hosts. Trypanosomes of marsupials, rodents, non-human primates and carnivores play a very relevant role in the evolution of *T. cruzi* and *T. rangeli*. Broader studies on trypanosomes of mammals in general from the New and Old Worlds using multilocus approaches are necessary to assess the wide spectrum of trypanosome genetic diversity, to resolve both the deepest branches (basal lineages) and the relationships among the species and lineages, and to trace back possible dispersion routes. The joint analyses of all these data are crucial to hypothesise on better supported evolutionary scenarios for the whole *T. cruzi* clade.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpara.2017.12.008.

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