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Mammals as engines of diversification: revisiting the evolutionary history of the *Trypanosoma cruzi* clade

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The *Trypanosoma cruzi* clade represents one of the most complex and ecologically diverse assemblages of mammalian trypanosomes. Although the group's human pathogenic member *T. cruzi* is best known as the etiological agent of Chagas disease, its evolutionary origins remain debated. Two main hypotheses attempt to explain the diversification of the *T. cruzi* clade: the supercontinent hypothesis, which proposes an ancient co-diversification with South American marsupials, and the bat-seeding hypothesis, which suggests a more recent origin through host-switching from bat trypanosomes. Here, we combined parasite and host phylogenies with global-fit cophylogenetic analyses to evaluate whether mammalian diversification has influenced the evolutionary history of the *T. cruzi* clade. Using PACo and ParaFit, we detected a weak but statistically significant global correspondence between mammal and trypanosome phylogenies. This correspondence was primarily associated with bat-trypanosome relationships, whereas associations involving non-bat mammals showed limited phylogenetic concordance. Together, these findings suggest that mammalian diversification may have influenced trypanosome evolution across multiple evolutionary contexts and timescales.

Keywords: bats, Chiroptera, Chagas disease, cophylogeny, host-switching, Trypanosomatids

El clado *Trypanosoma cruzi* representa uno de los ensambles más complejos y ecológicamente diversos de tripanosomas que infectan mamíferos. Aunque el miembro patógeno para humanos del grupo, *T. cruzi*, es ampliamente conocido como el agente etiológico de la enfermedad de Chagas, sus orígenes evolutivos continúan siendo motivo de debate. Dos hipótesis principales intentan explicar la diversificación del clado *T. cruzi:* la hipótesis del supercontinente, que propone una co-diversificación antigua con los marsupiales sudamericanos, y la hipótesis de "bat seeding", que sugiere un origen más reciente mediante cambios de hospedero a partir de tripanosomas asociados a murciélagos. En este estudio combinamos filogenias de parásitos y hospederos con análisis cofilogenéticos de ajuste global para evaluar si la diversificación de los mamíferos ha influido en la historia evolutiva del clado *T. cruzi*. Utilizando PACo y ParaFit, detectamos una correspondencia global débil pero estadísticamente significativa entre las filogenias de mamíferos y tripanosomas. Esta correspondencia estuvo asociada principalmente a las relaciones murciélago–*Trypanosoma*, mientras que las asociaciones que involucran a mamíferos no quirópteros mostraron una concordancia filogenética limitada. En conjunto, estos patrones sugieren que la diversificación de los mamíferos pudo haber influido en la evolución de los tripanosomas a través de múltiples contextos y escalas evolutivas.

Palabras clave: cambio de hospedero, Chiroptera, cofilogenia, enfermedad de Chagas, murciélagos, Tripanosomátidos

Mammals host a remarkable diversity of kinetoplastid parasites, among which the *Trypanosoma cruzi* clade (subgenus *Schyzotrypanum*) has received heightened attention due to its medical relevance. However, this attention from the medical community has nevertheless failed to resolve the evolutionary origin of *Schizotrypanum*. Determining how *T. cruzi* and its closest relatives emerged, either as relics of ancient Gondwanan lineages or as descendants of bat-associated trypanosomes, is essential for understanding how harmful human parasites originate, as well as reconstructing mammal-parasite coevolution more broadly (Stevens and Gibson 1999; Hamilton and Stevens 2012).

Genomic advances in molecular systematics have benefited our understanding of *Trypanosoma* evolution, revealing repeated transitions between aquatic and terrestrial hosts, frequent host-switching, and instances of recombination and hybridization (Westenberger *et al.* 2005; Simpson *et al.* 2006; Kaufer *et al.* 2017). However, parasite-based phylogenies must be contextualized within the broader evolutionary and biogeographic history of mammals, whose diversification and ecological variation fundamentally constrain and shape parasite radiation (Hamilton *et al.* 2012; Lima *et al.* 2015).

Although trypanosomes in general include taxa that can infect a variety of animals, all known species within *Schizotrypanum* exclusively infect mammals, suggesting a long and dynamic history of adaptation to mammal hosts (Hamilton and Stevens 2010; Lima *et al.* 2013; Cottontail *et al.* 2014; Juárez-Gabriel *et al.* 2024). Two major hypotheses

attempt to explain the origin of this mammal-exclusive clade. The supercontinent hypothesis proposes that the lineage diversified alongside South American marsupials and Triatominae vectors during the breakup of Gondwana, representing a deep Mesozoic ancestry (Stevens and Gibson 1999; Hamilton and Stevens 2012). In contrast, the bat-seeding hypothesis does not propose a single transmission event from bats to other mammals, but rather that bats acted as the primary ancestral hosts and evolutionary reservoir of the *T. cruzi* clade, from which multiple lineages diversified and subsequently colonized other mammalian groups through repeated host-switching events (Hamilton *et al.* 2012; Lima *et al.* 2013; Lima *et al.* 2015).

Aspects of mammalian diversification and observed trypanosome diversity correspond with both hypotheses. Marsupials retain long-standing associations with *T. cruzi*, a pattern historically interpreted as consistent with a Gondwanan origin of the clade [based largely on ecological persistence rather than explicit phylogenetic congruence] (Stevens and Gibson 1999; Hamilton and Stevens 2012). However, bats exhibit unparalleled trypanosome diversity and dispersal capacity, supporting their role as primary contributors to diversification within *Schizotrypanum* (Hamilton *et al.* 2012; Cottontail *et al.* 2014; Lima *et al.* 2015). Against the backdrop of both hypotheses, rodents, carnivores, and primates appear to have acquired *T. cruzi* through more recent and ecologically mediated host-switch events (Rocha *et al.* 2013; Jansen *et al.* 2015). By integrating molecular phylogenies and global-fit cophylogenetic analysis, the aim of this study was to analyze how mammalian evolution has influenced the origin and diversification of the *T. cruzi* clade.

Materials and methods

Trypanosoma spp. and mammalian hosts sequence dataset. We compiled publicly available sequences belonging to members of the *T. cruzi* clade. Sequences were retrieved from GenBank from studies that explicitly reported natural infections in mammals. Only accessions with confirmed host origin in the original publication were included. We used a fragment of the *18S rDNA* gene because it is widely available and informative for resolving relationships within *Trypanosoma*.

Redundant, low-quality (<500 bp), chimeric, or environmental sequences lacking host information were excluded. The final dataset consisted of 19 parasite species or lineages (see Supplementary Table S1).

To reconstruct host phylogeny, we assembled mitochondrial *cytochrome b* (*Cytb*) sequences exclusively from mammalian species in which trypanosomes have been molecularly confirmed. Sequences were obtained from peer-reviewed publications. When multiple sequences were available, we prioritized those from the same locality where the associated parasite was reported. The final host dataset included 58 species across different orders from Mammalia (see Supplementary Table S2).

Phylogenetic analysis and host-parasite matrix. Parasite and host datasets were aligned independently using MAFFT v7 with the L-INS-i algorithm. Resulting alignments were visually inspected and manually trimmed in AliView to remove ambiguously aligned regions and ensure positional homology.

Phylogenetic trees were inferred using IQ-TREE v2, implementing the best-fit substitution model selected by ModelFinder based on Bayesian Information Criterion (BIC). For *Trypanosoma* tree we used 19 sequences with 878 nucleotid sites, and the Best-fit model calculated was: TIM3e+I+G4. On the other hand, for host tree, we used 58 sequences with 1140 nucleotid sites, and the Best-fit model according to BIC was: GTR+F+I+G4. Nodal support was assessed using 1,000 ultrafast bootstrap replicates (UFBoot) and SH-aLRT tests. Trees were visualized and edited in FigTree v.1.4.4.

We constructed a binary host–parasite association matrix. Each parasite sequence was matched to its corresponding host species based on published reports. Only associations explicitly supported by primary literature were included; ambiguous or assumed associations were excluded (see Supplementary Table S3).

Cophylogenetic analyses. To assess whether mammal phylogeny influences the diversification of trypanosomes in the *T. cruzi* species complex, we tested whether there is a statistically significant global fit between host and parasite trees. To accomplish this, we first converted the phylogenetic trees of the *T. cruzi* species complex and their mammal hosts into distance matrices using the "*cophenetic.phylo*" function from the R package *ape* (v 5.8-1) (Paradis and Schliep 2019) as implemented in R Studio (v. 4.5.0), applying a cailliez correction to account for negative eigenvalues. We then tested the global congruence of these trees using two cophylogenetic fitting methods: PACo (Balbuena *et al.* 2013), in which we performed 10,000 permutations to generate a null distribution, and ParaFit (Legendre *et al.* 2002), in which we performed 999 permutations. For the PACo results, we also analyzed how strongly individual host-parasite relationships influenced the global congruence of host and parasite trees by performing jackknife estimation of their square residuals with the "*paco_links*" function. We assessed whether individual host-parasite relationships were statistically significantly correlated using the "*ParaFitLink1*" function in ParaFit.

To determine if bat:trypanosome relationships contributed disproportionately to the global signal of cophylogenetic concordance detected by PACo, we sorted the PACo residuals into two groups that represented bat:trypanosome and non-bat:trypanosome associations. We then performed a one-tailed two-sample *t*-test to assess whether bats and their trypanosomes had significantly lower residuals than non-bats, which would suggest they disproportionately drive the global cophylogenetic signal in the dataset.

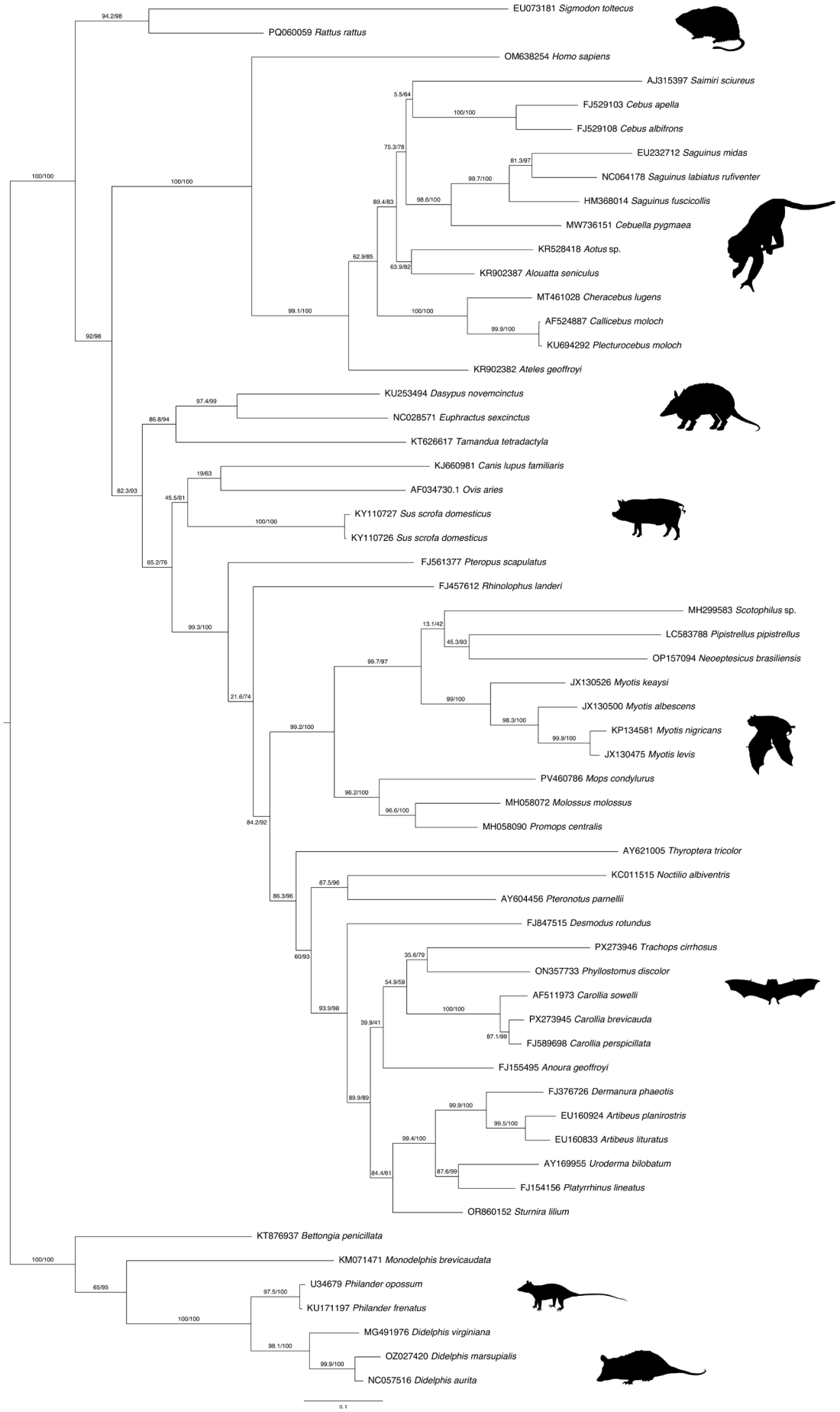

Figure 1. Phylogenetic relationships among mammalian hosts included in this study, inferred from mitochondrial (*Cytb*) sequences. Only ≥70 branch support values are shown.

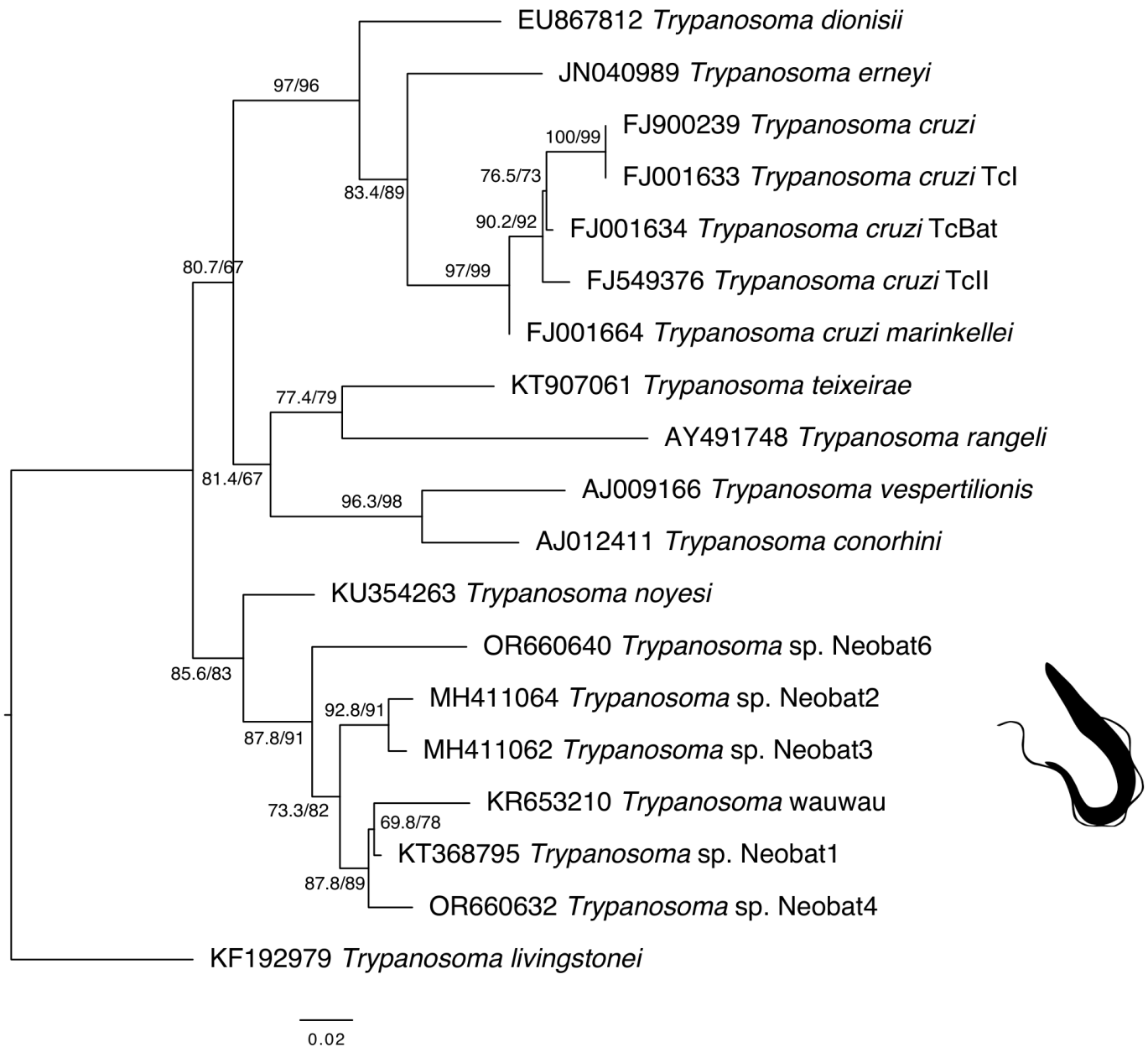

Figure 2. Phylogenetic relationships among *Trypanosoma cruzi* clade species inferred from partial *18S rDNA* sequences.

Results

The mammalian phylogeny inferred from the *Cytb* sequences recovered well-supported relationships across the major mammalian groups included in the analysis (Figure 1). These comprised Artiodactyla, Carnivora, Chiroptera, Marsupialia, Rodentia, and Xenarthra, each forming distinct and largely well-supported clades consistent with established mammalian phylogenies. Within Chiroptera, representatives of Phyllostomidae (*Artibeus, Carollia, Dermanura, Phyllostomus, Sturnira, Uroderma, Anoura, Trachops, Desmodus, Pteronotus*), Molossidae (*Molossus, Promops, Mops*), Vespertilionidae (*Myotis, Eptesicus, Pipistrellus, Scotophilus*), Rhinolophidae (*Rhinolophus*), Pteropodidae (*Pteropus*) and, Thyropteridae (*Thyroptera*), were recovered as monophyletic or near-monophyletic groups, with generally high branch nodal support value (>85%).

Marsupials clustered into a coherent clade including Didelphidae (*Didelphis, Philander, Monodelphis*), and Potoroidae (*Bettongia*), while placental mammals were distributed among expected orders. Although some deeper relationships showed moderated support (Figure 1), overall tree topology was congruent with previously published mitochondrial-based mammalian phylogenies, *i.e.* Primates were represented by multiple Neotropical lineages spanning Atelidae, Cebidae, Pitheciidae, Callitrichidae, Aotidae, and Hominidae, indicating that the host tree provides an appropriate framework for subsequent cophylogenetic analysis.

Phylogenetic reconstruction of trypanosomes based on *18S rDNA* sequences resolved the *T. cruzi* clade as a well-supported monophyletic group (Figure 2). Within this clade, bat associated trypanosomes occupied several basal

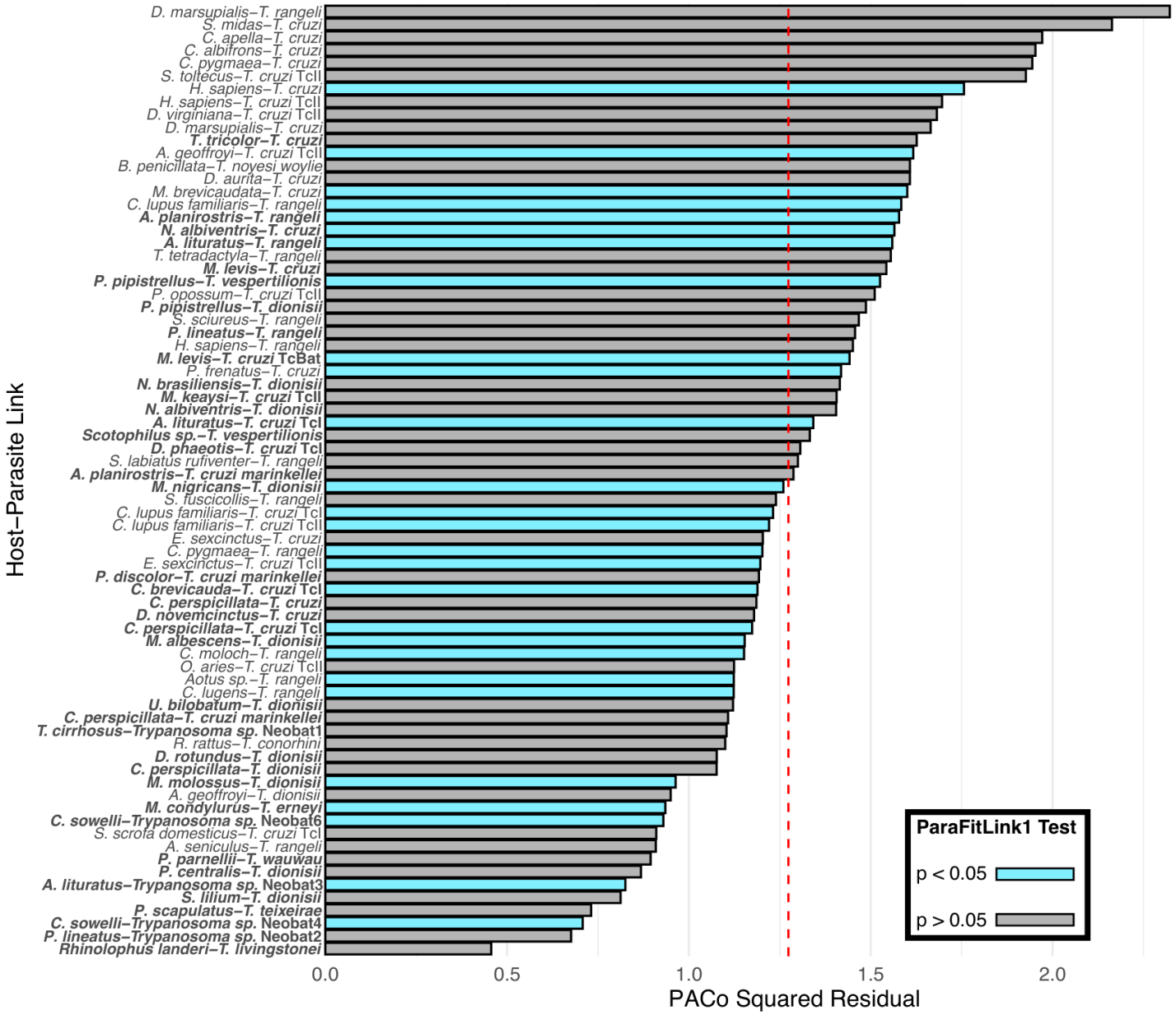

Figure 3. PACo jackknifed squared residuals of each host-parasite link between mammal hosts and trypanosome parasites included in this analysis. Lower residual values (bars) correspond to greater cophylogenetic signal of that host-parasite link. Bars are colored according to whether a given host-parasite link was found to exhibit statistically significant cophylogenetic signal according the ParaFitLink1 test. Red dashed line denotes the overall median PACo squared residual value.

and early diverging positions. Species such as *T. dionisii*, *T. erneyi*, *T. cruzi*, *T. livingstonei*, and multiple Neotropical bat-associated lineages formed distinct, well-supported subclades. Together, the reconstructed mammalian and trypanosome phylogenies provide the basis for evaluating patterns of host-parasite phylogenetic correspondence using global-fit cophylogenetic analyses.

Both PACo and ParaFit detected a weak but statistically significant global correspondence between host and parasite trees when compared to random permutations (PACo: m2 = 33.4040, p = 0.0004; ParaFit: ParaFitGlobal = 2.1457, p = 0.003). The weak global fit suggests that this statistical significance is driven by a few strongly correlated host-parasite associations amidst largely incongruent host-parasite trees, which was recapitulated by individual jackknifed squared residual values calculated in PACo, as well as tests of association significance in ParaFit. PACo residual values appeared to demonstrate that bat trypanosomes were the biggest drivers of this (Figure 3): of the 10 host-parasite associations contributing the most to the global cophylogenetic signal (lowest squared residuals), 8 involve bats, while none of the 10 associations with the highest squared residuals involve bat hosts. A one-tailed two sample *t*-test confirmed the statistical significance of this pattern, as bat:trypanosome relationships were characterized by PACo residuals that were significantly lower than those of non-bat:trypanosome relationships (p < 0.001, Cohen's d = 0.95) (Figure 4). This supports bats and their resident trypanosomes as the primary drivers of global cophylogenetic signal between these two trees.

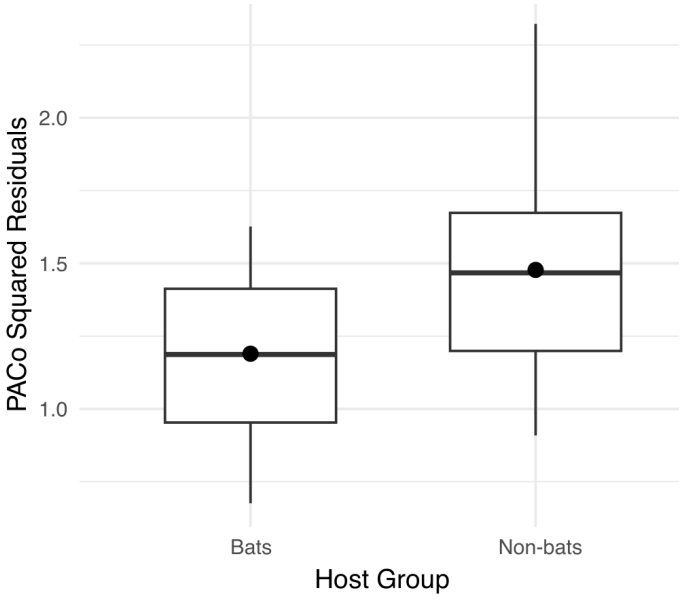

Figure 4. Distribution of PACo jackknifed squared residual values of host-trypanosome links in bat vs. non-bat hosts. Mean residual values added to plots as a black dot. Lower residuals indicate greater contribution to global cophylogenetic signals. Bats and their associated trypanosomes exhibit significantly lower residuals when compared to non-bat hosts ($p < 0.001$, Cohen's d = 0.95).

However, the identification of significant relationships using ParaFitLink1 did not strongly favor bat-trypanosome associations as drivers of the cophylogenetic signal. Instead, it was more likely to identify trypanosome taxa with very broad host ranges, such as *T. cruzi* infecting hosts as distantly related as the placental *Homo sapiens* (humans) and the marsupial *Monodelphis brevicaudata* (northern red-sided opossum), as exhibiting significant cophylogenetic concordance. Significant ParaFit *p*-values were also not strongly correlated with low squared PACo residuals (Figure 3), consistent with previous findings that ParaFit frequently identifies associations with very high PACo residuals to be nevertheless statistically significant.

Discussion

The evolutionary origin of the *T. cruzi* clade remains one of the most debated topics in kinetoplastid systematics, with two dominant competing hypotheses: a Gondwanan origin associated with early South American marsupials (Hoare 1972; Stevens and Gibson 1999; Hamilton and Stevens 2012), and a more recent origin arising from bat-associated trypanosomes (Hamilton *et al.* 2012). By explicitly testing patterns of host-parasite phylogenetic correspondence across mammalian hosts with confirmed infections, this study contributes new evidence to clarify how mammalian diversification shaped the evolutionary trajectory of the *T. cruzi* clade.

Our cophylogenetic analyses detected global cophylogenetic signal between host and parasite phylogenetic trees, suggesting mammal diversification does influence diversification of this trypanosome clade, as well as providing some support for the bat-seeding hypothesis. Although the program ParaFit did not find evidence of bats driving cophylogenetic signal, its assumption of cophylogenetic symmetry likely limits how well the analysis can assess biological reality. PACo, meanwhile, assumes asymmetric dependence, in which the parasite phylogeny is unidirectionally influenced by the host phylogeny (Balbuena *et al.* 2013), which better represents how host-parasite clades tend to evolve. This asymmetric analysis instead produced a strong and statistically significant finding that bat:trypanosome relationships are the primary drivers of cophylogenetic signal in this host-parasite assemblage. This concordance between bat and parasite phylogenies supports the hypothesis that bat diversification shaped trypanosome evolution, while the relative lack of cophylogenetic signal in non-bat:trypanosome associations is consistent with host switching leading to colonization of other hosts. Furthermore, marsupial:trypanosome links corresponded to some of the highest residuals in the PACo analysis, suggesting a lack of cophylogenetic signal and arguing against a marsupial-associated Gondwanan origin of *Schizotrypanum* from a cophylogenetic perspective.

Nevertheless, the disagreement between PACo and ParaFit results highlights the difficulties of applying traditional cophylogenetic analyses to systems that include broadly generalist parasites, such as *T. cruzi* and *T. rangeli,* which infect hosts as distantly related as marsupials and placentals. Such extreme generalists complicate cophylogenetic analysis and can lead to erroneous findings of congruence such as those presumably inferred by ParaFit in this work (Refrégier *et al.* 2008; de Vienne *et al.* 2013). It is also possible that sampling bias has made some taxa, such as the clade of novel Neotropical bat trypanosomes, appear more host-specific than they truly are (Sweet *et al.* 2016; Dallas *et al.* 2017). Further sampling of Mammalia may uncover these novel taxa in other hosts and dilute the cophylogenetic signal associated with bats. While we encourage improved sampling of trypanosomes across mammal diversity to address this concern, cophylogenetic analysis of all currently known taxa in the *Schizotrypanum* clade favors bat-seeded origins.

Multiple multilocus phylogenies have demonstrated that *Schizotrypanum* is a monophyletic group composed exclusively of mammalian parasites (Hamilton and Stevens 2010; Hamilton *et al.* 2012; Austen and Barbosa 2021). Within this clade, bat trypanosomes such as *T. dionisii*, *T. cruzi marinkellei*, *T. erneyi*, and *T. livingstonei* consistently occupy basal positions, indicating that the earliest diversification events occurred within bats rather than in marsupials or other terrestrial hosts (Marcili *et al.* 2009; Lima *et al.* 2013; Cottontail *et al.* 2014; Dos Santos *et al.* 2018). This recurrent phylogenetic pattern forms the conceptual core of the bat-seeding hypothesis, which proposes bats as the primary ancestral hosts rather than incidental reservoirs.

Furthermore, the discovery of *T. livingstonei* in African bats provides additional support for the idea that the origins of the *T. cruzi* clade may be older and geographically

broader than initially assumed (Lima *et al.* 2013; Pereira *et al.* 2022). Rather than being strictly Neotropical and tightly tied to South American marsupials, ancestral lineages may have diversified in bats across multiple continents, consistent with the high mobility and global distribution of these mammals. In addition, the persistence of the bat-restricted TcBat lineage, even in regions where other *T. cruzi* DTUs circulate broadly among terrestrial mammals, reinforces the notion that bats constitute an ancestral reservoir and continue to harbor unique parasite lineages (Marcili *et al.* 2009; Lima *et al.* 2015).

These phylogenetic insights are consistent with the ecological characteristics of bats. Their ability to fly, form dense colonies, migrate long distances, and exploit a wide range of habitats create ideal conditions for parasite transmission, diversification, and geographic expansion (Cottontail *et al.* 2014; Austen and Barbosa 2021; Peixoto *et al.* 2019). Consequently, bats act not only as hosts but also as evolutionary engines, repeatedly generating opportunities for host-switch events and shaping the structure of the *T. cruzi* clade.

On the other hand, marsupials, particularly didelphid opossums, are well established as key components of contemporary sylvatic cycles of *T. cruzi* in the Neotropics (Jansen *et al.* 2015; Jansen *et al.* 2018). Their high infection rates and capacity to support parasite development have long been cited as evidence for a deep evolutionary association. However, recent phylogenetic studies of didelphids show that most of their diversification occurred during the Miocene (Jansa *et al.* 2013), well after the breakup of Gondwana. This temporal mismatch weakens the plausibility of a strict Gondwanan co-diversification scenario, suggesting that marsupials are more likely to represent long-standing ecological reservoirs rather than ancestral hosts.

Similarly, numerous other mammalian groups, including rodents, carnivores, xenarthrans, and primates, are frequently infected by *T. cruzi* in sylvatic environments (Rocha *et al.* 2013; Gürtler *et al.* 2015; Jansen *et al.* 2018). While these associations highlight the ecological flexibility of the parasite, they do not imply a primary evolutionary role for these hosts. Instead, the available evidence suggests that these mammals' function mainly as secondary or bridge hosts, particularly in disturbed or anthropogenic landscapes, contributing more to parasite maintenance and spread than to its early diversification (Hamilton *et al.* 2012; Jackson 2015).

Recognizing bats as central to the origin of the *T. cruzi* clade has important implications for both evolutionary biology and disease ecology. It underscores the deep evolutionary flexibility of the parasite and suggests that novel host associations may continue to arise. It also highlights the need for expanded sampling of bat trypanosomes worldwide, particularly in Africa and Asia, where basal lineages remain poorly characterized (Pinto *et al.* 2015; Austen and Barbosa 2021).

Although this study refines the evolutionary context of the *T. cruzi* clade, several limitations remain. Genomic sampling of bat-associated trypanosomes is still incomplete, deep-time calibration of host and parasite phylogenies remains uncertain, and vector–host interactions are poorly resolved in many regions. Future work integrating genome-scale data, expanded host sampling, and time-calibrated cophylogenetic frameworks will be essential to fully reconstruct the origins and diversification of *T. cruzi* clade.

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Declaration of Artificial Intelligence use

Artificial intelligence was used only to assist with English grammar and manuscript wording (Grammarly Pro). All suggestions were carefully reviewed by the authors, who take full responsibility for the final version of the manuscript. The original manuscript, with all ideas, is original to the authors.

Author contributions

Javier Juárez-Gabriel: conceptualization, methodology, investigation, data curation, formal analysis, visualization. Spenser J. Babb-Biernacki: methodology, formal analysis and visualization. Ingeborg Becker: conceptualization and supervision. All authors made substantial contributions to the design, development, analysis, and writing, reviewing and editing of the manuscript, approved the final version, and agreed to be accountable for all aspects of the work.

Data availability

All DNA sequences used in this study were obtained from public databases and are available in GenBank. The corresponding accession numbers are listed in Supplementary Table S1.

The sequence alignments used for phylogenetic reconstruction of hosts (*Cytb*, Supplementary Data S1) and parasites (*18S rDNA*, Supplementary Data S2), as well as the host–parasite association matrices employed in the cophylogenetic analyses (Supplementary Table S3), are provided as Supplementary Material accompanying this manuscript.

Supplementary data

SDT1. Accession numbers of mammalian *Cytb* sequences used in this study.

SDT2. Accession numbers of trypanosomatid *18S rDNA* sequences used in this study.

SDT3. Host–parasite association matrices for cophylogenetic analyses.

SD1. FASTA-formatted alignment of mammalian *Cytb* sequences used in this study.

SD2. FASTA-formatted alignment of trypanosomatid *18S rDNA* sequences used in this study.

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