Trypanosoma rangeli is phylogenetically closer to Old World trypanosomes than to *Trypanosoma cruzi:* independent adaptation to different niches of mammals, even humans, and triatomine vectors

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

Note: Supplementary data associated with this article

Abstract

Trypanosoma rangeli and *T. cruzi* are generalist trypanosomes sharing a wide range of mammal 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. We explored these issues by comparing 20 trypanosome species from bats and terrestrial mammals, including new trypanosomes from bats and a bat-cimicid from Guinea Bissau, West Africa. In addition to the taxon-rich analyses using SSU rRNA and gGAPDH, HSP70 and SL RNA sequences, and multilocus phylogenetic using 11 single copy genes from x selected trypanosomes strongly supported two main sister lineages: *Schizotrypanum,* comprising *T. cruzi* and bat-restricted trypanosomes, and Tra[Tve-Tco], formed by *T. rangeli* (Tra), *T. vespertilionis* (Tve), and *T. conorhini* (Tco) lineages. Tve comprises European *T. vespertilionis* and African *T. vespertilionis*-like of bats and a bat-cimicid, and the *Trypanosoma* sp. Hoch of monkeys 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 greatly divergent from those of *Schizotrypanum*. We postulate the following evolutionary scenario: a bat trypanosome transmitted by cimicids gave origin to Tra[Tve-Tco] and *Schizotrypanum* lineages, and bat trypanosomes of diverse genetic backgrounds from these two deeply rooted lineages jumped to new hosts. Likely, *T. cruzi* diverged recently from a bat-restricted trypanosome, but the ancestors of *T. rangeli* remain unknown and may well be trypanosomes of hosts other than bats. The adaptation – independently and at different times – of trypanosomes lacking a recent common ancestor (long-established lineages) to different niches of shared mammals and vectors is consistent with marked differences in life-cycles and host-parasite interactions, resulting in *T. cruzi* (but not *T. rangeli*) being pathogenic to humans.

Keywords*:* bat; monkey; cimicid; DNA barcoding; multilocus phylogeny; evolution.

1. Introduction

Trypanosoma rangeli and *T. cruzi* are two-generalist trypanosomes of bats and all orders of terrestrial mammals, and are the only species infective to humans in the New World. *T. cruzi,* the agent of Chagas disease, occurs from 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. *T. rangeli* is prevalent from Central America to Amazonia, where *Rhodnius* spp. are highly abundant in palm trees, but it is also reported in different Brazilian biomes such as the Pantanal, Cerrado, and the Atlantic Forest. *T. rangeli* is a common parasite of xenarthrans, marsupials, rodents, carnivores and primates, and recent studies have identified a relevant prevalence of *T. rangeli* in chiropterans (Hoare, 1972; Maia da Silva et al., 2007, 2009; Vallejo et al., 2009; Dario et al., 2017a,b).

Mixed infections with *T. cruzi* and *T. rangeli* are common in triatomines and mammal hosts, including humans (Hoare, 1972; Maia da Silva et al., 2004,b, 2007, 2009; Ramirez et al., 2014b; Pinto et al., 2015; Dario et al., 2017). However, despite shared mammal hosts and vectors across Central and South America, life cycles of *T. cruzi* and *T. rangeli* differ significantly in both vertebrate and invertebrate hosts. *T. rangeli* is not pathogenic to its mammal 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 related to African trypanosomes transmitted by tsetse flies of the Salivaria section, and the subgenus *Tejeraia* was proposed to accommodate this species in this section (see Hoare, 1972; Maia da Silva et al., 2004a; Valejjo et al., 2009). However, based on molecular phylogeny, Stevens et al. (1999) suggested *T. rangeli* to be closely related to *T. cruzi*, thus distant from any species of the Salivaria Section. In more comprehensive phylogenies, *T. rangeli* and *T. cruzi* clustered with trypanosomes from diverse mammal 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 dionisi* of the New and Old Worlds, and *Trypanosoma erneyi* of African bats (Hamilton et al., 2007, 2012; Lima et al., 2012a, 2015a; Pinto et al., 2015). This 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. *T. 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 (Hemiptera: Reduviidae: Triatominae); its development is restricted to the digestive tract of insect vectors. All species of *Schizotrypanum* exhibit intracellular multiplication as amastigote forms, a trait unique to the subgenus (Molyneux, 1991; Cavazzana et al., 2010; Lima et al., 2012a). *T. cruzi* is a genetic complex, comprising at least six discrete taxonomic units (DTUs, TcI-TcVI), plus the Tcbat genotype tightly linked to bats. Bats are hosts of almost all DTUs (Marcili et al., 2009; Pinto et al., 2012; Ramirez et al., 2014a; Lima et al., 2015b; Dario et al., 2016, 2017). 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 the bat trypanosomes *T. dionisii* and *T. vespertilionis* in the Old World, and *T. hedricki* in North America; these ectoparasites are common in bat shelters (Molyneux, 1991; Bower and Woo 1981; Gardner and Molyneux 1988). 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 can transmit (cyclically or mechanically) these trypanosomes (Cavazzana et al., 2010; Lima et al., 2015a,b).

Trypanosoma vespertilionis appears to be bat-restricted 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. *T. 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). *T. conorhini* and *Tr. rubrofasciata* likely dispersed together with domestic rats carried by ships (Patterson et al., 2001; Hypsa et al, 2002; Dujardin et al., 2015). The presence of *T. conorhini* in field-collected monkeys and *Tr. rubrofasciata* have 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., 2004 a, b, 2007, 2009; Ortiz et al., 2009; Caballero et al., 2013). 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 SSU rRNA and gGAPDH have left uncertain the relationships of *T. rangeli* with *T. cruzi, T. conorhini,* European *T. vespertilionis* P14 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 *T. teixeirae* of Australian bats have challenged earlier hypotheses about the origin of this Neotropical trypanosome, hosts harbouring its recent ancestor, and the habitually assumed close relationships with *T. cruzi*. The addition of more taxa into the weakly supported lineage comprising *T. rangeli* appears critical to clarify these relationships. With this aim, in this study we characterised 8 new African (GW) trypanosomes from bats and one from a bat-cimicid related to *T. vespertilionis*, plus trypanosomes morphologically resembling *T. conorhini* isolates from *Rattus, 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 also with *T. cruzi* and other species of the subgenus *Schizotrypanum*, we performed taxon-rich phylogenetic analyses using SSU rRNA, gGAPDH and HSP70 sequences. Thereafter, trypanosomes from bats and terrestrial mammals representative of the genetic diversity of whole data set, transmitted by

triatomines and cimicids, from the New and Old Worlds, were selected for SL RNA and multilocus phylogenetic analyses.

2. Materials and Methods

2.1. Studied 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 DNA isolated from blood samples from monkeys and civets captured in Cameroon, Central Africa (Fig. 1) (Njiokou et al., 2004, 2006), that have previously been shown to be infected with the HochNdi1 and NanDoum1 trypanosomes (Hamilton et al., 2009). All trypanosomes included in the phylogenetic analyses, and their respective hosts and geographical origins are detailed in Supplementary Table 1. Bats were identified by morphological keys, and as previously specimens of each putative species of African bat were confirmed by cytochrome c oxidase subunit I (COI) 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 – Animal handling was performed in strict accordance with good animal practice and according to protocols of the institutions involved in this work. 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 (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., 2012a, 2013). Blood samples (~ 200 ul) 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℃. Five cimicid bugs taken from bats were dissected, examined microscopically for trypanosomes, and positive guts were inoculated into culture tubes as

described for haemocultures. Cultures were cryopreserved in the Trypanosomatid Culture Collection (TCC) of the University of São Paulo (Supplementary Table 1). 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., 2012a, 2013).

2.3. Trypanosome cultures and PCR-amplifications

Cultures of trypanosomes were grown in TC100 medium as described above, and DNA was extracted using the phenol-chloroform method. DNA samples were used for PCR amplification of the variable V7-V8 region of SSU rRNA (~ 800bp) 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 SSU rRNA 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 (TGATGCAGCTGGTGTCGGACTT) and HSP70R (CTGGTACATCTTCGTCATGATG). PCR reactions were performed using 100 ng of each primer, 200 µM of each dNTP, 1.5 mM of MgCl2, 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 1.

2.4. Phylogenetic analyses of SSU rRNA, gGAPDH and HSP70 gene sequences

To infer broad phylogenies based on conventional SSU rRNA and gGAPDH genes of a large set of samples from the *T. cruzi* clade, the following alignments of DNA sequences were created: a) V7-V8 SSU rRNA 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 *T.* sp TVY from the blood of lemurs (*Indri indri*) captured in Madagascar (Larsen et al., 2016) were included in the analysis; b) gGAPDH sequences of xxx new samples (from bats, rats, monkeys, and cimicid and triatomine bugs) aligned with xxx published *T. cruzi* clade

sequences (Supplementary Table S1); c) concatenated sequences of V7-V8 SSU rRNA and gGAPDH genes from trypanosomes of the *T. cruzi* clade, using *T. lewisi* and *T. microti* as outgroup taxa; d) HSP70 sequences from trypanosomes of the *T. cruzi* clade in isolation, or combined with V7-V8 SSU rRNA and gGAPDH sequences. All sequences determined in this study were submitted to GenBank (Accession numbers given in Supplementary Table 1).

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% 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 & Machado, 2011; Lima et al., 2015b) were performed using BLAST against genomes freely available in TriTrypDB and/or NCBI databanks of *T. cruzi* (Esmeraldo and CL Brener), *T. c. marinkellei* B7, *T. rangeli* SC58 and *T. b. 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 *T.* sp HochG3 (Table 1). The selected genes were also retrieved from draft genomes of *T. lewisi*, *T. theileri, T. cyclops* and *T. b. brucei,* and all these species were used as outgroup taxa of the *T. cruzi* clade. The 11 genes selected for this study were: GPI (Glucose-6-phosphate isomerase), GPX (Glutathione peroxidase), HMCOAR (3-Hidroxy-3-

187 metylglutaryl-CoA reductase), LAP (Leucine aminopeptidase), TcMPX (Mitochondrial peroxidase), PDH
188 (Pyruvate dehydrogenase E1 component alfa subunit), RB19 (RNA-binding protein-19), RHO1 (Rho-like GTP
189 binding protein), sodA (Superoxide dismutase A), sodB (Superoxide dismutase B) and STTP2
190 (Serine/threonine-protein phosphatase PP1) (Flores-López & Machado, 2011). Phylogenetic inferences were
191 performed using individual and combined genes as above. Access to the unpublished draft genomes analysed
192 in this paper can be obtained by contacting the corresponding author.
193
194 ***2.6. Spliced leader (SL) RNA sequences: amplification, sequencing, and data analysis.***
195 Entire SL RNA gene repeats were amplified and sequenced at both strands of 3-5 clones of each
196 trypanosome, and the secondary structures of SL transcripts were obtained using the RNAdraw program with
197 default settings (Maia da Silva et al., 2007; Lima et al., 2013, 2015a). The alignment of exon and intron
198 sequences was manually refined. Network analysis of SL RNA genes was inferred by SplitsTree v4.11.3 using
199 the neighbour-net method (Huson & Bryant, 2006). Internode supports were estimated by performing 100
200 bootstrap replicates using the same parameters optimized for network inferences.
201
202 **3. Results**
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204 ***3.1. African trypanosomes related to T. vespertilionis selected by V7-V8 SSU rRNA barcoding***
205 New trypanosomes were selected for this study by V7-V8 SSU rRNA barcoding of trypanosomes from
206 bats captured in Guinea Bissau (GW) in West Africa, and compared with bat trypanosomes from Mozambique
207 in East Africa, and Brazil, Colombia and Venezuela in South America (Fig.1). Eight new cultures of
208 trypanosomes obtained from bats (*Scotophilus* sp.), captured in GW, were characterized for the first time in
209 this study, as well as one culture obtained from the gut of a bat-cimicid (*Cacodmus* sp.) taken from one GW
210 bat (*Pipistrellus* sp.) (Table 1).
211 Phylogenetic analysis based on xxx sequences of V7-V8 SSU rRNA, including xx determined in the
212 present study (Supplemenary Table S1) revealed that the trypanosomes from GW bats are closely related –
213 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 (Hamilton et al., 2009), and due to the relevant sequence divergence separating this clade of trypanosomes from all species of the lineage [Tve-Tco], they represent a new trypanosome species that are hereafter referred as *Trypanosoma* sp. HochG3 (Table 1, Fig. 2). PCR-screening of GW bat blood samples revealed *T. vespertilionis*-like G1, *T*. 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 divergences separating *T. vespertilionis*-like G2 and *T.* sp. HochG3 from their closest relatives, formal description of these trypanosomes as new species will be done using a combined taxonomic approach based on multiloccus phylogenetic data, 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 *T.* sp. HochG3 with bat and cimicid species. Similarly to *T. rangeli* and *T. conorhini,* the newly characterized trypanosomes did not develop intracellularly (demonstrated by *in vitro* cultures), thereby more similar to *T. rangeli* and differing from *Schizotrypanum* species from the New and Old Worlds, all developing within mammalian cells (Molyneux, 1991; Cavazzana et al., 2010; Lima et al., 2012a).

The phylogenetic analysis presented in this study using V7-V8 SSU rRNA sequences is the most taxon-rich analysis of the *T. cruzi* clade to date, including sequences from 71 isolates from 20 different trypanosome species of several mammal 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 SSU rRNA analysis, trypanosomes from blood samples of lemurs from Madagascar (Larsen et al., 2016) were classified in the Australian clade, which is closely related to the Neobats clade formed by a diversity of trypanosomes so far

exclusively from Neotropical bats (Cottontail et al., 2014; Pinto et al., 2015; Lima et al., 2015a) (Fig. 2). The lineage composition within the *T. cruzi* clade revealed by the V7-V8 SSU rRNA phylogram (Fig. 2) was concordant with results from previous phylogenies (Lima et al., 2013, 2015a), and with phylogenetic relationships inferred in this study using other genes (Figs 3-4).

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

We compared one reference-isolate of *T. conorhini* (BR1) from *R. 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 (Table 1). These isolates of *T. conorhini* showed very similar but not identical SSU rRNA barcodes, and were separated by 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* and *Tr. rubrofasciata*.

Here we analyzed 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 Southeast Asia, the only place outside Latin America where triatomines occur (Patterson et al., 2001; Hypsa et al., 2002).

3.3. Phylogenies based on SSU rRNA, 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* 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., 2012a, 2013, 2015a). Here, the inclusion of 8 new trypanosomes from African (GW) bats and one from a bat-cimicid plus two additional isolates of *T.*

conorhini in combined (SSU rRNA, gGAPDH and HSP70 genes) phylogenetic analyses strongly supported a monophyletic assemblage comprising the lineage Tra sister to the clade clustering Tve and Tco lineages.

Here, our analyses allowed for better resolution of phylogenetic relationships among species within each lineages, and their sister relationships with *Schizotrypanum* species. Congruent phylogenies were inferred using concatenated gGAPDH and V7-V8 SSU rRNA (Fig. 3), and HSP70 (Fig. 4A) or concatenated HSP70, gGAPDH, and V7-V8 SSU rRNA (Fig. 4B) gene sequences. The analyses strongly supported Tve as a lineage formed by the European *T. vespertilionis* 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* may have dispersed in bats through the Mediterranean; whether its ancestral form originated in Africa or Europe remains unclear at this time. *T. 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). *T.* sp Hoch Ndi1 from African monkeys was the only trypanosome from non-bat hosts that nested into 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 *T.* sp Hoch Ndi1 are hereafter referred to as *T.* sp Hoch G3 (Figs 2 - 4; Table 1). The clade formed by three very similar isolates of *T. conorhini* included *T.* 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 a 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). *T. rangeli* was slightly more similar to *T. conorhini* and *T.* sp NanDoum1

(9.0% divergence) than to *T.* sp Hoch (9.4%) and *T. vespertilionis* (10%), while it diverged by 13% from *Schizotrypanum* spp.

In addition to Tra[Tve-Tco] and *Schizotrypanum,* the clade 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., 2015a). However, the relationships of Tra[Tve-Tco] with two trypanosomes from megabats, *T.* sp. bat from Gabon and *T. teixeirae* from Australia, remained unresolved. With a large gGAPDH sequence divergence (14%), the two trypanosomes from megabats 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 *T.* sp bat (Figs. 3, 4) produced more robustly supported phylogenies.

Trypanosoma wauwau and widespread trypanosomes from Neotropical bats (*T.* sp 1, 2 and 3) (Cottontail et al., 2014) clustered together forming a clade labelled as Neobats (Fig. 1) (Lima et al., 2015b), which was placed basal to the major phylogenetic lineage comprising Tra[Tve-Tco] and its sister *Shizotrypanum* clade. The clade Neobats was closely related to the Australian clade (Figs. 1, 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).

The phylogenetic analyses based on HSP70 sequences (Fig. 4A) or HSP70 combined with SSU rRNA and gGAPDH sequences (Fig. 4B), generate trees showing topologies largely congruent with those inferred using concatenated SSU rRNA 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 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. 5) retrieved from published and unpublished genomes of the following trypanosomes: *T. rangeli* (AM80, SC 58), *T. verpertilionis*-like G1, *T. conorhini* BR1, *T.* sp HochG3, *T. erneyi*, *T. dionisii*, *T. c. marinkellei (*B7, 344), *T. cruzi* (Esmeraldo, CL Brener, G), *T. wauwau*, *T. noyese* 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 sister clade to *Schizotrypanum* – a finding highly congruent with those generated by conventional SSU rRNA 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 SSU rRNA+gGAPDH genes using much larger taxon coverage (Figs. 3, 4). Multilocus phylogenetic analysis generated trees that strongly supported the placement of Tra sister to [Tve-Tco] lineage, and Tra[Tve-Tco] as sister to the *Schizotrypanum* lineage (Fig. 5 A, B).

Our multilocus analyses included *T. cruzi* of DTUs TcVI (CL Brener), TcII (Esmeraldo), and TcI (G). One 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 2015b). Isolates of TrB (AM80) and TrD (SC58), the two main evolutionary lineages of *T. rangeli,* were included in our multilocus analyses (Maia da Silva et al., 2007; Caballero et al., 2015). Previously, another study compared the genomes of *T. rangeli* strains assigned to TrA (Chachi strain, genome data not available) and the closely related TrD (SC58 strain) (Stocco et al., 2014). Isolates of all lineages examined clustered tightly together forming a clade exclusive of *T. rangeli* isolates.

3.5. T. rangeli share 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, *T.* sp HochNdi1 and HochG3, *T.* 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 characterized by our group (Lima et al., 2013, 2015a). The analyses of SL RNA primary (Figs 7A, B) and secondary (Fig. 6) structures of trypanosomes in the [Tve-Tco] clade corroborated *T. rangeli* as being

their closest relative. In addition, results supported *T.* sp bat from African megabat as being closely related to Tra[Tve-Tco] (Figs 6, 7A). In the present study, SL transcript sequence of *T.* sp bat, which positioning remain uncertain in most phylogenetic analyses, could be confidently aligned and share highly similar secondary structures (Fig. 6) with SL RNA from Tra[Tve-Tco] lineage (Fig. 7A), corroborating the close relationships of this African bat trypanosome with this lineage. Regarding the species of *Schizotrypanum*, although the alignment was relatively reliable including sequences of *T. dionisii* (Fig. 7A), very large polymorphisms precluded trustworthy alignments using *T. cruzi* sequences (data not shown). In addition, highly divergent SL RNA sequences of *T. wawau* and *T. noyesi,* which are basal species at the clade formed by Tra[Tve-Tco] and *Schizotrypanum*, both sharing very similar secondary structure, could not be included in the alignment (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 to 975 bp) of isolates representing all Tra lineages (Fig. 7C). In agreement with its basal phylogenetic positioning, TraB exhibited the most divergent sequences when compared to the other lineages (Figs. 7A, B). Similar to *T. rangeli* of the different lineages, all trypanosomes of the [Tve-Tco] clade had a copy of the 5S RNA inserted into the intergenic region (Fig. 7C). Although SL gene repeats of [Tve-Tco] trypanosomes varied in length from 631 to 1180 bp (Fig. 7C), they shared highly conserved transcript sequences, comprising an almost identical exon (39 nt), very conserved introns (110 nt), and variable intergenic sequences containing blocks of conserved sequences unique to each species (Fig. 7A, B). SL transcripts and blocks of intergenic sequences of Tra[Tve-Tco] species could be aligned with high confidence (Fig. 7A).

Results from this study corroborated SL RNA sequences as valuable markers for the differentiation of trypanosomes, with intron sequences (Fig. 7A) and intergenic sequences (Fig. 7B) 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* species compared to the apparently more

slowly evolving Tra[Tve-Tco] species. Therefore, their use as evolutionary and taxonomic markers requires comparison with data obtained using conventional SSU rRNA and gGAPDH genes.

4. Discussion

4.1. Triatomines and cimicids play important roles in the evolution of T. rangeli, T. vespertilionis, T. conorhini and T. cruzi, potentially shaping their geographical and vertebrate host ranges

Our data suggest an important association of trypanosomes nested into the Tra[Tve-Tco] clade with haematophagous hemipterans of the Triatominae (Reduviidae) and Cimicidae of the Cimicoidea superfamily. An evolutionary hypothesis of relatively younger age of triatomines (~ 32 Ma for Triatomini, and ~ 27.5 Ma for Rhodniini + Cavernicolini) compared to ancient cimicids (~ 100 Ma) (Schuh et al., 2009; Hwang & Weirauch, 2012) is consistent with a bat trypanosome transmitted by cimicids as the last common ancestor of the *T. cruzi* clade. 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) colonize and undergo metacyclogenesis (in the digestive tract) in diverse species of triatomine genera. A notable exception appears to be Tcbat, for which experimental infection failed in triatomines from laboratory colonies. However, infectivity of Tcbat to triatomines that share shelters with bats remains to be investigated (Marcili et al., 2009). In contrast, metacyclogenesis in *T. rangeli* occurs exclusively in the salivary glands of determined *Rhodnius* complex depending on the parasite lineage, whereas development restricted to the digestive tract occur in triatomines of other genera. *T. 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 of *T. rangeli* (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 have been linked to the complexes *prolixus* and *pallescens,* respectively. Nevertheless, a much more tangled vector-lineage association has been suggested by studies of TrB, which although earlier associated with the Amazonian *R. brethesi* (*pictipes* complex)*,* has been found in *R. robustus* (*prolixus*), many times mixed with TrA. The lineage TrE has been associated with *R. stalli* and *R. pictipes* (*pictipes*), and the lineage TrD with *R. domesticus* (*prolixus*), although field isolates of TrD were restricted to the guts of *Panstrongylus megistus,* a

widespread species of triatomine (Maia da Silva et al., 2004 a,b, 2007, 2009, Vallejo et al. 2009; Ortiz 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 relationships have been suggested by phylogenetic studies of *Rhodniini*: one suggesting 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 relationships support a single *cis*-Andean (*pictipes* + *prolixus*) lineage sister to the *trans*-Andean (*pallescens*) lineage (Justi et al., 2014, 2016). This alternative phylogeny agrees with previous evidence that all lineages of *T. rangeli* develop in *cis*-Andean *Rhodnius* spp. The only exception appears to be isolates of the lineage TrC, which salivary gland invasion appear to be restricted to *trans*-Andean *Rhodnius* spp. *T. rangeli* of different lineages infects mammals of diverse orders. For instance, humans have been reported to be infected with TrA, TrB and TrC, monkeys with TrA and TrB, and bats with TrA, TrC, TrD, and TrE. The basal TrB lineage has been found in monkeys, sloths, anteater and bats of extended geographical range, from Amazonia to the Atlantic Forest (Maia da Silva et al., 2004 a,b, 2007, 2009; Ortiz et al., 2009; Pinto et al., 2012, 2015; Sincero et al., 2015; Dario et al., 2017). Consistent with TrB transmission by different vectors, we gathered experimental evidences demonstrating that isolates of TrB lineage develop in salivary glands of both cis (Maia da Silva et al., 2004b, 2007, 2009) and trans-Andean species of *Rhodnius* (unpublished data).

Results obtained in the present study demonstrated, for the first time, the ~~existence~~ presence of *T. vespertilionis* in African bats and in the gut of bat-cimicids. 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 molecular surveys (Molyneux, 1991; Cavazzana et al., 2010; Pinto et al., 2012; Ramirez et al., 2014 a,b; Cottontail et al., 2014; 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.

Trypanosoma vespertilionis, apparently found only in the Old World, is 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*, *C. 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 (human-adapted species) and overwater dispersal (Schuh et al., 2009), their spreading 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 characterized 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 characterized *T.* sp HochG3 from bat is very closely related genotype of *T.* sp HochNdi1 from a monkey (*Cercopithecus nictitans*), being isolates of the first African trypanosome in the *T. cruzi* clade parasitizing both bats and primates (Figs 2, 3; Table 1). *Cercopithecus* spp. from Cameron, Tanzania and Congo were previously reported as being infected with *Trypanosoma primatum* (Hoare, 1972), a species not available for molecular studies. Vectors of these two trypanosomes from African monkeys are unknown. Interestingly, bats share palms used as nests for monkeys (*Cercopithecus* spp. and chimpanzees) in the area where *T.* sp. HochG3 was isolated in Guinea Bissau; 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 & Timm, 1985; Tapanes et al., 2016), favouring trypanosome host-switching by oral infection.

The strong 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 – 10 Mya, with probable dispersal via the Bering Land Bridge (Hypsa et al., 2002; Hwang & Weirauch, 2012; Justi et al., 2016).

The origin of *T. conorhini* remains far from clear, but its closer relationships with both the African *T.* sp NanDoum1 from a civet and *T. vespertilionis* (Old World) than to 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 hypothesized that original hosts of *T. conorhini* were Asian monkeys (Deane et al., 1986). This hypothesis was reinforced by 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 was not molecularly confirmed 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 distribution will be valuable to clarify this history.

The vector of *T.* sp NanDoum1 from African civet and closely related to *T. conorhini* is unknown. Interestingly, another trypanosome of African carnivores, *Trypanosoma helogalei* from mongoose, shares with *T. conorhini* morphological features of culture and blood forms, 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. lectularius* (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 *T.* 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 tested (Cavazanna et al., 2010; Lima et al. 2012a,

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 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 and ecosystems where bats have been found infected with *T. dionisii*, *T. c. marinkellei*, and *T.* spp of the Neobats clade in the New Word (Cavazanna et al., 2010; Pinto et al., 2015; Ramirez et al., 2014; Lima et al. 2015a; Hodo et al., 2015; 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) including a range of ectoparasites (flies, ticks, mites, cimicids and fleas) feed on bats (Obame-Nkoghe et al., 2017), and all these arthropods should allow for both cyclical and mechanical trypanosome transmission. In addition to abundance of bat species living in diverse environments, gregariousness behaviour of bats favour host switching of ectoparasites. In addition, grooming (with the ingestion of ectoparasites that may carry trypanosomes) and sharing of regurgitated food (including blood), may all facilitate mechanical transmission of bat trypanosomes.

4.2. Evolutionary hypothesis of deep-rooted T. cruzi and T. rangeli lineages, and their evolution in the Neotropics adapted to different niches in triatomines and mammals

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 hypothesized in this study reaffirm that the majority of bat trypanosomes identified to date throughout the world cluster in the *T. cruzi* clade. To date, only *T. vegrandis*, a species found infecting bats and a range of marsupials in Australia, did not cluster in the *T. cruzi* clade (Austen et al., 2015). Our phylogenetic analyses strongly supported the monophyly of Tra[Tve-Tco] clade, and greatly increased the resolution of the relationships within the [Tve-Tco] clade, and strongly supported the clade Tra sister to [Tve-Tco] clade forming a monophyletic lineage sister to the *Schizotrypanum* lineage. In a plausible (most parsimonious) evolutionary scenario, all these trypanosomes evolved from within a lineage of bat-restricted

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 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 allied species able to infect Old World monkeys can also infect humans.

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 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 (Neobats clade) closely related to Australian trypanosomes from marsupials, rodents and bats, all placed at a basal position (clade Neobats) 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).

All data gathered to date strongly supported a bat trypanosome as the last ancestor of the *Schizotrypanum* lineage in which all currently recognised species are bat-restricted, except the generalist *T. cruzi*. Likely, a bat trypanosome adapted to triatomines gave origin to two lineages, one that remained evolving in bats represented by *T. c. marinkellei,* and other 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 mammal 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 hypothesized (Ramirez 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

probably the most basal species of the *Schizotrypanum* clade (Lima et al., 2012a, 2013, 2015a; Hamilton et al., 2012). Despite the findings of bats infected with different lineages of *T. rangeli*, relatively low prevalence of this species compared to high infection rates of *Schizotrypanum* spp. in bats, and the fact that *T. rangeli* appear to be more common in other animals such as monkeys and xenartrans (Hoare, 1972; Maia da Silva et al., 2004a,b, 2007, 2009) do not permit one reliable hypothesis about the origin and vertebrate species that harboured the ancestor of *T. rangeli.* The answer to these questions may rely on trypanosomes of many other hosts such as marsupials, xenarthrans, rodents, and non-human primates, which are known to have played important roles in the evolution of *T. cruzi* clade trypanosomes in the Neotropics.

Our findings strongly support the hypothesis that 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 mammal and insect hosts may have led to *T. cruzi* and *T. rangeli* evolutionary lineages. *T. cruzi* and *T. rangeli* differ greatly in life cycles, and 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] than to *Schizotrypanum* lineage 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 hypothesized evolutionary scenario, bat trypanosomes of very different genetic backgrounds successively adapted to a range of mammalian hosts lacking host-specificity. To our knowledge, *T. cruzi, T. rangeli, T. conohrini* and *T.* sp. Hoch 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 trypanosomes of different lineages 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 non-human and human primates. Bat-borne parasites (mainly viruses) are well known for their high 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 also occur

562 (Han et al., 2015). Infections with *T. lewisi*, a rat-borne zoonosis transmitted by fleas, have been diagnosed by
563 microscopic detection of blood trypomastigotes and species-specific PCR in children, immune-depressed
564 adults, and non-human-primates. This trypanosome belong to the subgenus *Hepetosoma*, a clade currently
565 positioned as the outgroup more related to the *T. cruzi* clade, and is currently being considered a human
566 emergent pathogen in Asia and Africa (Maia da Silva et al., 2010; Truc et al., 2013). Children infected with *T.*
567 *cruzi* Tcbat and *T. dionisii*, which are both tightly linked to bats, were recently reported by PCR blood-
568 screening (Ramirez et al., 2014a; Dario et al., 2016) (Table 1). However, active infection, even transient, with
569 the detection of live parasites in humans as showed in infections caused by *T. lewisi*, still need to be
570 confirmed.

571 In conclusion, taxon-rich and multilocus phylogenetic analysis of *T. cruzi* clade trypanosomes reconstructed
572 in the present study, together with biogeographical data about mammaln hosts and vectors, permitted to
573 hypothesize one plausible evolutionary scenario for *T. rangeli*. All analyses suggested a long-established
574 divergence, at different times, from an common Old World trypanosome ancestor giving origin to Tra, [Tve-
575 Tco] and *Schizotrypanum* lineages. In addition, we discuss how the deeply rooting of these two main lineages
576 relates to their independent evolution over a time horizon that ~~that~~ begins well before the dispersal from the
577 Old World of the ancestors of *T. cruzi* and *T. rangeli*. The knowledge of deep-rooted lineages is particularly
578 informative in the reconstruction of any evolutionary scenario. However, this study focused only on the
579 Tra[Tve-Tco] and *Schizotrypanum* lineages, and the deepest branches in the whole *T. cruzi* clade still remains
580 not very well-resolved. Our analyses evidenced that the placement of the basal clades depended on the genes
581 and taxa employed for phylogenetic inferences (Supplementary Fig. S1). Therefore, the phylogenetic
582 relationships between lineages could be reordered, including the placement of the root, with the inclusion of
583 additional taxa.

584 The evolutionary history of the whole *T. cruzi* clade is still very fragmented and biased. Available data is
585 concentrated on bat trypanosomes, mainly from the Neotropics, with very few data from African, European and
586 Australian bats. Broader studies on trypanosomes of bats and mammals in general ~~orders~~, of the New and Old
587 Word, are necessary to assess the wide spectrum of trypanosome genetic diversity, to resolve both the

deepest branches (basal lineages) and the relationships among species and lineages, and to trace back possible dispersion routes. The joint analyses of all these data are crucial to hypothesize on better supported evolutionary scenarios for the whole *T. cruzi* clade.

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Legends to Figures

Figure 1. Geographical distribution of the *T. cruzi* clade trypanosomes. Isolates characterized 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.

Figure 2. Unrooted phylogram of the *T. cruzi* clade based on V7-V8 SSU rRNA barcode sequences. Dendrogram (P) inferred using V7-V8 SSU rRNA barcode sequences (~800 bp) from 73 trypanosomes (most from bats) from the Neotropics, Afrotropics and Australia, showing the phylogenetic positioning of 8 new trypanosomes from bats and one from a bat-cimicid from Guinea Bissau, West Africa, and new isolates of *T. conorhini* (in bold), in the *T. vespertilionis* and *T. conorhini* clades, respectively. Numbers at the nodes are support values from 500 replicates.

Figure 3. Phylogeny of *Trypanosoma* species with focus in the *T. cruzi* clade and inferred using combined SSU rRNA and gGAPDH sequences. Inferred ML phylogenetic tree (1.548 characters, –Ln = 8659.072660) supporting a major assemblage comprising the lineages Tra, Tve and Tco, all clustering together forming the major clade Tra[Tve-Tco] and its sister *Schizotrypanum* (harbouring *T. cruzi*) clade. New trypanosomes from bats and a bat-cimicid (Tve-like G1 and G2 and *T.* sp HochG3) were placed within the [Tve-Tco] clade.

818 Trypanosomes of *T. lewisi* clade (*T. lewisi* and *T. microti*) were used as outgroup of the *T. cruzi* clade.
819 Numbers at the nodes are respectively ML/P (500 replicates) and BI support values.
820
821 **Figure 4.** Phylogeny of Tra[Tve-Tco] and *Schizotrypanum* trypanosomes inferred using HSP70 sequences.
822 ML analysis of **(A)** HSP70 gene sequences (~800 characters, −Ln = 2442.385253), **(B)** concatenated HSP70,
823 SSU rRNA and gGAPDH sequences (2.246 characters, −Ln = 10446.636791). *T. lewisi, T. theileri, T. cyclops*
824 and *T. b. brucei* were used as outgroups. Numbers at the nodes correspond respectively to ML (500
825 replicates) and BI support values.
826
827 **Figure 5**. Multilocus phylogenetics of Tra[Tve-Tco] and *Schizotrypanum* trypanosomes. ML analyses of
828 concatenated sequences from: **(A)** 11 single-copy protein-coding genes: GPI, GPX, HMCOAR, LAP, TcMPX,
829 PDH, RB19, RHO1, sodA, sodB and STTP2 (4.396 characters, −Ln = 31062.587854). **(B)** gGAPDH, HSP70
830 plus sequences from the 11 genes included in the analysis showed in A (5.782 characters, −Ln =
831 37115.145486). *T. lewisi, T. theileri, T. cyclops* and *T. b. brucei* were used as outgroups. Numbers at the
832 nodes are ML (500 replicates) and BI support values, respectively.
833
834 **Figure 6.** Network and predicted secondary structure of Spliced Leader (SL) RNA sequences. Highly similar
835 primary and secondary structures of SL RNA transcripts (~200 bp) are shared by trypanosomes of the
836 Tra[Tve-Tco] lineage, and their closely related *Trypanosoma* sp bat from African megabat. Numbers at nodes
837 correspond to bootstrap values estimated with 500 replicates using the same parameters optimized for
838 network inference.
839
840 **Figure 7.** Length and sequence polymorphisms of SL RNA sequences of trypanosome species of the Tra[Tve-
841 Tco] and *Schizotrypanum* lineages. Alignment of SL intron sequences **(A)** and conserved block of the
842 intergenic region **(B),** selected to illustrate the very close relationships of all Tra[Tve-Tco] species, and their
843 great divergence compared with the species of the *Shizotrypanum* lineage. **(C)** Intra-specific and intra-lineage

length polymorphisms of SL repeat units. Each species is represented by dots and bars of the specific colour shown in **(A)**.

Supplementary Data

Table S1: Trypanosomes, host and geographical origin, and Genbank accession numbers of V7-V8 SSU rRNA, gGAPDH, HSP70 and SL RNA gene sequences.

Table S2: GenBank accession numbers of trypanosome sequences from 11 single copy genes, retrieved from genome data banks, selected for multilocus phylogenetic of *T. cruzi* clade trypanosomes (showed in Fig. 5).

Figure S1. Phylogenetic trees inferred by maximum likelihood (ML) based on single copy gene sequences selected to illustrate different topologies obtained using different genes.