Molecular Phylogenetics and Evolution 145 (2020) 106705

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Editor's Choice Article

Out of Africa: The origins of the protozoan blood parasites of the *Trypanosoma cruzi* clade found in bats from Africa

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ARTICLE INFO

Keywords:
Trypanosome parasites
Chiropterans
OTU analyses
Species delimitation
Phylogeny
Parasite dispersal

ABSTRACT

Understanding geographic patterns of interaction between hosts and parasites can provide useful insight into the evolutionary history of the organisms involved. However, poor taxon sampling often hinders meaningful phylogenetic descriptions of groups of parasites. Trypanosome parasites that constitute the *Trypanosoma cruzi* clade are worldwide distributed infecting several mammalian species, especially bats. Diversity in this clade has been recently expanded by newly discovered species, but the common ancestor and geographical origins of this group of blood parasites are still debated. We present here results based on the molecular characterization of trypanosome isolates obtained from 1493 bats representing 74 species and sampled over 16 countries across four continents.

After estimating the appropriate number of hypothetical species in our data set using GMYC models in combination with Poisson Tree Processes (mPTP) and ABGD, the 18S rRNA and gGAPDH genes were used for phylogenetic analyses to infer the major evolutionary relationships in the *T. cruzi* clade. Then, biogeographical processes influencing the distribution of this cosmopolitan group of parasites was inferred using BioGeoBEARS. Results revealed a large lineages diversity and the presence of trypanosomes in all sampled regions which infected 344 individuals from 31 bat species. We found eight *Trypanosoma* species, including: five previously known; one subspecies of *Trypanosoma livingstonei* (*Trypanosoma* cf. *livingstonei*); and two undescribed taxa (*Trypanosoma* sp. 1, *Trypanosoma* sp. 2), which were found exclusively in bats of the genus *Miniopterus* from Europe and Africa.

The new taxa discovered have both an unexpected position in the global phylogeny of the *T. cruzi* clade. *Trypanosoma* sp. 1 is a sister lineage of *T. livingstonei* which is located at the base of the tree, whereas *Trypanosoma* sp. 2 is a sister lineage of the *Shizotrypanum* subclade that contains *T. c. cruzi* and *T. dionisii*. Ancestral areas reconstruction provided evidence that trypanosomes of the *T. cruzi* clade have radiated from Africa through several dispersion events across the world. We discuss the impact of these findings on the biogeography and taxonomy of this important clade of parasites and question the role played by bats, especially those from the genus *Miniopterus*, on the dispersal of these protozoan parasites between continents.

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https://doi.org/10.1016/j.ympev.2019.106705
Received 12 September 2019; Received in revised form 26 November 2019; Accepted 6 December 2019
Available online 09 December 2019

1. Introduction

Phylogenetic analysis of molecular data is fundamental as it allows insights into evolutionary history of all forms of life, including parasites and lay the foundation for recognizing and diagnosing species (De Meeus and Renaud, 2002). Moreover, in the case of parasitic relationships, phylogenies of both hosts and parasites, associated with a good knowledge of host ecological relationships, may help to formulate predictions of future outbreaks by identifying evolutionary lineages with significant host-switching potential (Alcala et al., 2017; Guth et al., 2019; Wells and Clark, 2019). Complete phylogenies of parasites can provide hypotheses on their origin, their diversification as well as changes of their life cycle (Suzán et al., 2015). Common drawbacks of phylogenetic analyses, however, include inappropriate taxon sampling, wrong outgroup assignment or overlooked differences in rates of evolutionary change across phylogenetic lineages (Nabhan and Sarkar, 2012). Such problems could hinder accurate phylogenetic reconstruction.

Trypanosomes (genus *Trypanosoma*) are flagellated, kinetoplastid protozoan parasites that have complex lifecycles, with a transmission to a final vertebrate host by a blood sucking arthropod or leech vector (Simpson et al., 2006). Over the last 20 years, our understanding of the evolutionary origin of the genus Trypanosoma has gained considerable insights through the application of molecular methods (Hamilton and Stevens, 2017). Recent studies provide strong evidence that trypanosomes are monophyletic, having diversified to parasitize all vertebrate classes as reservoir hosts, using certain blood-feeding invertebrates as vectors (Hamilton et al., 2004) such as Cimicidae (bed bugs) and Triatominae (kissing bugs), Glossinidae (tsetse flies) or leeches in aquatic areas. Trypanosomes are widespread in all continents except Antarctica, although most of the inferred clades are associated with a single type of vertebrate or invertebrate vector (Hamilton et al., 2007). Two major clades of *Trypanosoma* are present in mammals, the *"Trypanosoma brucei"* and the *"Trypanosoma cruzi"* clades. The *"Trypanosoma brucei"* clade is associated with tsetse flies and is mostly restricted to the African continent, infecting livestock, wildlife and humans and is responsible for African trypanosomiasis (Stevens et al., 1998). On the other hand, in Central and South America, the deadly parasite *T. c. cruzi* (subgenus *Schizotrypanum*) is the causative agent of Chagas disease (American trypanosomiasis) and it is transmitted to humans as well as domestic and wild mammals by triatomine bugs (Donadeu et al., 2009).

The parasite *Trypanosoma cruzi cruzi* is part of the larger "T. cruzi" clade, together with trypanosomes from a wide range of mammals from both the Old and New Worlds. Evidence on the origin of the *T. cruzi* clade suggests that the ancestor currently found in terrestrial mammals was a bat trypanosome (*T. cruzi sensu stricto*), which evolved within a broader clade of other species of bat trypanosomes (Hamilton et al., 2012b). Since the Early Eocene (54 to 48 million years ago), bats and their various ectoparasites were involved in at least five host-switching events to terrestrial mammals and several trans-continental transfers (Hamilton et al., 2012a; Lima et al., 2012). It seems likely that early trypanosomes colonised the South American continent from the Old World through several independent colonisation events (Cottontail et al., 2014).

The *T. cruzi* clade is composed by a few generalist species (e.g. *T. c. cruzi* and *T. rangeli*) but most species are restricted to bats and have a broad distribution across the world. Phylogenetic relationships in this clade reveal major subdivisions, which contain several described species but also undescribed cryptic taxa. The *Schizotrypanum* subgenus comprising *T. c. cruzi* also includes *T. cruzi marinkellei,* which is hosted by bats in Central-South America (Franzén et al., 2012), *T. erneyi* hosted by African bats (Lima et al., 2012) and *T. dionisii,* which is distributed worldwide in bats (Cavazzana et al., 2010; Gardner and Molyneux, 1988; Hamilton et al., 2012a; Mafie et al., 2018). Another distinct subclade of the *T. cruzi* radiation is represented by *T. rangeli,* which is hosted by different South American mammals and clusters with an undescribed taxon "*T.* sp. bat" and with *T. teixeria* found respectively in African (*Rousettus aegyptiacus*) and Australian (*Pteropus scapulatus*) pteropid bats (Stevens and Gibson, 1999; Barbosa et al., 2016). Furthermore, *T. vespertilionis,* hosted by various European and African bats (Stevens et al., 1998; Espinosa-Álvarez et al., 2018) is closely related to "*T.* sp. HochG3" and "*T.* sp. HochND1", both found respectively in African bats (*Scotophilus* sp.) and monkeys (*Cercopithecus nictitans*) (Espinosa-Álvarez et al., 2018; Hamilton et al., 2009). This second subclade also contains *T. conorhini* which is distributed worldwide in rodents (Stevens and Gibson, 1999) and closely related to a species informally named "*T.* sp. NanDoum" as it infects the African civet (*Nandina binotata*) (Hamilton et al., 2009). The third subclade comprises several species of trypanosomes from Neotropical bats that include: *T. madeirera, T. wauwau* and the unnamed lineage "*T.* sp. Neobats" (Barros et al., 2019; Cottontail et al., 2014; Lima et al., 2015). Together, these three species cluster with *Trypanosoma noyesi* isolated from Australian marsupials (Botero et al., 2016). Finally, basal to the whole clade is *T. livingstonei*, hosted by African bats (Lima et al., 2013).

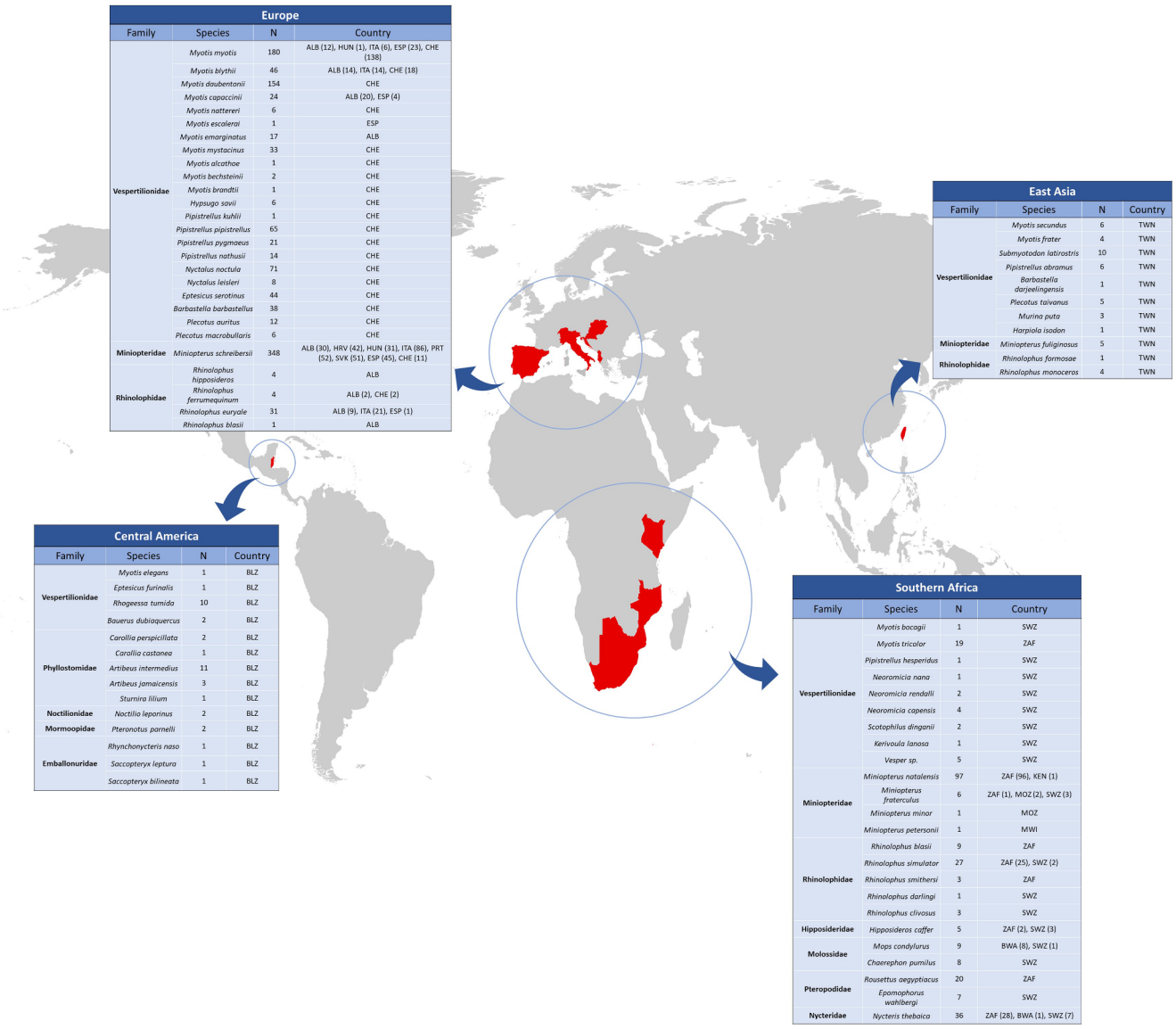
Despite having dramatically increased our knowledge of species in the *T. cruzi* clade, its overall diversity and biogeographic origins are far from complete. Indeed, many hosts and geographical regions including several parts of Africa, Asia and Europe remain unsampled. Additionally, many species have been confirmed phylogenetically to belong to this clade, but it is uncertain how many more remain to be discovered. A detailed understanding of the diversity, biogeographic patterns and phylogenetic relationships is crucial for a better comprehension of the evolutionary history of the *T. cruzi* trypanosomes. In this study, we present a novel phylogenetic resolution for the *T. cruzi* clade based on bats sampled across the world. We collected data on the diversity and the distribution of trypanosomes on a large sample of individuals and bat species from four continents. We combined these new data with an extensive literature survey of the *T. cruzi* clade from several mammal species and additional bat species to revaluate the major evolutionary relationships among taxa in this clade. Finally, we performed biogeographical analysis to reconstruct ancestral areas in order to understand the origin and the radiation pattern of this largely dispersed group of parasites.

2. Materials and methods

2.1. Sampling and sequencing

A total of 1493 bats representing 74 species from 11 families were sampled between 1998 and 2017 in several regions of the world (Fig. 1). Of these, 1139 samples came from European countries (Portugal, Spain, Switzerland, Italia, Slovakia, Croatia, Hungary and Albania), 267 samples from southern African countries (South-Africa, Eswatini, Mozambique, Botswana, Kenya and Malawi), 46 samples from East Asia (Taiwan) and 39 samples from Central America (Belize). Bats were captured in caves, forests, buildings and bat boxes using harp traps (Faunatech, Australia), mist nests or hand nets. Animals were handled according to the guidelines of the American Society of Mammalogy (Sikes and Gannon, 2011). Each individual was identified with conventional morphological keys and their identification confirmed with genetic analyses of the *Cytochrome b* gene in cases of uncertainty (Irwin and Kocher, 2016). Blood samples (20-50 μL) were taken by a small venipuncture in the uropatagium and conserved on ice or on filter paper (WhatmanMM) until further processing. To facilitate healing, haemostatic cotton was applied to the wound and the bat was returned to the tissue bag for 15 min until its release.

A different method was used for the individuals sampled in South Africa as we took advantage of a larger study performed on viral zoonotic disease. Captured bats were euthanized using a mixture of ketamine and xylazine (1:2; a volume of 0.05–0.1 mg/g body mass) for frugivorous bats and isophore for insectivorous bats before bleeding. Blood was collected *via* cardiac puncture using a 1 ml (25G) syringe for fruit bats, with a maximum volume of 600–800 μL collected. For



Europe

Family	Species	N	Country
Vespertilionidae	*Myotis myotis*	180	ALB (12), HUN (1), ITA (6), ESP (23), CHE (138)
	Myotis blythii	46	ALB (14), ITA (14), CHE (18)
	Myotis daubentonii	154	CHE
	Myotis capaccinii	24	ALB (20), ESP (4)
	Myotis nattereri	6	CHE
	Myotis escalerai	1	ESP
	Myotis emarginatus	17	ALB
	Myotis mystacinus	33	CHE
	Myotis alcathoe	1	CHE
	Myotis bechsteinii	2	CHE
	Myotis brandtii	1	CHE
	Hypsugo savii	6	CHE
	Pipistrellus kuhlii	1	CHE
	Pipistrellus pipistrellus	65	CHE
	Pipistrellus pygmaeus	21	CHE
	Pipistrellus nathusii	14	CHE
	Nyctalus noctula	71	CHE
	Nyctalus leisleri	8	CHE
	Eptesicus serotinus	44	CHE
	Barbastella barbastellus	38	CHE
	Plecotus auritus	12	CHE
	Plecotus macrobullaris	6	CHE
Miniopteridae	*Miniopterus schreibersii*	348	ALB (30), HRV (42), HUN (31), ITA (86), PRT (52), SVK (51), ESP (45), CHE (11)
Rhinolophidae	*Rhinolophus hipposideros*	4	ALB
	Rhinolophus ferrumequinum	4	ALB (2), CHE (2)
	Rhinolophus euryale	31	ALB (9), ITA (21), ESP (1)
	Rhinolophus blasii	1	ALB

East Asia

Family	Species	N	Country
Vespertilionidae	*Myotis secundus*	6	TWN
	Myotis frater	4	TWN
	Submyotodon latirostris	10	TWN
	Pipistrellus abramus	6	TWN
	Barbastella darjeelingensis	1	TWN
	Plecotus taivanus	5	TWN
	Murina puta	3	TWN
	Harpiola isodon	1	TWN
Miniopteridae	*Miniopterus fuliginosus*	5	TWN
Rhinolophidae	*Rhinolophus formosae*	1	TWN
	Rhinolophus monoceros	4	TWN

Central America

Family	Species	N	Country
Vespertilionidae	*Myotis elegans*	1	BLZ
	Eptesicus furinalis	1	BLZ
	Rhogeessa tumida	10	BLZ
	Baueurs dubiaquercus	2	BLZ
Phyllostomidae	*Carollia perspicillata*	2	BLZ
	Carollia castanea	1	BLZ
	Artibeus intermedius	11	BLZ
	Artibeus jamaicensis	3	BLZ
	Sturnira lilium	1	BLZ
Noctilionidae	*Noctilio leporinus*	2	BLZ
Mormoopidae	*Pteronotus parnelli*	2	BLZ
Emballonuridae	*Rhynchonycteris naso*	1	BLZ
	Saccopteryx leptura	1	BLZ
	Saccopteryx bilineata	1	BLZ

Southern Africa

Family	Species	N	Country
Vespertilionidae	*Myotis bocagii*	1	SWZ
	Myotis tricolor	19	ZAF
	Pipistrellus hesperidus	1	SWZ
	Neoromicia nana	1	SWZ
	Neoromicia rendalli	2	SWZ
	Neoromicia capensis	4	SWZ
	Scotophilus dinganii	2	SWZ
	Kerivoula lanosa	1	SWZ
	Vesper sp.	5	SWZ
Miniopteridae	*Miniopterus natalensis*	97	ZAF (96), KEN (1)
	Miniopterus fraterculus	6	ZAF (1), MOZ (2), SWZ (3)
	Miniopterus minor	1	MOZ
	Miniopterus petersonii	1	MWI
Rhinolophidae	*Rhinolophus blasii*	9	ZAF
	Rhinolophus simulator	27	ZAF (25), SWZ (2)
	Rhinolophus smithersi	3	ZAF
	Rhinolophus darlingi	1	SWZ
	Rhinolophus clivosus	3	SWZ
Hipposideridae	*Hipposideros caffer*	5	ZAF (2), SWZ (3)
Molossidae	*Mops condylurus*	9	BWA (8), SWZ (1)
	Chaerephon pumilus	8	SWZ
Pteropodidae	*Rousettus aegyptiacus*	20	ZAF
	Epomophorus wahlbergi	7	SWZ
Nycteridae	*Nycteris thebaica*	36	ZAF (28), BWA (1), SWZ (7)

Fig. 1. Sample location of the 74 bat species included in this study. The bat family and species, number of individual sampled (N), and the country of origin are specified. Abbreviation codes for each country are: Albania (ALB), Croatia (HRV), Hungary (HUN), Italy (ITA), Portugal (PRT), Slovakia (SVK), Spain (ESP), Switzerland (CHE), South-Africa (ZAF), Eswatini (SWZ), Mozambique (MOZ), Botswana (BWA), Kenya (KEN), Malawi (MWI), Taiwan (TWN), and Belize (BLZ).

insectivorous bats, we used a 0.3 ml (30G) syringe and collected 50 µL maximum. Blood was collected in Serum separator tubes, inverted after collection, and kept after centrifugation in a cooler box with an icepack in the field and then 4 °C.

Total DNA was extracted from blood using Qiagen® standard DNeasy Blood & Tissue Kits (Hombrechtikon, Switzerland). To detect trypanosomes, diagnosis of infection was conducted by carrying out at least three independent nested-PCR for each blood sample and for each gene. One is a partial 642 bp fragment of the small subunit 18S ribosomal RNA gene (18S rRNA) which was amplified following the protocol described by Noyes and colleagues (Noyes et al., 1999). The second gene consisted of 786 bp of the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene and was obtained using the pairs of primers G5-G3 for the first PCR round and G1-G4A for the second (Hamilton et al., 2004). We used a high grade Taq polymerase (AmpliTaqGold Applied biosystem®, Switzerland) and increased the number of cycles up to 30 for the first PCR and 40 cycles for the nested PCR to improve yield of the PCRs. Sequences of 18S rRNA and gGADPH gene were obtained by direct sequencing of PCR fragments of both strands, assembled and submitted to a BLAST analysis in NCBI data base (http://www.ncbi.nlm.nih.gov/blast).

2.2. Taxonomic assessment of Trypanosoma species

To assign newly sequenced trypanosome genes to known taxonomic lineages (i.e. Operational Taxonomic Units, OTU), we implemented the General Mixed Yule Coalescent (GMYC) (Fujisawa and Barraclough, 2013) and the multiple rate Poisson Tree Processes (mPTP) (Zhang et al., 2013) methods using the 18S rRNA gene data set. These are two similar approaches aimed to bridge the gap between species-tree and distance-based methods. Both are appropriate methods for species delimitation based on single-locus data. However, PTP often yields more accurate delimitations than GMYC (Kapli et al., 2017). Sequences of 18S rRNA bat trypanosomes generated in this study were compared with 35 sequences from GenBank issued from all known species or lineages from the *T. cruzi* clade (Table 1). The gene tree phylogeny was estimated using BEAST v.1.8.2 (Drummond et al., 2012) and PhyML (Gouy et al., 2010), with the same parameters as in the phylogenetic analyses detailed hereafter, but by removing the outgroups and sequences within less than 1% of difference for each parasite species. The

Table 1
Trypanosoma isolates composing the *T. cruzi* clade and their respective sequences of 18S rRNA and gGADPH genes retrieved from GenBank database. Mammalian host species, year of collection and geographical origins are provided.

Trypanosome isolates	Host origin	Infected species	Year	Geographic origin	18S rRNA	gGADPH
Trypanosoma cruzi						
TCC793	Chiroptera	*Myotis levis*	2004	Brazil	FJ900241	GQ140358
TCC507	Chiroptera	*Carollia perspicillata*	2002	Brazil	FJ900240	GQ140352
TCC34 Y	Primate	*Homo sapiens*	1953	Brazil	AF301912	GQ140353
TCC844	Primate	*Homo sapiens*		Brazil	AF303660	GQ140355
G	Didelphimorphia	*Didelphis marsupialis*	1983	Brazil	AF239981	GQ140351
Trypanosoma marinkellei						
B7	Chiroptera	*Phyllostomus discolor*	1974	Brazil	AJ009150	AJ620270
TCC344	Chiroptera	*Carollia perspicillata*	2001	Brazil	AJ001664	GQ140360
Trypanosoma erneyi						
TCC1294	Chiroptera	*Tadarida* sp.	2006	Mozambique	JN040988	JN040965
TCC1934	Chiroptera	*Mops condylurus*	2009	Mozambique	JN040991	JN040967
Trypanosoma dionisii						
TCC211	Chiroptera	*Eptesicus braziliensis*	2000	Brazil	FJ001666	GQ140362
TCC495	Chiroptera	*Carollia perspicillata*	2002	Brazil	FJ001667	GQ140363
Trypanosoma vespertilionis						
TCC2045_G1	Chiroptera	*Scotophilus* sp.	2010	Guinea-Bissau	MF144887	MF144726
TCC2099_G1	Chiroptera	*Scotophilus* sp.	2010	Guinea-Bissau	MF144889	MF144728
Trypanosoma rangeli						
AM80	Primate	*Homo sapiens*	1996	Brazil	AY491766	JN040973
328	Primate	*Homo sapiens*		El Salvador	AY491738	KT368808
TCC643	Chiroptera	*Platyrrhinus lineatus*	2003	Brazil	FJ900242	GQ140364
23-SC58	Rodentia	*Echimys dasythrix*		Brazil	AY491745	KT368804
Trypanosoma sp. bat						
TryCC60	Chiroptera	*Rousettus aegyptiacus*	1997	Gabon	AJ012418	GQ140365
Trypanosoma teixeirae						
	Chiroptera	*Pteropus scapulatus*	2014	Australia	KT907061	KT907062
Trypanosoma noyesi						
AB-2016a woylie	Diprotodontia	*Bettongia penicillata*	2016	Australia	KU354263	KU354264
H25	Diprotodontia	*Macropus giganteus*	1997	Australia	AJ009168	AJ620276
AP-2011a D64	Diprotodontia	*Trichosurus vulpecula*	2009	Australia	JN315383	JN315397
AB-2013 G8	Diprotodontia	*Bettongia penicillata*	2013	Australia	KC753537	KC812988
Trypanosoma sp. NeoBats						
BACO44	Chiroptera	*Artibeus lituratus*	2014	Colombia	KT368797	KT368800
BACO46	Chiroptera	*Artibeus lituratus*	2014	Colombia	KT368798	KT368801
T. wauwau						
TCC411	Chiroptera	*Pteronotus parnellii*	2002	Brazil	KT030810	KT030800
TCC1022	Chiroptera	*Pteronotus parnellii*	2005	Brazil	KT030830	KT030805
Trypanosoma livingstonei						
TCC1298	Chiroptera	*Rhinolophus landeri*	2006	Mozambique	KF192982	KF192961
TCC1935	Chiroptera	*Rhinolophus landeri*	2006	Mozambique	KF192987	KF192965
TCC1953	Chiroptera	*Hipposideros caffer*	2006	Mozambique	KF192984	KF192969
Trypanosoma conorhini						
TCC1452	Rodentia	*Rattus rattus*		Brazil	MF144891	MF144730
USP	Rodentia	*Rattus rattus*	1947	Brazil	AJ012411	AJ620267
Others – *Trypanosoma* sp.						
*T.*sp. NanDoum1	Carnivora	*Nandinia binotata*	2004	Cameroon	FM202492	FM164793
T. sp. HochNdi1	Primate	*Cercopithecus nictitans*	2004	Cameroon	FM202493	FM164794
TCC2041- *T.* sp. Hochlike G3	Chiroptera	*Scotophilus* sp.	2010	Guinea-Bissau	MF144893	MF144732

maximum clade credibility (MCC) of the Bayesian analysis tree was implemented in the GMYC species delimitation tool and it was tested using the SPLITS and APE package in the R statistical software (R Developpement Core Team, 2015) for the single and multiple-threshold options. The online version of Poisson Tree Processes http://mptp.h-its.org (Kapli et al., 2017) was used to confirm this partition. It was tested with multiple rate analyses on a Netwick formatted tree using the maximum likelihood tree from PhyML. Finally, the online version of the Automatic Barcode Gap Discovery (ABGD) method (Puillandre et al., 2012) was used to partition the number of possible groups in our data set. We ran the ABGD analysis with the 18S rRNA alignment applying both K2P and Jukes-Cantor (JC) distance models, applying a gap width of 0.5, with 1000 steps and the rest of the values as default.

2.3. Phylogenetic analyses

Each sequence was aligned using the program Clustal X (Larkin et al., 2007), manually edited and visualized with MEGA v6.0 (Tamura et al., 2013). The most appropriate model of nucleotide substitution for each gene was chosen with the Akaike information criterion (AIC) by using hierarchical likelihood ratio test (hLRTs) performed with R 3.2.0 and the package APE (Paradis et al., 2004). The best model for both 18S rRNA and gGADPH was GTR + I + G (Posada and Crandall, 1998). We then ran both maximum likelihood (ML) and Bayesian phylogenetic reconstruction methods on the partitioned concatenated sequences. Phylogenetic inferences by ML were performed with PhyML (Gouy et al., 2010) using 10′000 replicates. For the Bayesian analysis, it was

implemented in BEAST v.1.8.2 (Drummond et al., 2012) and XML file was made with BEAUti v1.7.1 interface with the following settings: the MCMC chain was 50 million generations using empirical base frequencies, four gamma categories, all codon positions partitioned with unlinked base frequencies and substitution rate, uncorrelated relaxed clock using coalescent tree as prior. Trees were sampled every 2000 generations, and we discarded the first 10% as a burn-in. From the post burn-in tree samples, we constructed a maximum clade credibility (MCC) phylogeny using TreeAnnotator and then visualized it on FigTree (Rambaut, 2007). Nucleotide diversity and polymorphic sites were obtained via DNAsp 5.10.01 (Librado and Rozas, 2009). Finally, we computed uncorrected pairwise distances with the 'dist.dna' command in the APE package in R.

To infer phylogenies based on 18S rRNA and gGADPH genes from a larger set of samples from the *T. cruzi* clade, we used the 41 distinct concatenated sequences of 18S rRNA and gGADPH obtained here along with 35 others from known species and lineages from the *T. cruzi* clade and issued from GenBank (Table 1). We used *T. lewisi* and *T. microti* hosted by rodents as outgroup taxa (Zhang et al., 2013).

2.4. Ancestral area reconstruction

We reconstructed ancestral areas on internal nodes of the trypanosome parasites trees using the package BioGeoBEARS (Matze, 2014) implemented in RASP 4.0 (Yu et al., 2015). The reconstructions were made with 1000 trees randomly sampled from the posterior probability distribution of the Bayesian analysis. We evaluated the fit of our data to three distinct biogeographic models (DEC, DIVALIKE, BAYAREALIKE) using likelihood ratio tests based on Akaike information criterion corrected for small sample sizes (AICc). For all the three models, we compared the fit with and without a founder event speciation parameter "J" (DEC + J, DIVALIKE + J, BAYAREALIKE + J). The jump dispersal parameter, J, allows for a daughter lineage to immediately occupy a new region that is different from that of the parental lineage. We defined five biogeographic regions: Africa, Asia, Australia, Europe and South & Central America. Parasite lineage distributions were coded as present or absent in each of the five regions. We removed the outgroup from the trees prior to analysis and have fixed the maximum number of regions to five. Analyses were implemented without constraints. Dispersal events among regions were plotted into a Chord diagram using the circlize package (https://github.com/jokergoo/circlize) in R 3.6.1 (http://www.cran.r-project.org/).

3. Results

3.1. Trypanosoma species prevalence and distribution

The species and number of bats infected, parasite distribution and parasite prevalence are reported in Table 2. Of the 1'493 bat sampled, 354 (24%) were positive for *Trypanosoma* infection. All 354 infected

Table 2
Trypanosoma distribution in 31 bat species infected across the world. N-bats = number of bat individuals sampled; N-inf = number of bat individuals infected; % Prevalence = proportion of individuals infected when N-bats > n 5; *Trypanosoma* lineages = Parasite lineages and distribution using concatenated sequences of 18S rRNA and gGADPH genes, number in brackets represents the number of times this lineage has been found for each infected bat species.

Region	Bat family	Bat species	N-bats	N-infected	% Prevalence	*Trypanosoma* lineages 2019 (18S rRNA + gGADPH genes)
Europe	Vespertilionidae	*Myotis myotis*	180	63	35.0	*T. dionisii*_EU2 (1); *T. dionisii*_EU5 (62)
Europe		*Myotis blythii*	46	12	26.1	*T. dionisii*_EU5
Europe		*Myotis daubentonii*	154	10	6.5	*T. dionisii*_EU1(6); *T. dionisii*_EU2 (3); *T. dionisii*_EU3 (1)
Europe		*Myotis emarginatus*	17	1	5.9	*T. dionisii*_EU1
Europe		*Myotis mystacinus*	33	8	24.2	*T. dionisii*_EU4 (7); *T. dionisii*_EU5 (1)
East Asia		*Myotis frater*	4	1		*T. dionisii*_AS1
East Asia		*Submyotodon latirostris*	10	2	20.0	*T. dionisii*_AS2
Europe		*Hypsugo savii*	6	2	33.3	*T. dionisii*_EU1
Europe		*Pipistrellus kuhlii*	1	1		*T. dionisii*_EU1
Europe		*Pipistrellus pipistrellus*	65	32	49.2	*T. dionisii*_EU3
Europe		*Pipistrellus pygmaeus*	21	5	23.8	*T. dionisii*_EU1 (1); *T. dionisii*_EU3 (4)
Europe		*Pipistrellus nathusii*	14	4	28.6	*T. dionisii*_EU3
Southern Africa		*Pipistrellus hesperidus*	1	1		*T. vespertilionis*_AF1
Southern Africa		*Neoromicia capensis*	4	2		*T. dionisii*_AF5 (1); *T. vespertilionis*_AF2 (1)
Europe		*Nyctalus noctula*	71	21	29.6	*T. dionisii*_EU2 (19); *T. vespertillionis*_EU (2)
Europe		*Nyctalus leisleri*	8	3	37.5	*T. dionisii*_EU2
Europe		*Eptesicus serotinus*	44	19	43.2	*T. dionisii*_EU1 (18); *T. dionisii*_EU2 (1)
Europe		*Barbastella barbastellus*	38	2	5.3	*T. dionisii*_EU5
Europe		*Plecotus auritus*	12	1	8.3	*T. dionisii*_EU1
Europe		*Plecotus macrobullaris*	6	1	16.7	*T. dionisii*_EU1
Central America		*Rhogeessa tumida*	10	3	30.0	*T. dionisii*_CA1 (1); *T. dionisii*_CA2 (2)
Southern Africa		*Scotophilus dinganii*	2	2		*T. vespertilionis*_likeG1_AF3
Europe	Miniopteridae	*Miniopterus schreibersii*	348	113	32.5	*T.* sp. 1_EU (1 0 9); *T. dionisii*_EU1 (2); *T. dionisii*_EU2 (2)
Southern Africa		*Miniopterus natalensis*	97	12	12.4	*T.* sp. 1_AF (2); *T.* sp. 2_A (2); *T.* sp. 2_B (3); *T. dionisii*_AF1-AF4 (4); *T.* cf. *livingstonei*_likeH_G1 (1)
Southern Africa, Europe	Rhinolophidae	*Rhinolophus blasii*	10	1	10.0	*T. livingstonei*_A
Southern Africa		*Rhinolophus simulator*	27	2	7.4	*T. livingstonei*_A; *T. livingstoneii*_C
Southern Africa	Hipposideridae	*Hipposideros caffer*	5	1		*T. livingstonei*_B
Southern Africa	Pteropodidae	*Rousettus aegyptiacus*	20	3	15.0	*T.* sp_AF
Southern Africa	Nycteridae	*Nycteris thebaica*	36	24	66.7	*T.* cf. *livingstonei*_likeA, likeB(5), likeC(5), likeD, likeE, likeF, likeG, likeI, likeJ, likeK(3), likeL, likeM, likeN(2)
Central America	Phyllostomidae	*Artibeus intermedius*	11	1	9.1	*T.* sp. Neo Bats_CA
Central America		*Artibeus jamaicensis*	3	1		*T.* sp. Neo Bats_CA
				Total = 354	**Mean = 24%**	

individuals were sequenced for both genes and resulted in 27 distinct haplotypes of 574 bp for the 18S rRNA gene and 35 haplotypes of 786 bp for the gGADPH gene. 18% of the gGADPH sequences represented heterozygotes were phase out of the analysis. *Trypanosoma* were detected in 31 bat species from seven families and were present in all geographic regions sampled (Table 2). When trypanosome was found in a bat species for which we have more than 5 individuals sampled, we calculated the intra-specific prevalence, which ranged between 5.3% and 66.7%. The highest rates of infection were evidenced in *Nycteris thebaica* from South Africa (66.7%, n-infected/n = 24/36), and in *Pipistrellus pipistrellus* (49.2%, n-infected/n = 32/65) and *Eptesicus serotinus* (43.2%, n-infected/n = 19/44) from Europe.

According to the primary results from the sequencing of 18S rRNA and gGADPH, eight distinct *Trypanosoma* taxa were found, including the following five corresponding to known lineages (97–100% identity): *T. dionisii* infecting Vespertilionidae and Miniopteridae and present in all regions sampled; *T. vespertilionis* infecting Vespertilionidae from Europe and Africa; *T. livingstonei* infecting Rhinolophidae and Hipposideridae from Africa; *T.* sp. bat infecting African fruit bats of the family Pteropodidae; and *T.* sp. Neobats infecting exclusively Phyllostomidae from Central America.

Three other parasite taxa presenting less than 97% identity with any known species, *(Trypanosoma* sp. 1*, Trypanosoma* sp. 2 and *Trypanosoma* cf. *livingstonei),* could not be assigned to any named species or lineage. *T.* sp. 1 occurred in Miniopteridae from both Europe and Africa; sequences of 18S rRNA representing this unknown lineage showed 94.1% identity with *T. livingstonei* while sequences of gGADPH showed 88.81% similarity with *Trypanosoma noyesi*. *T.* sp. 2 was detected in Miniopteridae from Africa only and representative sequences of 18S rRNA showed 89% identity with those of *T. dionisii*, and 93.57% for the gGADPH gene. Finally, *T.* cf. *livingstonei* was detected in Nycteridae and Miniopteridae bats from Africa and sequences showed 96.4% identity with *T. livingstonei* for the 18S rRNA gene and 93.7% for the gGADPH gene.

3.2. Trypanosoma species delimitation

The OTUs issued from the GMYC, mPTP and ABGD analyses based on the 18S rRNA genes were remarkably congruent (Fig. 2). For GMYC, the likelihood of the null model was significantly lower than the maximum likelihood of the single-threshold and in the multiple threshold version of the model ($P\text{-value}_{single} = 0.000954$; $P\text{-value}_{multiple} = 0.001$). Considering the single-threshold option, 17 distinct clusters were defined by the analyses, which include the 8 lineages found in this study in addition to the other lineages issues from GenBank database. The mPTP method also found that the best score for multi coalescent rate was significantly lower than the null model (P-value = 0.001) and resulted in 16 distinct clusters. Finally analyses performed with ABGD suggested the existence of 15 clusters.

The resulting OTUs suggest that *Trypanosoma* sp. 1 and *Trypanosoma* sp. 2 represent two new species. *T.* sp. 1 appeared genetically very distinct from its closest relative, the African *T. livingstonei*. It was found in 109 individuals of *Miniopterus schreibersii* sampled across Europe and in two *M. natalensis* from South Africa. On the other hand, *T.* sp. 2 was most closely related to *T. dionisii,* then to the other taxa of the *Shizotrypanum* subgenus *(T. cruzi cruzi*, *T. cruzi marinkelleii* and *T. erneyi)*. *T.* sp. 2 was found in five *M. natalensis* from South Africa. A third unknown lineage, *T.* cf. *livingstonei* was related to *T. livingstonei* but genetically quite divergent. GMYC models considered *T.* cf. *livingstonei* and *T. livingstonei* as different species, while mPTP and ABGD analyses considered the two species like one single taxon. As these results depended to the analyses performed, we considered *T.* cf. *livingstonei* as a subspecies of *T. livingstonei* and not as a species on its own.

3.3. Parasite phylogenies

The combinations of 18S rRNA and gGADPH resulted in 41 distinct concatenated sequences of 1360 bp (Supplementary Fig. S1) in length (see Supplementary Table 1). This alignment included 357 variable and 288 parsimony-informative sites. Both ML and Bayesian phylogenetic analyses resulted in similar tree topologies. The complete phylogeny using all sequences is presented in Fig. 3.

The newly acquired sequences from this study fall within four major genetic groups. The cosmopolite *T. dionisii* falls in a first group corresponding to the subgenus *Shizotrypanum*, and contained *T. erneyi*, *T. cruzi marinkelleii* and *T. cruzi cruzi*. A large diversity of sequences was found within *T. dionisii* as it was represented by five distinct European lineages (EU1-EU5*),* two Asian (AS1-AS2), two Central American (SA1-SA2) and five African ones (AF1-AF5). Average genetic distance within *T. dionisii* was about 10% and the following two subgroups were identified: the European *T. dionisii* EU1_2_4_5*,* the Asian and the Central American lineages clustered together whereas lineages AF1-AF5 from Africa clustered with *T. dionisii*_EU3 from Europe. Basal to all species of the subgenus *Schizotrypanum*, *T.* sp. 2 was represented by two distinct lineages, *T.* sp. 2_A and *T.* sp. 2_B with an average distance of 0.8% between them and at 27% distanced from its closest relative *T. dionisii*. *T. vespertilionis* appeared within a second distinct group with a large variety of other species, including *T.* sp bat from the African pteropid *Rousettus aegyptiacus*. *T. vespertilionis* is represented by one lineage in Europe (*T. vespertilionis*_EU) and three in Africa (*T. vespertilionis*_AF1-AF3), with 15% of divergence between the different lineages. The third major group contains the undescribed species "*T.* sp. Neobats" and clustered closely with *T. wauwau*; both species are known to be hosted by Neotropical bats and are related to the Australian *T. noyesi* found in marsupials (Botero et al., 2016). Finally, the last group contains *T. livingstonei* and is represented here by three lineages called *T. livingstonei* A to C*,* with mean of 16% divergence among them. The taxon *T.* cf. *livingstonei,* represented by 17 lineages is divided in three distinct groups called G1, G2 and G3 which differ by 14% divergence between them and at 23% distanced from *T. livingstonei.* Finally, *T.* sp. 1 is basal to the *T. cruzi* clade and is related to the *Livingstonei* group with an average sequence's divergence of 31%. It was represented by two distinct lineages (0.4% divergence between them), one in Europe and infecting *Miniopterus schreibersii* (*T.* sp. 1_A) and one in Africa infecting *M. natalensis (T.* sp. 1_B*)*.

3.4. Ancestral area reconstruction

The AIC model comparison supported the BAYAREALIKE + J model as the best fitting model in the BioGeoBEARS analyses (Supplementary Table 2). BAYAREALIKE + J model estimated 29 dispersion events (including 15 dispersion events between different biogeographic regions, Fig. 3B), 14 vicariant events and one extinction event to explain current distribution of the species in trypanosome parasite clade. Through dispersal events, Africa was the most important region for the radiation of Trypanosomes, acting as the primary source of lineage dispersal across the world. At least five dispersion events took place from Africa to South-America, three from Africa to Europe and one from Africa to Australia (Fig. 3B).

Analysis identified Africa as the ancestral area for the *Trypanosoma cruzi* parasite clade with 95% of probability (Supplementary Fig. 1; Supplementary Table 3). Moreover, the major phylogenetic groups composing the *T. cruzi* clade all have an African origin, with more than 93% of probability.

4. Discussion

Using an extensive sampling of bat trypanosomes from four different continents (Fig. 1), we provide here novel insights into their evolution, their dispersion and their origin. We also expand

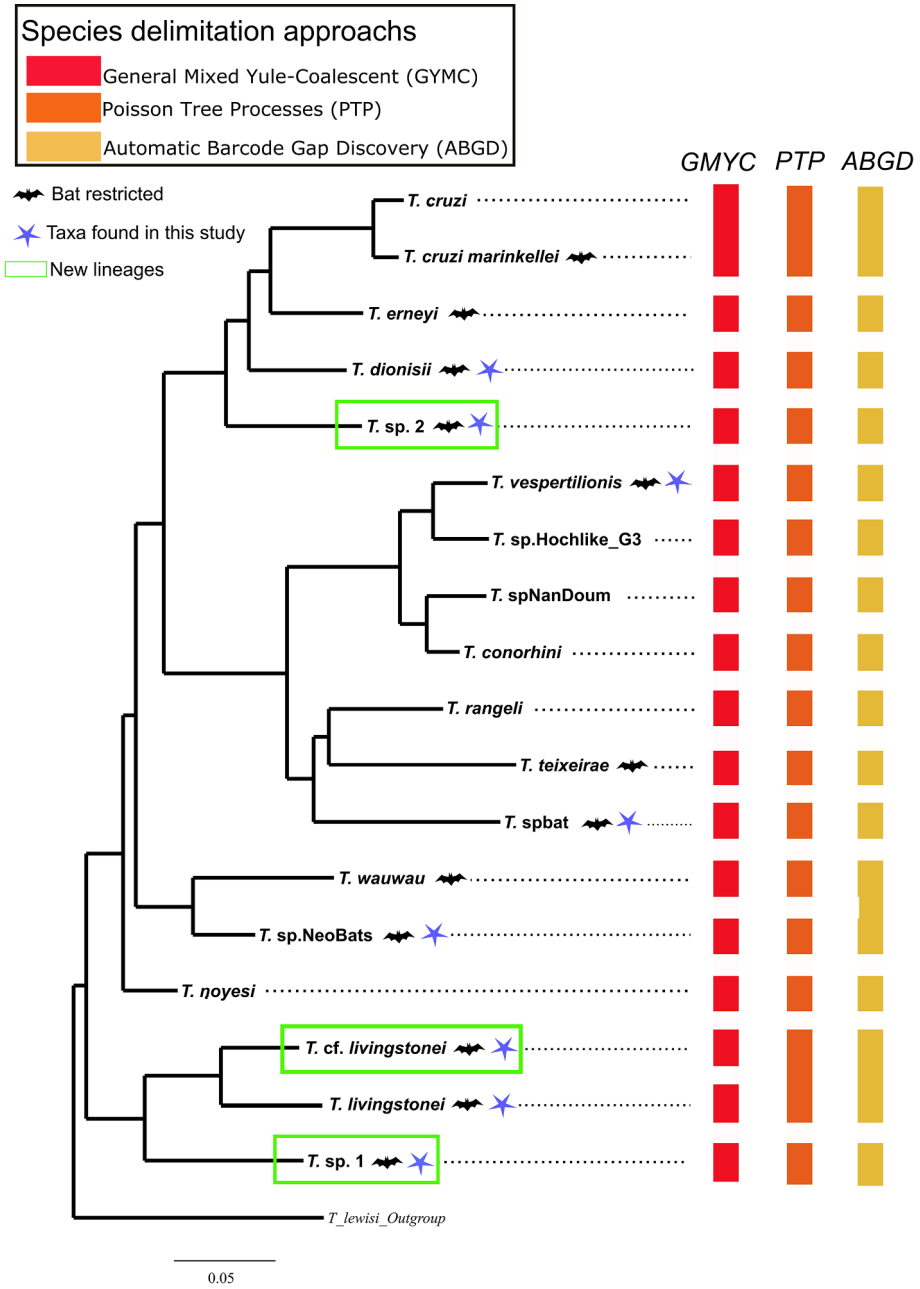


Fig. 2. Results of species delimitation analyses on the 574 bp of 18S rRNA fragment using General Mixed Yule Coalescent (GMYC), Poisson Tree Processes and Automatic Barcode Gap Discovery (ABGD) methods. Each taxa found is presented on this simplified phylogenetic tree of the *T. cruzi* clade (Maximum likelihood-10000 replicates). The eight taxa found here are marked by a blue star and the new ones are surrounded by a green square. Trypanosome species that are bat restricted are indicated with a bat symbol.

considerably the geographic coverage of several parasite species and reconstruct the phylogenetic relationships of several bat trypanosomes of the *T. cruzi* clade (Fig. 3).

Our broad sampling demonstrated that a large number of bat species from different families (Vespertilionidae, Miniopteridae, Pteropodidae, Rhinolophidae, Hipposideridae, Nycteridae, Phyllostomidae) were detected positive for *Trypanosoma* infection. This extensive taxonomic coverage of *Trypanosoma* hosts suggests that the high vagility of chiropterans might be an important factor in the biogeographic history of these protozoan parasites (Hamilton et al., 2012a) and explain their very large distribution across the world. The presence of the trypanosomes across the bat species diversity (over 1400 species worldwide (Burgin et al., 2018)) is probably still largely underestimated due to insufficient sampling. Of the eight trypanosome taxa detected in the sampled bats (Table 2), only five were already known from bats and while three were unknown, but for most of them, we considerably increased their phyletic diversity and expanded the number of hosts and geographic range occupied by those parasites. Ancestral area reconstruction analysis bring strong evidences that *T. cruzi* clade parasites radiated numerous times from the African continent and that most species composing the tree have an African origin.

4.1. Increased phyletic diversity within *T. dionisii, T. vespertilionis* and *T. livingstonei*

We found many distinct lineages within *T. dionisii* in all regions sampled (Table 2, Fig. 3). This species was found in Africa, Asia, Central America and in all infected bats species from Europe, including in

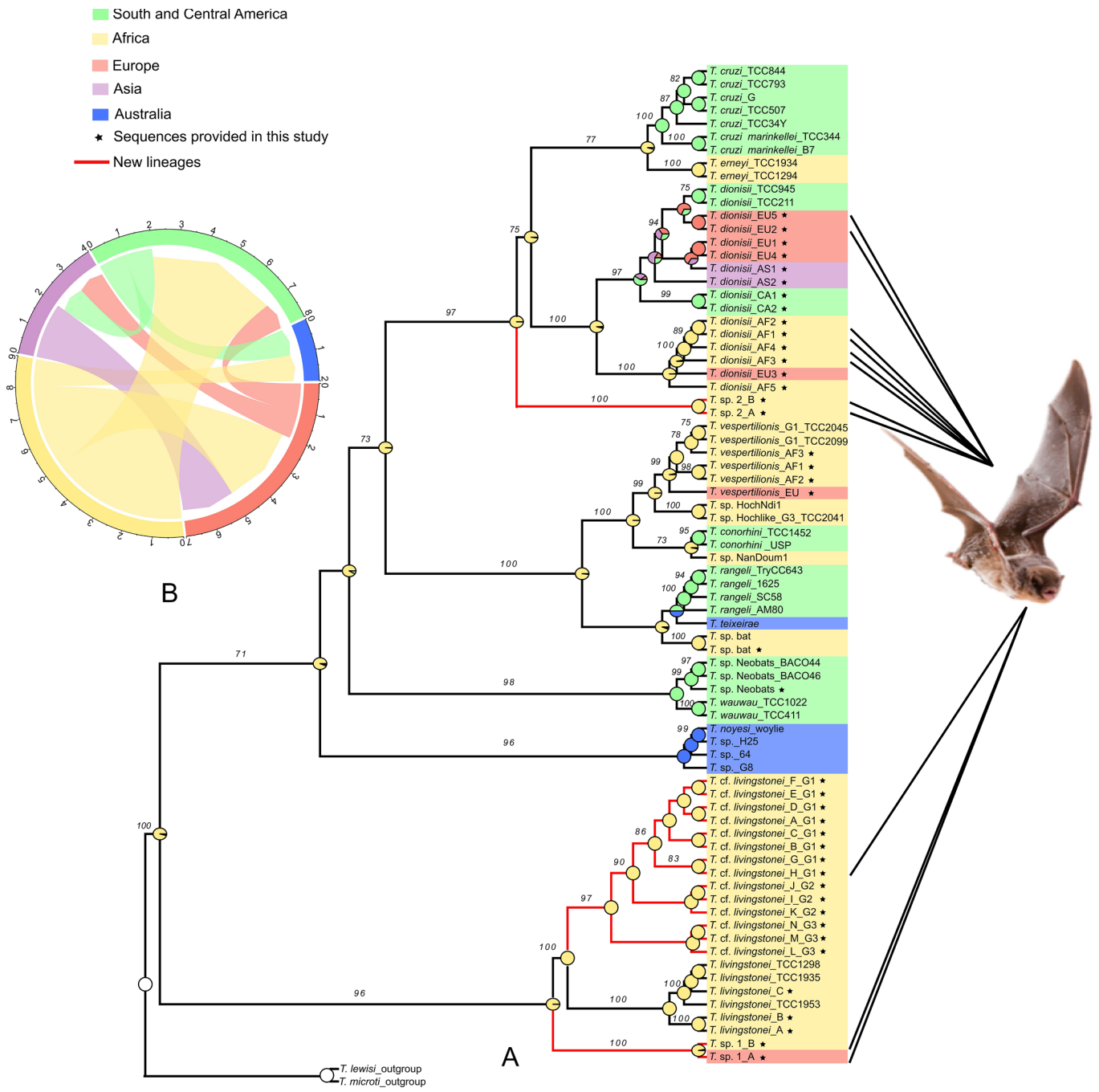


Fig. 3. (A) Complete phylogeny of the *Trypanosoma cruzi* clade inferred by Bayesian and Maximum likelihood analysis from a partitioned concatenated sequence of the 18S rRNA and gGADPH genes (1360 bp) using GTR + I + G model. The 41 lineages found in this study are marked with a small star. Numbers at nodes are ML bootstrap value (> 70) using 10,000 replicates. New trypanosomes from African and European bats (*T.* sp. 1, *T.* sp. 2, *T.* cf. *livingstonei*) are indicated by red branches. Parasite species and lineages infecting *Miniopterus* bat genus are specified by the photo of this species. Coloured pie charts at interior nodes of the phylogeny correspond to ancestral area reconstructions (most likely biogeographic areas inferred by the BAYAREALIKE + J algorithm in RASP). Colours corresponding to biogeographic regions are illustrated in the figure legend. Coloured squares indicate the current parasite species distribution. (B) Chord diagram depicting the dispersal events among biogeographical regions. Chord thickness indicates the frequency of dispersal events and chord colours indicate the region of origin for the dispersal events. Arrows represent migration directions (dispersal events were inferred by the BAYAREALIKE + J algorithm in RASP).

Miniopterus schreibersii. This wide distribution contrasts with rather limited nucleotide variation across continents (Mafie et al., 2018). We report the presence of the widespread *T. dionisii* for the first time from the African continent where it was parasitizing *Miniopterus natalensis* and *Neoromicia capensis*. This occurrence coincides with the discovery of a related species of *T. dionisii (Trypanosoma* sp. sequences type 2*)* that was detected in Zambia and infecting another bat species, *Hipposideros vittatus* (Qiu et al., 2019). Biogeographical results show that the widespread *T. dionisii* originated from Africa (94% probability), then dispersed to South-America and to Europe through several dispersal events. The presence of *T. dionisii* in the Americas (Cavazzana et al., 2010; Hamilton et al., 2012a) also suggests that chiropterans were able to disperse the parasite over large aquatic areas owing to their important potential for colonising new regions.

Until recently *T. vespertilionis* was only known from European bat, but a first trypanosome isolate identified molecularly to this species was

detected in a *Scotophilus* sp. bat from West Africa (Guinea-Bissau) (Espinosa-Álvarez et al., 2018). Authors suggested that this African *T. vespertilionis* may have dispersed across the Mediterranean. We found here additional phyletic diversity of *T. vespertilionis* in the Southern African region, on three different Vespertilionidae bat species (*Pipistrellus hesperidus, Neoromicia capensis* and *Scotophilus dinganii*) and a larger divergence among African lineages compared to the European ones. Biogeographical analysis supports an African origin (97% probability) of *T. vespertilionis* and subsequent colonization of the European bat fauna probably through some Vespertilionidae bats.

For *T. livingstonei*, which has originally been described in bats from Mozambique (Lima et al., 2013), we extended its lineage diversity and found a particularly divergent lineage we called *T.* cf. *livingstonei*. A high prevalence of this lineage was detected in *Nycteris theibaica*, a species that to our knowledge has not been tested for the presence of trypanosome. Species-delimitation analyses were ambiguous in identifying this divergent lineage as distinct from the true *T. livingstonei* (Fig. 2) and we consider it consequently as subspecies of this one. Representative samples of this divergent lineage, as well as the entire Livingstonei group, originated from Africa. Moreover, *T.* cf. *livingstonei* exhibited high nucleotide diversity, which reinforced the position of the Livingstonei group at the base of the *T. cruzi* tree.

4.2. New Trypanosoma species in Europe and Africa

Two new *Trypanosoma* species were inferred consistently by all methods of tree reconstructions (Fig. 2), and both were only detected in species of *Miniopterus* (Table 2), indicating that they may be restricted to that type of host. *T.* sp. 1 was found in all sampled European countries infecting *M. schreibersii*, whereas it was also present in *M. natalensis* from South Africa. Despite the considerable geographic distance separating these two sampling areas, DNA sequences differed minimally (< 1%). On the other hand, *T.* sp. 2 was found only in *M. natalensis* from South Africa.

Besides increasing the diversity of trypanosomes from Africa and Europe, these two new species occupy an unsuspected position in the phylogenetic tree of the *T. cruzi* clade (Fig. 3). *T.* sp. 1 is related to the Livingstonei group which is placed at the base of the tree. Given its current exclusive occurrence in Miniopteridae bats, *T.* sp. 1 likely dispersed recently via some *Miniopterus* host from Africa across Europe. The South African *T.* sp. 2 is firmly placed at the base of the *Shizotrypanum* radiation and it represents an ancestral African lineage that led to *T. dionisii, T. erneyi, T. cruzi marinkellei* and *T. cruzi cruzi.*

Obviously, further analyses on the life cycle, morphology and vector transmission are needed to fully understand the evolution of *T.* sp. 1 and *T.* sp. 2. Before a full biogeographic scenario for these new *Trypanosoma* species can be inferred, other species of Miniopteridae bats must be investigated, particularly from Asia and Australia.

4.3. Multiple parasite infection in the Miniopteridae family

The Miniopteridae specimens analyzed so far were the only bats infected by multiple parasite species (i.e. by *T. dionisii, T.* sp. 1, *T.* sp. 2, *and T.* cf. *livingstonei*, Table 2; Fig. 3), while bats from other families were infected by one or rarely two taxa and show an apparent specificity between the bat family and the trypanosome species. High apparent host specificity could be due to inadequate spatial sampling (Poulin et al. 2006), but because many bat species were sampled in the same locations as the *Miniopterus*, and often within the same roosts living in sympatry (e.g. with *Myotis* or *Rhinolophus* spp. in Europe), biased sampling is not likely to affect this pattern. We rather hypothesise that the major factor driving the distribution of trypanosomes, at least in Europe and Africa, is truly specific to bats.

However, even if some parasites are apparently specific to Miniopteridae (i.e. *T.* sp. 1, *T.* sp. 2) it seems that this family has the potential to be infected by multiple parasite species and this could be explained by its ecology which may favor parasite dispersion. The genus *Miniopterus* (Bonaparte 1837) is widely distributed across the Old World, ranging through the majority of the Afrotropic, Palaearctic, Indomalayan and Australasian ecozones (Miller-Butterworth et al., 2007). Most of the *Miniopterus* species are highly social, as for example *M. schreibersii* forming the largest bat aggregation known in Europe (Ramos Pereira et al., 2009; Rodrigues et al., 2010). *M. natalensis* also has a wide geographic distribution across several biomes in Southern Africa and occurs in large colonies numbering thousands of individuals (Monadjem et al., 2010). These bat species often form mixed-species colonies with various other species of *Myotis* or *Rhinolophus* (Dietz and vonHelversen, 2004; Monadjem et al., 2010), opening the possibility for extensive inter-specific parasitic exchanges (Dick et al., 2009) and in the case of trypanosome parasite, transmission of the arthropod vector. Cimicid bugs were identified as the vector of trypanosomes in European Vespertilionidae bats (Gardner and Molyneux, 1988), while triatomine bugs are the vector in South America (Gaunt and Miles, 2000; Gourbière et al., 2012). The vectors of *T.* sp. 1 and *T.* sp. 2 identified in *Miniopterus* bats as well as many other *Trypanosoma* vectors remain unknown and possibly do not belong to cimicid bugs, as to our knowledge, this arthropod has rarely been found on Miniopteridae. Nevertheless, *Trypanosoma spp* in *Schizotrypanum* subclade were described only once in the literature from a bat bug (*Stricticimex brecispinosus*) in Burundi, Africa (Van den Berghe et al., 1963). Thenceforth, we can also suspect other arthropods, to be potential vectors.

5. Conclusion

Our results indicate that trypanosomes of the *T. cruzi* clade contain more species and are phylogenetically more diverse than previously reported, especially in the Old World, and this high diversity warrants further surveys and taxonomic scrutiny. The discovery of new *Trypanosoma* parasites in African and European bats give new insights into the evolutionary history of the *T. cruzi* and underscores the importance of thorough sampling when assessing the origin of epidemics of pathogens or parasites. Ancestral analyses reconstruction in accordance with the high diversity of *Trypanosoma* parasites in Sub-Saharan Africa and the basal position of the new taxa suggest that most clades have evolved in an African bat ancestor.

A broad phylogeographical analysis of trypanosomes in bats and other mammals especially from Asia, Australia and other part of Africa is still required to resolve taxonomic issues within this clade. Further surveys may help discovering more basal trypanosome lineages that might allow a finer inference of the ancestral distribution area of this clade.

CRediT authorship contribution statement

L. Clément: Conceptualization, Methodology, Software, Formal analysis, Resources, Writing - original draft, Visualization. **M. Dietrich:** Resources, Writing - review & editing. **W. Markotter:** Resources, Writing - review & editing. **N.J. Fasel:** Resources, Writing - review & editing. **A. Monadjem:** Resources, Writing - review & editing. **A. López-Baucells:** Resources, Writing - review & editing. **D. Scaravelli:** Resources, Writing - review & editing. **P. Théou:** Resources, Writing - review & editing. **R. Pigeault:** Methodology, Software, Writing - review & editing. **M. Ruedi:** Resources, Writing - review & editing. **P. Christe:** Conceptualization, Writing - review & editing, Visualization, Supervision, Funding acquisition.

Acknowledgment

We would like to warmly thank all the people that help us collecting data in the field in the different countries sampled in Europe and in Botswana. In Europe we particularly thank François Biollaz, Laura Bonny, Marina Durovic, Ludovic Dutoit, Peter Estok, Carles Flaquer,

Eleonore Genzoni, Nicolas Hazi, Ervis Loce, Igor Pavlinic, Jorge Palmeirim, Pierre Perreaz, David Progin, Pamela Priori, Xavier Puig, Albertine Roulet, Tamara Szentivanyi, Fardo Witsenburg. In Botswana we thank Raphaël Arlettaz, Michel Genoud and Olivier Glaizot. We also would like to thank technicians of the University in Lausanne, Céline Simon, Céline Stoeffel and Christine La Mendola for their help and advices during the lab work.

In South Africa, we are particularly grateful to all the staff and students from the Biosurveillance and Ecology of Emerging Zoonoses (BEEZ) Research Group in the Centre for Viral Zoonoses of University of Pretoria (UP-CVZ), Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) at the National Institute for Communicable Diseases (NICD), AfricanBats NPC and Ditsong National Museum of Natural History who assisted with field work pertaining to this research project. We would also like to thank the Ga Mafefe community in the Limpopo Province for supporting our research at Matlapitsi cave and the landowner Aquila Steel (Thabazimbi) and Motjoli Resources for permission to sample at Madimatle cave.

Likewise, we thank Sandile Gumedze, Senior Ecologist for the Eswatini National Trust Commission, who provided research permits to AM and NJF for capturing bats in Eswatini and for conducting the fieldwork in Mlawula Nature Reserve. Tal Fineberg allowed us to survey Mbuluzi Game Reserve. The Savanna Research Center and Mnqobi Mamba provided AM and NJF with logistic support. We thank the Staff from Shipstern Conservation & Management Area, Chunox-Sarteneja Road, Corozal District, Belize for their logistic support.

Ethics and regulatory approvals:

South African Study:

We obtained permission to conduct research under Section 20 of the Animal Disease Act (Act No. 35 of 1984) from the Department of Agriculture, Forestry and Fisheries of South Africa. This research was conducted with the approval of the University of Pretoria Animal Ethics committee (Project no. EC054-14 and EC059-14). Permits were obtained for bat sample collection from the South African provinces involved: the Department of Economic Development, Environment and Tourism Limpopo province directorate- wildlife permit no.CPM006806; Premier of the Province of Gauteng Nature conservation permit no. CPF6 no. 0027/ no.0109.

Switzerland:

Bat capture Authorization have been allowed by the CCO (Centre de Coordination Ouest pour l'étude et la protection des chauves-souris) and the DGE (Direction Générale de l'Environnement).

Funding

This work was financially supported in part by the National Research Foundation of South Africa under grant numbers UID 92524, 85756, and 91496 (held by Prof W. Markotter) as well as the South African Research Chair in Animal Infectious Diseases (Zoonoses) held by Prof W. Markotter, grant no. 98339 and the Swiss National Science Foundation grant 31003A-159600 held by Prof P. Christe.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106705.

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