Cytochrome c oxidase subunit 1 gene as a DNA barcode for discriminating *Trypanosoma cruzi* DTUs and closely related species

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**Abstract**

**Background:** The DNA barcoding system using the cytochrome c oxidase subunit 1 mitochondrial gene (cox1 or COI) is highly efficient for discriminating vertebrate and invertebrate species. In this present study, we examined the suitability of cox1 as a marker for *Trypanosoma cruzi* identification from other closely related species. Additionally, we combined the sequences of cox1 and the nuclear gene glucose-6-phosphate isomerase (GPI) to evaluate the occurrence of mitochondrial introgression and the presence of hybrid genotypes.

**Methods:** Sixty-two isolates of *Trypanosoma* spp. obtained from five of the six Brazilian biomes (Amazon Forest, Atlantic Forest, Caatinga, Cerrado and Pantanal) were sequenced for cox1 and GPI gene fragments. Phylogenetic trees were reconstructed using neighbor-joining, maximum likelihood, parsimony and Bayesian inference methods. Molecular species delimitation was evaluated through pairwise intraspecific and interspecific distances, Automatic Barcode Gap Discovery, single-rate Poisson Tree Processes and multi-rate Poisson Tree Processes.

**Results:** Both cox1 and GPI genes recognized and differentiated *T. cruzi*, *Trypanosoma cruzi marinkellei*, *Trypanosoma dionisii* and *Trypanosoma rangeli*. Cox1 discriminated Tcbat, TcI, TcII, TcIII and TcIV. Additionally, TcV and TcVI were identified as a single group. Cox1 also demonstrated diversity in the discrete typing units (DTUs) TcI, TcII and TcIII and in *T. c. marinkellei* and *T. rangeli*. Cox1 and GPI demonstrated TcI and TcII as the most genetically distant branches, and the position of the other *T. cruzi* DTUs differed according to the molecular marker. The tree reconstructed with concatenated cox1 and GPI sequences confirmed the separation of the subgenus *Trypanosoma* (*Schizotrypanum*) sp. and the *T. cruzi* DTUs TcI, TcII, TcIII and TcIV. The evaluation of single nucleotide polymorphisms (SNPs) was informative for DTU differentiation using both genes. In the cox1 analysis, one SNP differentiated heterozygous hybrids from TcIV sequences. In the GPI analysis one SNP discriminated Tcbat from TcI, while another SNP distinguished TcI from TcIII.

**Conclusions:** DNA barcoding using the cox1 gene is a reliable tool to distinguish *T. cruzi* from *T. c. marinkellei*, *T. dionisii* and *T. rangeli* and identify the main *T. cruzi* genotypes.

**Keywords:** Cytochrome c oxidase subunit 1, *Trypanosoma cruzi*, Discrete typing units, Glucose-6-phosphate isomerase, Barcoding, Subgenus *Trypanosoma* (*Schizotrypanum*)
Background
How many species are there on Earth? Estimates suggest that approximately 90% of species remain undescribed [1]. The identification and classification of biodiversity is a practice that has always fascinated humankind. The Greek philosopher Aristotle (4th century BC) developed the first classification system, which was used for nearly 2000 years [2]. In the 1700s, Carl Linnaeus [3] developed the concept of binomial nomenclature using Latin, which was the language of educated men at his time. Binomial nomenclature is a standard method for scientists that speak different languages to classify living things to clearly communicate their discoveries. Recently, DNA sequences have been applied in the classification of life forms. However, different methods and DNA regions have been used to compare the same taxonomic groups, frequently leading to conflicting results.

In the search for a simple method to identify and compare species, Hebert et al. [4] proposed DNA barcoding, a new system of species identification using the cytochrome c oxidase subunit 1 mitochondrial gene (cox1 or COI) as a standardized single molecular marker for the classification of animal species. One of the requirements of the DNA barcoding approach is that species identification is associated with a voucher belonging to a curated biological collection, enabling follow up and a strategy for corroborating species identification [5]. Until recently, national barcode networks have been established in 11 countries, including Brazil, which uses the Brazilian Barcode of Life (BrBOL) [6].

In addition to the identification of known and new species, barcoding with the cox1 gene is suggested as a standard for cryptic taxa discovery, association of different life stages of the same species and wildlife conservation genetics [5]. Cox1 appears to have a better phylogenetic signal than the other mitochondrial genes [7]. Some authors argue that the evolution of the cox1 gene is sufficiently rapid to distinguish between closely related species and investigate intraspecific diversity [4, 8]. The cox1 region is highly efficient for discriminating vertebrate and invertebrate species [5, 9, 10], but is not suited for plants and some fungal species [4, 11, 12]. The use of cox1 for identifying protozoa and other unicellular organisms is still in its initial stage but has been demonstrated as a promising barcode marker for dinoflagellates, Paramecium sp., Nemertea [13–15]. Moreover, joint analyses of nuclear and mitochondrial markers may be necessary to distinguish species [1].

Therefore, the small subunit ribosomal DNA (SSU rDNA) has been proposed as a first step for a protist barcode, followed by the use of a second marker specific for each group [6]. To confirm a gene as a barcode marker, it is essential to calculate the “barcoding gap”, that is, the gap between the maximum intraspecific and minimum interspecific genetic distances that establish the limits between species [16]. Several molecular targets for trypanosomatid identification have previously been used: internal transcribed spacer (ITS) [17], mini-exon [18], glucose-6-phosphate isomerase (GPI) [19–21], cytochrome b (cytb) [22, 23], multilocus sequencing typing (MLST) [24], the variable regions V7 V8 of SSU rDNA, and glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) genes [22, 25, 26]. However, there is no consistency concerning which DNA region to use as a trypanosomatid barcode, and none of these markers were evaluated as a universal marker for biodiversity analysis.

With the emergence of molecular techniques with higher analytical power, an increasing number of new species of Trypanosoma have been reported [26, 27] and it is becoming increasingly clear that there is a high diversity of Trypanosoma spp. within the subgenus Schizotrypanum of Trypanosoma. The extreme morphological similarity and phylogenetic proximity of these species, and the use of tools with lower analytical power, resulted in the isolation of numerous species of T. (Schizotrypanum) from the wild transmission cycle remaining only at the generic level or wrongly diagnosed as Trypanosoma cruzi, highlighting the importance of a universal method to characterize T. cruzi, its subpopulations and representatives of the subgenus Schizotrypanum.

Trypanosoma cruzi (Trypanosomatida: Trypanosomatidae) is a successful parasite that is capable of establishing an extracellular cycle in dozens of triatomine species (Hemiptera: Reduviidae) and infecting almost every cellular type from more than 100 mammal species distributed from the south of the USA to the south of Latin America [28, 29]. Although described as one taxon, T. cruzi has a remarkable genetic heterogeneity that was already recognized by the very first authors who researched it [30–32]. The T. cruzi population structure has been classified as clonal or, at least, primarily clonal [32, 33] and hybridization events have also been observed, suggesting that genetic recombination occurs in this group [33, 34]. Moreover, the extent to which these hybridization events are responsible for the enormous heterogeneity of this parasite remains a controversial issue [19, 35–37]. Several techniques, (biological, biochemical and molecular) applied to define T. cruzi subpopulations have led to different designations (Fig. 1) [25, 30, 35, 38–51]. Presently, T. cruzi subpopulations are assembled in six discrete typing units (DTUs) - named Tcl, TcII, TcIII, TcIV, TcV, TcVI [29] - and a genotype called Tcbat, initially described as being associated with Chiroptera [25], although it was previously isolated from a child [52] and from mummified human tissue [53]. However, Barnabé et al. [51] recently proposed the subdivision of T. cruzi into three mitochondrial clades (mtTcl, mtTcII and mtTcIII) based on the analysis of the nucleotide sequences available in the GenBank database, showing that
the classification of *T. cruzi* subpopulations remains a debatable issue.

In the present study, we tested *cox1* as a DNA barcode to identify *T. cruzi* from other closely related species belonging to the subgenus *Schizotrypanum* and to examine the genetic diversity within *T. cruzi* and its DTUs to further understand the ecology of the species of *T. (Schizotrypanum)*. Additionally, we evaluated *cox1* as a target to also identify *Trypanosoma (Tejeraia) rangeli*, as this trypanosome shares the geographical distribution, vectors and mammal hosts of *T. cruzi* and is also included in the *T. cruzi* clade [54]. To evaluate the occurrence of mitochondrial introgression events and the presence of hybrid genotypes we tested a combination of *cox1* (uniparental inheritance) and the nuclear gene glucose-6-phosphate isomerase (*GPI*) (biparental inheritance). *GPI* is one of the genes sequenced for an extensive number of *T. cruzi* isolates distributed over several geographic regions [51], enabling a comparison of the sequences generated in the present study. Thus, the precise identification of these *Trypanosoma* species is of the utmost importance. In addition, the present study will enhance the amount of nucleotide sequences available for comparison, since the GenBank database still lacks a broader deposit on trypanosomatid sequences for the *cox1* gene.

**Methods**

**Samples**

The present study included 62 isolates of *Trypanosoma* spp. obtained from 16 different genera of free-ranging wild mammals and from triatomines of the genera *Rhodnius* and *Triatoma*, distributed in five of the six Brazilian biomes (Amazon Forest, Atlantic Forest, Caatinga, Cerrado and Pantanal) (Table 1 Fig. 2b). Isolates were cryopreserved and deposited in the Coleção de *Trypanosoma* de Mamíferos Silvestres, Domésticos e Vetores, Fiocruz - COLTRYP (Oswaldo Cruz Foundation, Rio de Janeiro - RJ/Brazil) in previous studies. Details on animal capture and parasite culture methods are described elsewhere [55–57]. After thawing, the cells were sown in axenic culture media (NNN and LIT). When the cultures reached the exponential growth phase, DNA was extracted using the phenol-chloroform method, as described elsewhere [58]. The *T. dionisii* samples used in the present study were molecularly identified using 18S (SSU) and gGAPDH sequences and subsequently deposited in COLTRYP.

Nine *T. cruzi* references were also genotyped and used as DTU standards. Colombiana (COLPROT 004), Dm28c (COLPROT 010), Y (COLPROT 106), 3663 (COLPROT 608), 4167 (COLPROT 607) DNA samples obtained from the Protozoa Collection - COLPROT (FIOCRUZ, Rio de Janeiro, Brazil); Sc43cl1, Bug2148cl1, CLBrener and Tulacl2 DNA samples were a kind gift from Dr. Michael Miles from the London School of Hygiene & Tropical Medicine (London, UK). The *T. rangeli* lineage R1625 DNA sample (COLPROT 002) was obtained from COLPROT (Table 1). In addition, *cox1* and *GPI* sequences were retrieved from the GenBank database and used as references (Table 1).

**PCR and sequencing**

A fragment of the mitochondrial gene *cox1* was amplified using a set of forward (5’-CCA WAC AAC AAA CAT ATG ATG CTG C-3’) and reverse (5’-TCC HGA TAT GGT WTT KCC YGC-3’) primers. Polymerase chain reaction (PCR) was conducted in a 50 μl final reaction volume containing 2.5 mM MgCl₂ (Invitrogen, Carlsbad, California, USA), 0.25 mM of each nucleotide (Thermo Scientific, Waltham, Massachusetts, USA), 0.25 mM of each primer (IDT, Coralville, Iowa, USA), 1.0 U of Platinum Taq DNA polymerase (Invitrogen), and 50 ng of DNA template. The amplification was performed with a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, California, USA) with the following cycle conditions: denaturation at...
| Strain code     | Host or Vector | State/Biome | Lineage | Hap (cox1)^a | GenBank accession number (cox1, GPI) |
|-----------------|----------------|-------------|---------|--------------|------------------------------------|
| TCC 1994        | Myotis levis   | SP/Brazil   | Tcbat   | hap9         | KT327226^b KT327312^b              |
| COLTRYP 038     | Rattus rattus  | CE/Caatinga | Tcl     | hap3         | KU145414 KT390200                  |
| COLTRYP 039     | Rattus rattus  | CE/Caatinga | Tcl     | hap3         | KU145415 KT390201                  |
| COLTRYP 048     | Didelphis albiventris | CE/Caatinga | Tcl     | hap3         | KU256221 KU256227                  |
| COLTRYP 087     | Didelphis albiventris | CE/Caatinga | Tcl     | hap3         | KU145426 KT390212                  |
| COLTRYP 128     | Didelphis albiventris | CE/Caatinga | Tcl     | hap3         | KU145433 KT390219                  |
| COLTRYP 018     | Thylamys macrurus | MS/Pantanal | Tcl     | hap1         | KU256219 KU256225                  |
| COLTRYP 084     | Oecomys sp.    | MS/Pantanal | Tcl     | hap1         | KU145425 KT390211                  |
| COLTRYP 103     | Monodelphis domestica | MS/Pantanal | Tcl     | hap3         | KU145428 KT390214                  |
| COLTRYP 115     | Thylamys macrurus | MS/Pantanal | Tcl     | hap1         | KU145430 KT390216                  |
| COLTRYP 368     | Nasua nasua    | MS/Pantanal | Tcl     | hap3         | KU145441 KT390227                  |
| COLTRYP 468     | Oecomys mammorae | MS/Pantanal | Tcl     | hap1         | KU145443 KT390229                  |
| COLTRYP 053     | Rhodnius pictipes | PA/Amazon   | Tcl     | hap2         | KU145418 KT390204                  |
| COLTRYP 055     | Didelphis marsupialis | PA/Amazon   | Tcl     | hap2         | KU145419 KT390205                  |
| COLTRYP 126     | Didelphis marsupialis | PA/Amazon   | Tcl     | hap2         | KU145432 KT390218                  |
| COLTRYP 220     | Didelphis marsupialis | PA/Amazon   | Tcl     | hap5         | KU145435 KT390221                  |
| COLTRYP 339     | Rhodnius pictipes | PA/Amazon   | Tcl     | hap2         | KU145438 KT390224                  |
| Epinet 88,115   | Rhodnius robustus | PA/Amazon   | Tcl     | hap3         | KU145452 KT390238                  |
| COLTRYP 356     | Akodon robustus     | RJ/Atlantic Forest | Tcl | hap3         | KU145459 KT390225                  |
| Epinet 88,127   | Rhodnius sp.     | RJ/Atlantic Forest | Tcl | hap4         | KU145454 KT390240                  |
| Epinet 88,132   | Philander frenatus | RJ/Atlantic Forest | Tcl | hap4         | KU145456 KT390242                  |
| Epinet 88,135   | Philander frenatus | RJ/Atlantic Forest | Tcl | hap4         | KU145457 KT390243                  |
| COLTRYP 003     | Didelphis aurita  | SC/Atlantic Forest | Tcl | hap1         | KU145410 KT390196                  |
| COLTRYP 036     | Didelphis albiventris | GO/Cerrado   | Tcl     | hap8         | KU145413 KT390199                  |
| COLTRYP 042     | Desmodus rotundus | TO/Cerrado   | Tcl     | hap4         | KU145416 KT390202                  |
| COLTRYP 136     | Phyllostomus abilica | TO/Cerrado   | Tcl     | hap4         | KU145434 KT390220                  |
| COLTRYP 224     | Carollia perspicillata | TO/Cerrado   | Tcl     | hap4         | KU145436 KT390222                  |
| COLTRYP 305     | Philander opossum | TO/Cerrado   | Tcl     | hap6         | KU145437 KT390223                  |
| COLTRYP 362     | Gracilinanus sp.  | TO/Cerrado   | Tcl     | hap6         | KU145440 KT390226                  |
| Colombiana      | Homo sapiens    | Colombia     | Tcl     | hap7         | KU168553 KU168558                  |
| Dm28c           | Didelphis marsupialis | Venezuela   | Tcl     | hap4         | KU168554 KU168559                  |
| Silvio          | Homo sapiens    | PA/Amazon    | Tcl     | hap2         | FJ203996^b                        |
| OPS21c111       | Homo sapiens    | Venezuela    | Tcl     |              | AY484472^b                        |
| COLTRYP 061     | Leontopithecus chrysomelas | BA/Atlantic Forest | TcIl | hap11        | KU145420 KT390206                  |
| COLTRYP 062     | Leontopithecus chrysomelas | BA/Atlantic Forest | TcIl | hap11        | KU145421 KT390207                  |
| COLTRYP 063     | Leontopithecus chrysomelas | BA/Atlantic Forest | TcIl | hap11        | KU145422 KT390208                  |
| COLTRYP 072     | Leontopithecus chrysomelas | BA/Atlantic Forest | TcIl | hap11        | KU145423 KT390209                  |
| COLTRYP 081     | Leontopithecus chrysomelas | BA/Atlantic Forest | TcIl | hap11        | KU145424 KT390210                  |
| COLTRYP 099     | Leontopithecus chrysomelas | BA/Atlantic Forest | TcIl | hap11        | KU145427 KT390213                  |
| COLTRYP 006     | Thrichomys aperiodes | PI/Caatinga | TcIl | hap10        | KU256218 KU256224                  |
| COLTRYP 021     | Leontopithecus rasalia     | RJ/Atlantic Forest | TcIl | hap12        | KU145411 KT390197                  |
### Table 1: Molecular identification, geographical and host origin of the COLTRYP isolates and reference stocks under study (Continued)

| Strain code | Host or Vector | State/Biome | Lineage | GenBank accession number |
|-------------|----------------|-------------|---------|-------------------------|
| COLTRYP 121 | Leontopithecus rosalia | RJ/Atlantic Forest | TcII | KU145431 KT390217 |
| Epinet 88,130 | Philander frenatus | RJ/Atlantic Forest | TcII | KU145455 KT390241 |
| COLTRYP 043 | Triatoma tibiamaculata | SC/Atlantic Forest | TcII | KU145417 KT390203 |
| Epinet 88,121 | Triatoma tibiamaculata | SC/Atlantic Forest | TcII | KU145453 KT390239 |
| Y | Homo sapiens | SP/Brazil | TcII | KU168555 KU168560 |
| Esmeraldo | Homo sapiens | BA/Brazil | TcII | DQ343646b |
| Tu18c12 | Triatoma infestans | Bolivia | TcII | AY484477b |
| COLTRYP 113 | Monodelphis domestica | GO/Cerrado | TcIII | KU145429 KT390215 |
| COLTRYP 370 | Rhodnius pictipes | PA/Amazon | TcIII | KU145442 KT390228 |
| COLTRYP 029 | Galictis vittata | RJ/Atlantic Forest | TcIII | KU145412 KT390198 |
| 3663 | Panstrongylus geniculatus | AM/Brazil | TcIII | KU168556 KU168561 |
| M6241 cl6 | Homo sapiens | PA/Brazil | TcIII | AY484478b |
| COLTRYP 041 | Thrichomys pachyurus | MS/Pantanal | TcIV | KU256220 KU256226 |
| COLTRYP 471 | Oecomys marmoreae | MS/Pantanal | TcIV | KU145444 KT390230 |
| COLTRYP 524 | Triatoma sp. | MS/Pantanal | TcIV | KU145445 KT390231 |
| COLTRYP 526 | Triatoma sp. | MS/Pantanal | TcIV | KU145446 KT390232 |
| COLTRYP 527 | Triatoma sp. | MS/Pantanal | TcIV | KU145447 KT390233 |
| COLTRYP 528 | Triatoma sp. | MS/Pantanal | TcIV | KU145448 KT390234 |
| COLTRYP 529 | Triatoma sp. | MS/Pantanal | TcIV | KU145449 KT390235 |
| COLTRYP 531 | Triatoma sp. | MS/Pantanal | TcIV | KU145450 KT390236 |
| COLTRYP 532 | Triatoma sp. | MS/Pantanal | TcIV | KU145451 KT390237 |
| 4167 | Rhodnius brethesi | AM/Brazil | TcIV | KU168557 KU168562 |
| CANIlIlc1 | Homo sapiens | PA/Brazil | TcIV | AY484474b |
| Sc43c11 | Triatoma infestans | Bolivia | TcV | KU145429 KT390215 |
| Bug2148 | Triatoma infestans | RS/Brazil | TcV | KU145442 KT390228 |
| CLBrener | Triatoma infestans | SP/Brazil | TcVI | KU145446 KT390232 |
| Tulacl2 | Homo sapiens | Chile | TcVI | KU145447 KT390233 |
| CLBrener | Triatoma infestans | SP/Brazil | TcVI | KU145448 KT390234 |
| Bug2148_1 | Triatoma infestans | RS/Brazil | TcV | KU145449 KT390235 |
| Bug2148_2 | Triatoma infestans | RS/Brazil | TcV | KU145450 KT390236 |
| CLBrener_1 | Triatoma infestans | SP/Brazil | TcVI | KU145451 KT390237 |
| CLBrener_2 | Triatoma infestans | SP/Brazil | TcVI | KU145452 KT390238 |
| 4167 | Rhodnius brethesi | AM/Brazil | TcV | KU168557 KU168562 |
| CANIlIlc1 | Homo sapiens | PA/Brazil | TcIV | AY484474b |
| Sc43c11 | Triatoma infestans | Bolivia | TcV | KU145429 KT390215 |
| Bug2148 | Triatoma infestans | RS/Brazil | TcV | KU145442 KT390228 |
| CLBrener | Triatoma infestans | SP/Brazil | TcVI | KU145446 KT390232 |
| Tulacl2 | Homo sapiens | Chile | TcVI | KU145447 KT390233 |
| CLBrener | Triatoma infestans | SP/Brazil | TcVI | KU145448 KT390234 |
| Bug2148_1 | Triatoma infestans | RS/Brazil | TcV | KU145449 KT390235 |
| Bug2148_2 | Triatoma infestans | RS/Brazil | TcV | KU145450 KT390236 |
| CLBrener_1 | Triatoma infestans | SP/Brazil | TcVI | KU145451 KT390237 |
| CLBrener_2 | Triatoma infestans | SP/Brazil | TcVI | KU145452 KT390238 |
| T. c. marinkellei | Phyllostomus discolor | GO/Cerrado | TcVI | KU145458 KT390244 |
| COLTRYP 107 | Phyllostomus discolor | GO/Cerrado | TcVI | KU256222 KU256228 |
| COLTRYP 117 | Phyllostomus discolor | GO/Cerrado | TcVI | KU256223 KU256229 |
| COLTRYP 143 | Phyllostomus discolor | GO/Cerrado | TcVI | KU256233 KU256239 |
| COLTRYP 576 | Phyllostomus hastatus | AC/Amazon | TcVI | KX620471 KX620476 |
| COLTRYP 577 | Phyllostomus hastatus | AC/Amazon | TcVI | KX620472 KX620477 |
| B7 | Phyllostomus discolor | BA/Brazil | TcVI | KC427240b AY484485b |
| TCC 344 | Carollia perspicillata | RO/Brazil | TcVI | KT327227b KT327313b |
| T. dionisi | Anoura geoffroyi | ES/Atlantic Forest | TcVI | KX274234 KX274236 |
95 °C for 3 min; followed by 35 cycles at 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; and a final elongation step at 72 °C for 10 min. Nucleotide sequences were also determined using a fragment of the nuclear gene GPI. The primers and cycling conditions are described elsewhere [20]. The PCR products were separated on 1.5% agarose gels and stained with GelRed (Biotium Inc., Fremont, California, USA). The fragments were purified using the Wizard Genomic DNA Purification Kit, according to manufacturer’s instructions (Promega, Madison, Wisconsin, USA), and direct sequencing of both strands of DNA was performed with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using an ABI 3730 DNA sequencer available at the RPT01A/FIOCRUZ sequencing facilities.

**Data analysis**

The sequences were manually edited using Geneious software version 8.1.6. (Biomatters, Auckland, New Zealand) and aligned using the CLUSTAL X version 2.1 multiple alignment program [59]. All sequences were translated to amino acid (Table 1).

The heterozygous hybrid lineages (TcV and TcVI) amplified for GPI were subjected to haplotype reconstruction using the PHASE algorithm implemented in DnaSP v5.10.01 [60].

The neighbor-joining (NJ) method and Kimura 2-parameters (K2P) model were applied for both cox1 and GPI genes according to the barcode approach [4]. NJ analyses were performed with MEGA version 6 [61]. For each node, bootstrap percentages (BP) were computed after 1000 resamplings.

The maximum likelihood (ML) method was also applied to each topology. The model of nucleotide substitution that best fitted the cox1 data was the Hasegawa-Kishino-Yano’s model (HKY), with a gamma-distributed rate (Γ). For GPI, the best-fit model was the Tamura-Nei model, with a gamma-distributed rate. These models were selected using the Akaike Information Criterion corrected for small samples (AICc) approach implemented in the program jModelTest [62].

ML analyses were performed using PhyML 3.0 [63]. For each node, BP were computed after 1000 resamplings.

Maximum Parsimony (MP) analyses were performed using PAUP* 4.0b10 [64]. For the tree search and bootstrap we used a heuristic search with 100 random sequence addition replicates through tree bisection and reconnection (TBR) branch-swapping algorithm. Bayesian inference (BI) was run in MrBayes v3.2.6 [65] with a general time reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites (GTR + Γ + I). The runs converged after 1,000,000 generations, by sampling every 100th generation and discarding the first 25% of the trees as ‘burn-in’. Cox1 and GPI sequences were concatenated in SequenceMatrix 1.8 [66] and submitted to NJ, ML, MP and BI analysis as described above.

The number of haplotypes, nucleotide diversity (n) and haplotype diversity (Hd) were calculated for both genes, except for Tcbat, which had a single sequence available in GenBank. The analyses were run in DnaSP v5.10.01 [60].

Molecular species delimitation was evaluated using distance-based methods and coalescent-based models. Distance-based analyses included the pairwise intraspecific and interspecific distances calculated using MEGA version 6 [61] and the Automatic Barcode Gap Discovery (ABGD) method, which detects a gap in the distribution of pairwise distances and uses this information to partition the sequences into groups of hypothetical species [67]. ABGD analysis was conducted in the web version: Jukes Cantor,
K2P and p distances were calculated, and the remaining parameters were used as default [67]. Coalescent-based analysis included the single-rate Poisson Tree Processes (PTP) model [68], which considers that every species evolved at the same rate, and the multi-rate Poisson Tree Processes (mPTP) [69], which assumes a different evolution rate for each species. PTP and mPTP analyses were conducted using the web version of this software [69].

**Fig. 2** Phylogenetic tree based on **cox1** and the geographical origin of the isolates under study. **a** The **cox1** gene differentiates *T. cruzi* DTUs TcI, TcII, TcIII and TcIV, *T. c. marinkellei*, *T. dionisi* and *T. rangeli*. The heterozygous hybrids TcV and TcVI cannot be differentiated and were placed into the same cluster as TcIV. The numbers at the nodes correspond to NJ, ML, MP and BI support values, respectively (only values >60 are shown). The scale-bar shows the number of nucleotide substitutions per site. The different haplotypes correspond to the diversity observed in **cox1** sequences and are represented by colors in the tree. **b** The map represents the distribution of the characterized isolates among Brazilian biomes. Each colored circle indicates a different haplotype. Circle size represents the number of haplotypes. **Abbreviations:** Brazilian states: AC, Acre; BA, Bahia; CE, Ceará; ES, Espírito Santo; GO, Goiás; MS, Mato Grosso do Sul; PA, Pará; PI, Piauí; RJ, Rio de Janeiro; SC, Santa Catarina; TO, Tocantins.
Results

Cox1 and GPI gene fragments were successfully amplified for a panel of 62 Trypanosoma spp. isolates and ten reference strains (Table 1). All sequences were translated to amino acids and compared to cox1 and GPI proteins. No indels (insertions/deletions) or stop codons were detected. No pseudo genes or contaminants were observed.

Phylogenetic tree reconstruction using cox1 as barcode

The clusters observed in the cox1 trees were the same for all methods tested, indicating that these groups are robust and do not depend on the evolutionary methods selected (Fig. 2a). Cox1 discriminated species belonging to the subgenus Schizotrypanum and T. cruzi DTUs. TcI and Tcbat were closely related but clearly constitute two different DTUs with a statistical support of 99, 70, 92 and 100 in NJ, ML, MP and BI analysis, respectively (Fig. 2a). TcIII and TcIV sequences were separated into two different clusters with bootstrap values of 99, 87, 93 and 100 in NJ, ML, MP and BI analysis, respectively. For the heterozygous hybrid lineages, TcV and TcVI formed an indistinguishable group in the same cluster as TcIV.

Phylogenetic tree reconstruction using GPI

Both GPI and cox1 helped recognize Schizotrypanum species, but not T. cruzi DTUs. Depending on the method used for the phylogenetic tree reconstruction there was a slightly different topology. Tcbat and TcI clustered together and could not be differentiated in the tree (Fig. 3). In NJ analysis TcIII constituted a separate DTU close to TcI (Fig. 3a). However, with ML, MP and BI methods TcI, Tcbat and TcIII clustered together (bootstrap of 80, 77 and 82, respectively) (Fig. 3b). GPI sequences generated for TcV and TcVI presented electropherograms with double peaks (i.e. with two bases at the same position) and were submitted to haplotype reconstruction prior to use in the final alignments and tree reconstructions. This analysis resulted in two sequences for each hybrid sample corresponding to alleles. One allele was closer to TcII, and the other allele was closer to TcIII (Fig. 3).

The geographical distribution of the trypanosomatid isolates under study is represented in Fig. 2b. Both cox1 and GPI sequences demonstrated the differences between T. cruzi, T. c. marinkellei, T. dionisii and T. rangeli and also, to some degree, T. cruzi lineages. Cox1 and GPI phylogenies equally demonstrated that TcI and TcII are the most genetically distant branches, but showed differences concerning the positions of the DTUs TcIII, TcIV, TcV, TcVI and Tcbat in the phylogenetic trees. The mitochondrial gene cox1 may be a better discriminator of T. cruzi lineages, identifying five DTUs and TcV/TcVI as a single group (Fig. 2a). Additionally, these differences between mitochondrial and nuclear tree topologies, no incongruence was observed in DTU assignment (Table 1), and mitochondrial introgression events were absent in the present sample set.

Identification of T. cruzi DTUs through single nucleotide polymorphisms (SNPs)

Some T. cruzi sequences were not clearly assigned to a DTU based solely on information from the trees. In the cox1 tree, TcIV and TcV/TcVI sequences were arranged in the same cluster (Fig. 2a), whereas in GPI analyses, TcI, TcIII and Tcbat separation was blurred (Fig. 3). Therefore, the multiple sequence alignment of cox1 and GPI data was considered for the evaluation of single nucleotide polymorphisms (SNPs) using T. cruzi sequences only. These polymorphisms were informative to DTU differentiation for both genes.

In the cox1 gene fragment analysis, we identified 84 polymorphic sites. We observed a single nucleotide polymorphism (SNP) that differentiates the heterozygous hybrids from TcIV sequences. A T (thymine) was present at position 1264 of the cox1 gene in all ten TcIV sequences analyzed, whereas TcV and TcVI sequences display a C (cytosine) at the same site. No polymorphism differentiating TcV from TcVI was observed (Additional file 1: Figure S1).

In the GPI analysis we identified 20 polymorphic sites concerning all T. cruzi sequences. A thymine at position 315 separates Tcbat from TcI (cytosine) and one guanine to adenine change separates TcI from TcIII at position 396 of the gene (Additional file 2: Figure S2). No polymorphism discriminating TcV from TcVI sequences was observed.

Phylogenetic tree reconstruction using concatenated data

The concatenation of cox1 and GPI gene fragments confirmed, with robust statistical support values, the separation of species belonging to the subgenus Schizotrypanum and the T. cruzi DTUs.

TcI, TcII, TcIII and TcIV sequences constituted clearly separated clades. In addition, concatenated data supported Tcbat as a sister clade to TcI. The heterozygous hybrids TcV and TcVI could not be differentiated and formed a cluster separate from TcIV. The topologies observed in the trees were the same for the four methods tested (NJ, ML, MP and BI) and were supported by values above 80 in the main branches (Fig. 4).

Genetic diversity evaluated with cox1 and GPI genes

Although the cox1 gene did not distinguish TcV from TcVI, this gene demonstrated diversity in T. cruzi DTUs TcI, TcII and TcIII and T. c. marinkellei and T. rangeli (Additional file 3: Table S1, Fig. 2b). GPI sequences also...
displayed distinct haplotypes in *T. c. marinkellei* and *T. rangeli*, but lower intra-DTU diversity. The correlation between haplotype and geographical area or host species was not evident.

In the *cox1* analysis, Tcl was demonstrated as the most diverse DTU with the highest nucleotide diversity and haplotype diversity of all DTUs, followed by TcII and TcIII. The Tcl isolates and reference strains in the present study were distributed in eight haplotypes throughout five Brazilian biomes (Fig. 2b) and six different host orders (Carnivora, Chiroptera, Didelphimorphia, Hemiptera, Primates and Rodentia) (Table 1). In the Amazon, we observed the highest number of different Tcl haplotypes in the Para state (hap 2, 3 and 5). Haplotypes 2 and 5 were observed only in the Para State, while haplotype 3 was widely distributed and detected in four...
Fig. 4 (See legend on next page.)
different biomes (Amazon, Atlantic Forest, Caatinga and Pantanal). Additionally, in Para, we observed two different haplotypes in three Tcl isolates derived from *Didelphis marsupialis* (Table 1). Thus, establishing a correlation between the Tcl haplotype and location or host species would be premature. Substantial genetic diversity was also observed in TcII sequences. In four TcIII isolates derived from *Monodelphis domestica* (Cerrado biome), *Galictis vittata* (Atlantic Forest), *Rhodnius pictipes* (Amazon biome) and a reference strain from *Panstrongylus geniculatus* (Amazon biome), we identified three different haplotypes (Table 1, Fig. 2b). These TcII isolates were divided into three haplotypes and originated from primates, a marsupial and triatomines captured in the Atlantic Forest and from a rodent captured in the Caatinga biome. The reference strains were isolated from humans in the Atlantic Forest. The three TcII haplotypes were distributed in the state of Rio de Janeiro, isolated from a *Philander frenatus* and two *Leontopithecus rosalia* (Table 1). Genetic diversity was not detected among the TcIV, TcV and TcVI isolates. Diversity could not be evaluated for TcBat, reflecting the unique sequence available for this DTU.

The seven *T. c. marinkellei* isolates displayed three haplotypes (Additional file 3: Table S1, Fig. 2b). One haplotype was formed by isolates originated from *P. discolor* from the Goiás state (Cerrado) and the Bahia state (Atlantic Forest); another haplotype comprised isolates originated from *P. hastatus* from the Acre State (Amazon biome); and a third group was formed by the reference strain TCC 344, isolated from *C. perspicillata* (Amazon) (Table 1, Fig. 2a). Despite the low number of isolates, an apparent correlation between haplotype and host species was observed.

We compared two *T. rangeli* isolates previously identified as lineages C and D [54, 70, 71]. Sequences generated with *cox1* exhibited two different haplotypes (Table 1, Additional file 3: Table S1). Trypanosoma dionisii sequences showed no diversity. Isolates derived from two different species of phyllostomid bats from the same geographical area and collected during the same field expedition (Table 1, Additional file 3: Table S1).

In the GPI analysis, intra-DTU diversity was lower than observed with *cox1*. Tcl, TclI, TclIII and TclIV sequences displayed two different haplotypes each. In TclIV, one haplotype was formed by the nine isolates identified herein and the reference strain 4167, while the other haplotype only comprised the reference strain CANIII, which was not available for *cox1* analysis. No diversity within TcV/TcVI sequences was detected.

The highest diversity in GPI sequences was observed in *T. c. marinkellei* with five haplotypes. One haplotype comprised three isolates from the Goiás state (Cerrado) and the other haplotypes corresponded to the other four isolates (Additional file 3: Table S1, Fig. 3). The two *T. rangeli* sequences generated with GPI were identified elsewhere as lineage C [71]. However, these sequences displayed distinct haplotypes (Additional file 3: Table S1, Fig. 3). No diversity within *T. dionisii* sequences was observed. No correlation between haplotype, host or geographical location could be established.

Barcoding gap and species delimitation with *cox1* and GPI sequences

Based on the analysis of the barcode gaps we assessed and compared the efficiency of *cox1* and GPI for the identification of trypanosomatids. In *cox1* the mean genetic distance between *T. cruzi* sequences and *T. c. marinkellei* was 13%. The mean intraspecific divergence for *T. cruzi* was 7.6%, a value lower than the mean interspecific value. However, comparing the minimum and maximum values, we observed an overlap of the genetic distances between *T. cruzi* and *T. c. marinkellei* (10.1–15.3%) and the intraspecific divergence for *T. cruzi* (0–12.7%), indicating the absence of a limit that separates these two subspecies (i.e. absence of a barcoding gap). The divergence between *T. cruzi* and *T. dionisii* limits was 13.0–16.0%, while the divergence between *T. cruzi* and *T. rangeli* was 14.7–21.3%, indicating that the genetic distance separating *T. cruzi*, *T. dionisii* and *T. rangeli* as different species is 0.3 and 2.0%, respectively (Additional file 4: Table S2).

GPI was not as discriminative as *cox1*. The mean interspecific divergence between *T. cruzi* sequences and *T. c. marinkellei*, *T. dionisii* and *T. rangeli* was 3.6, 7.8 and 13.7%, respectively.

We observed differences between *T. c. marinkellei* sequences in the trees (Fig. 3) confirmed by an intraspecific distance ranging from 0 to 1.0% (Additional file 5: Table S3). Distance within *T. rangeli* sequences was 0.7% and there was no genetic difference between *T. dionisii* sequences. For *T. cruzi*, the intraspecific genetic distance ranged from 0 to 3.0%, i.e. lower than the mean interspecies values (Table 2). Similar to *cox1*, we observed an overlap of the genetic distances between *T. cruzi* and *T. c.
Trypanosoma cruzi and T. c. marinkellei were separated into distinct groups according to ABGD, PTP and mPTP analysis using cox1 and GPI data. Here, we report the results for the three substitution models and both initial and recursive partitions in the output of ABGD. The results varied from 6 to 21 for cox1 depending on the substitution model used, confirming diversity in T. cruzi sequences and the separation of T. cruzi, T. c. marinkellei, T. rangeli and T. dionisii (Table 2). Trypanosoma cruzi sequences were divided into a minimum of three groups in the three models tested. One group corresponded to Tcbat and TcI; another group corresponded only to TcII sequences only; and a third group comprised TcIII, TcIV, TcV and TcVI sequences. A maximum of 13 different groups were observed, with TcI sequences divided into eight different groups, showing the higher intra-DTU diversity of TcI compared to the other DTUs (Additional file 6: Table S4). In all models and partitions, T. dionisii sequences were arranged in one group.

The number of ABGD groups for GPI data varied from 2 to 14 depending on the model applied (Table 2). Trypanosoma cruzi sequences were separated into different groups. TcI sequences were grouped together, showing less variability with GPI. TcII sequences were divided into two groups: one group with only TcII sequences and another group with TcII, TcV (allele 1) and TcVI (allele 1). TcIII sequences were grouped together with sequences representing the other TcV and TcVI alleles. TcIV sequences were combined in one group, except for the reference strain CANIII, which was placed in a separated group. Trypanosoma cruzi marinkellei and T. rangeli sequences were divided into groups, reaffirming their diversity (Additional file 7: Table S5), while T. dionisii sequences formed one group in all tests (Table 2, Additional file 7: Table S5).

The number of groups recovered by ABGD was higher than the number of species studied. However, this finding confirms the genetic diversity within T. cruzi DTUs, T. c. marinkellei and T. rangeli observed in the phylogenetic trees (Figs. 2a and 3).

The PTP and mPTP models identified, respectively, a total of 10 and 7 putative species in the cox1 dataset (Table 3). Four of these putative species were subdivisions of T. cruzi, indicating the heterogeneity of this taxon. The PTP model also recognized diversity within T. c. marinkellei and T. rangeli sequences.

PTP and mPTP provided a similar number of putative species for GPI sequences (Table 3). Trypanosoma cruzi was divided into three groups, and T. c. marinkellei sequences were allocated into one group. The difference between models was observed in the T. rangeli sequences, separated into two groups or placed into one group.

In cox1 and GPI analysis using both models, T. cruzi, T. c. marinkellei, T. rangeli and T. dionisii were recognized as different species. The diversity of T. cruzi was confirmed, and no diversity was observed in T. dionisii sequences.

**Discussion**

In the present study, the DNA barcoding approach using the cox1 gene has been demonstrated to be efficient at

| Table 2 | cox1 and GPI sequences division in groups based on ABGD analysis |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Substitution model | Xa Partition | Prior intraspecific divergence (P) | 0.059948 | 0.035938 | 0.021544 | 0.012915 | 0.007743 | 0.004642 | 0.002783 | 0.001668 | 0.001000 |
| cox1 | | | | | | | | | |
| Jukes Cantor | 1.5 | Initial | 6 | 6 | 6 | 10 | 10 | 10 | 10 | 10 | 10 |
| | | Recursive | 8 | 11 | 14 | 14 | 14 | 21 | 21 | |
| K2P | 1.5 | Initial | 6 | 6 | 6 | 11 | 11 | 11 | 11 | 11 | 11 |
| | | Recursive | 8 | 14 | 14 | 21 | 21 | 19 | 19 | |
| p-distance | 1.5 | Initial | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| | | Recursive | 0 | 9 | 9 | 9 | 11 | 11 | 11 | |
| GPI | | | | | | | | | |
| Jukes Cantor | 1.5 | Initial | 2 | 2 | 3 | 3 | 5 | 7 | 3 | 14 | 14 |
| | | Recursive | | | | | | | 7 | |
| K2P | 1.5 | Initial | 2 | 2 | 3 | 3 | 5 | 7 | 3 | 14 | 14 |
| | | Recursive | | | | | | | 7 | |
| p-distance | 1.5 | Initial | 2 | 2 | 3 | 3 | 5 | 5 | 5 | 7 | 7 |

*a* X, relative gap width  
*b* K2P Kimura 2-parameter
recognizing *Trypanosoma* species and their major sub-populations. With *cox1*, we distinguished *T. cruzi* from *T. c. marinkellei, T. donisii* and *T. (Tejeraia) rangeli*, fulfilling the main DNA barcode demands of a short gene fragment that can be sequenced in diverse sample sets and generating comparable sequences that enable the distinction of species from each other [4]. We also generated a library of trypanosome sequences for *cox1* and *GPI* genes. Each specimen analyzed is linked to an identification number, collection date, country, region and host of origin, geographical coordinates and other information that enable the tracking of the origin of the specimen and ensure the reproducibility of subsequent experiments.

*Trypanosoma cruzi* is currently divided into seven DTUs [29]. Using *cox1*, we identified five *T. cruzi* groups (TcI, TcII, TcIII, TcIV and Tcbat). The DTUs TcI and TcII are consistently shown as the most genetically distant groups, well separated by *cox1* in all four methods tested (Fig. 2a). This structure has been observed by other authors in trees with high bootstrap support values, sustaining TcI and TcII as the two discernible DTUs, independently of gene or method used [25, 51, 72, 73]. Furthermore, this system showed the potential for separating genetically closer DTUs. We observed Tcbat as a separated cluster within *T. cruzi* and its proximity to TcI, consistent with Marcili et al. [25]. According to other studies using *cytb*, *V7 V8* rRNA and *gGAPDH* genes this relationship is unanimous [26, 51, 74, 75]. However, the data on Tcbat are still limited, as only one sequence was generated with *cox1* available in GenBank. Our *cox1* sequences also showed the homozygous hybrids TcIII and TcIV forming distinct groups in all methods tested (Fig. 2a). These DTUs are proposed to have been originated from the genetic exchange between TcI and TcII and evolved separately giving origin to TcIII and TcIV [76, 77]. The genetic proximity between TcIII and TcIV is undeniable, and their separation and position in the phylogenetic trees is altered by the gene and method of inference selected. Based on *cytb* (inferred by MP), *V7 V8*, *gGAPDH*, *GPI* genes and MLST approaches, TcIII and TcIV were identified as two separate DTUs [24–26, 28, 51]. However, in other studies using the *cytb* gene (inferred by neighbor-joining and maximum likelihood), TcIII and TcIV were identified as a single group [34, 51, 77]. TcV and TcVI sequences were indistinguishable and clustered with TcIV (Fig. 2a), consistent with previous *cytb* mitochondrial gene results [25]. In some studies, independently of the molecular marker and phylogenetic method applied, TcV and TcVI were also indistinguishable from each other. However, these hybrids clustered together with TcII or TcIII when analyzed using nuclear markers [25, 26, 34, 78]. In contrast, in studies using 4 to 10 gene fragments and neighbor-joining trees, TcV and TcVI appeared as two distinct DTUs [24, 79]. In the *cox1* analysis, we observed one SNP that differentiates TcIV sequences from TcV/ TcVI (Additional file 1: Figure S1). This nucleotide polymorphism, combined with the phylogenetic tree, was demonstrated as relevant to DTU assignment. However, TcV and TcVI are the less conspicuous lineages, and their separation remains an issue.

*cox1* was also suitable to determine diversity within DTUs TcI, TcII and TcIII (Additional file 3: Table S1). The number of sequences classified as TcI and the number of different haplotypes in these DTU sequences were the highest, compared to the other DTUs. The diversity within TcI is consistent with previous studies and may be explained by TcI being a multi-host lineage widely distributed throughout Brazilian biomes, representing the DTU with the largest set of samples analyzed, and consequently, the DTU with the most published studies compared to the other DTUs [20, 51, 52, 80, 81]. The nucleotide and haplotype diversity of the TcII sequences generated using *cox1* were lower. However, this effect may not reflect the reality, but rather may show subsampling. In the Rio de Janeiro State, we observed one TcII haplotype circulating in a specimen of *L. rosalia* and a different haplotype in another specimen of *L. rosa- lia*. This observed diversity may reflect primates captured in different years and the changes in TcII haplotype circulation in that area. Nevertheless, the same host can harbor different haplotypes from the same parasite, and one haplotype can prevail over another in different moments of isolation. Diversity within TcII has previously been demonstrated through the sequencing of the glycoprotein 72 gene (*gp72*) and showed that this DTU has a higher distribution range than

### Table 3 Number of species according to PTP and mPTP delimitation methods

| Gene and taxon | PTP | mPTP |
|---------------|-----|------|
| *cox1*        |     |      |
| T. cruzi      | 4   | 4    |
| T. c. marinkellei | 3 | 1    |
| T. donisii    | 1   | 1    |
| T. rangeli    | 2   | 1    |
| Total         | 10  | 7    |
| *GPI*         |     |      |
| T. cruzi      | 3   | 3    |
| T. c. marinkellei | 1 | 1    |
| T. donisii    | 1   | 1    |
| T. rangeli    | 2   | 1    |
| Total         | 7   | 6    |
previously been observed based on \( V_7 \) \( V_8 \). Its dispersion is yet to be explored. Diversity in TcIII has previously been observed based on \( V_7 \) \( V_8 \), cytb, GPI, MLST approaches, but no correlation with geographical area or host species was evident [24, 25, 28]. In the present study, TcIV samples were isolated from triatomines and rodents from the same geographical area (Table 1). This aspect might explain the observation of only one haplotype in TcIV sequences. However, isolates from rodents were collected 11 years before the parasites isolated from the intestinal content of triatomines. Thus, we propose that TcIV haplotype circulation in the Pantanal area was at least equally predominant throughout more than a decade. However, the TcIV reference was isolated from a triatomine in the Amazon region (Table 1) and had the same haplotype as the Pantanal isolates. This finding could reflect the conservation of the \( \text{cox1} \) region in TcIV. A correlation between TcIV diversity and the geographical region has been suggested by other studies based on mitochondrial genes cytb and cytochrome c oxidase subunit 2-NADH dehydrogenase subunit 1 (cox2-nad1) [28, 34, 77].

The \( \text{cox1} \) tree topologies, independently of the method applied, showed \( T. \text{c. marinkellei} \) as a sister clade to the monophyletic clade formed by all \( T. \text{cruzi} \) DTUs (Fig. 2a); we also observed genetic diversity within \( T. \text{c. marinkellei} \) (Additional file 3: Table S1). Even with the characterization of a low number of isolates, the samples were separated into two groups, and a sequence retrieved from GenBank was positioned in a third group (Fig. 2a). Heterogeneity within \( T. \text{c. marinkellei} \) has previously been reported [75, 82, 83]. Subdivision into two major groups (T.c.m.I and II) and a potential third group (lineage Z) was proposed using multilocus enzyme electrophoresis (MLEE) and random amplified polymorphic DNA (RAPD). No association with a host or geographical distribution was confirmed [82]. This lack of evidence for an association and the different markers used prevented the comparison of these data.

We also observed genetic differences between \( T. \text{rangeli} \) reference sequences R1625 and SC58 classified, respectively, as lineage C and lineage D [54, 70]. Previous studies have proposed the subdivision of \( T. \text{rangeli} \) in five lineages (A–E), based on spliced leader and SSU rDNA [54, 70, 71]. Even with only two sequences, we suggest that \( \text{cox1} \) can distinguish different \( T. \text{rangeli} \) lineages and is a promising tool for use in species identification.

In the present study, we showed the first \( T. \text{dionisii} \) sequences for the \( \text{cox1} \) gene. The nucleotide sequences were deposited in GenBank, contributing to the enhancement of the barcode public library for \( \text{Trypanosoma} \) species. We did not observe diversity among these sequences (Fig. 2a, Additional file 3: Table S1), likely because the samples were collected from bats of the same area. No subdivisions in groups or subpopulations have been proposed for \( T. \text{dionisii} \) until recently. Although potential diversity within this species can be observed in cytb and 18S (SSU) phylogenetic trees, these data were not reported [84].

The concomitant analysis of the mitochondrial gene \( \text{cox1} \) and the nuclear gene GPI enable the confirmation of the absence of mitochondrial introgression events in the sample set. The frequency of this genetic phenomenon is unknown and has primarily been observed in heterozygous hybrid DTUs TcV and TcVI, where uniparental inheritance of maxicircle kDNA is the rule [28, 36, 56, 85]. \( \text{cox1} \) has limitations and does not work as a single barcode in all situations [1], and since \( T. \text{cruzi} \) possesses heterozygous hybrid lineages and mitochondrial introgression events have previously been reported, we proposed this \( \text{cox1-GPI} \) barcoding system. Additionally, the concatenated analysis of \( \text{cox1} \) and GPI confirmed \( T. \text{cruzi} \) DTU separation (Fig. 4).

Consistent with the \( \text{cox1} \) results, GPI distinguished \( T. \text{cruzi} \) from \( T. \text{c. marinkellei} \), \( T. \text{dionisii} \) and \( T. \text{(Tejeraia)} \) \( \text{rangeli} \). Additionally, with \( \text{GPI} \), TcI and TcII were separated into two conspicuous groups. This nuclear gene recognizes a lower number of \( T. \text{cruzi} \) groups (Fig. 3) and considerably lower intra-DTU diversity compared to \( \text{cox1} \) (Additional files 4 and 5: Tables S2 and S3). In contrast to the \( \text{cox1} \) results, Tcbat and TcI were clustered together. The lower power of resolution to discriminate DTUs and intra-DTU diversity might reflect the fact that \( \text{GPI} \) is a housekeeping nuclear gene, which shows a lower evolution rate than mitochondrial genes [28, 85]. Furthermore, we compared the \( \text{GPI} \) sequences with the single Tcbat sequence available in GenBank and the results may change depending on the number of sequences available. It is likely that a larger set of Tcbat sequences would resolve the incongruence between the mitochondrial and nuclear gene trees. In addition, we also observed differences in DTU placement in the trees according to the method of inference in the \( \text{GPI} \) analyses. TcIII sequences formed a cluster separate from TcI when we applied the neighbor-joining method and clustered together with TcI when maximum likelihood, parsimony and Bayesian inference were used. This effect may reflect the fact that the neighbor-joining method was based on the genetic distance matrix, where a pairwise distance matrix is produced, and the tree is inferred from this matrix; maximum likelihood, parsimony and
Bayesian inference were character-based methods of inference where each position of the alignment is analyzed [86]. However, in the GPI analysis, we detected SNPs that enable the differentiation of TcI, TcIII and Tcbat (Additional file 2: Figure S2). We observed diversity within T. c. marinkellei and generated the first five T. dionisií sequences for GPI. We could not compare T. rangeli sequences generated with GPI, as both sequences analyzed belonged to lineage C [54, 70].

Barcoding gap in trypanosomatids is still an unresolved issue. Therefore, there are no parameters or cutoff values available to compare with these results. As expected, we did not observe a barcoding gap between T. cruzi and T. c. marinkellei because T. c. marinkellei is considered a subspecies of T. cruzi [87] (Additional files 4 and 5: Tables S2 and S3).

The debate concerning the definition of species will always exist since species are not discrete units, but rather continuous entities.

Conclusions

The use of partial sequences of cox1 and GPI genes can clearly identify and separate T. cruzi samples from T. c. marinkellei, T. dionisií and T. rangeli. The two-locus barcoding system using cox1 and the nuclear gene GPI revealed that mitochondrial introgression was absent from the sample set. Additionally, the resolution of cox1 at the intraspecific level shows great potential for DTU characterization, separating five DTUs and recognizing the heterozygous hybrids TcV and TcVI as one group different from all the other DTUs; the resolution of cox1 at the intraspecific level also demonstrates intra-DTU genetic diversity. Moreover, with cox1, we evaluated the diversity within T. c. marinkellei sequences and identified two T. rangeli lineages. Therefore, the cox1 gene is a promising DNA barcode for studying the genus Trypanosoma and represents a simple, fast and reliable marker.

Additional files

Additional file 1: Figure S1. Comparison between TdV and TcV/TcVI nucleotide sequences generated with the cox1 barcode. a Alignment of TdV sequences with TcV and TcVI shows one single nucleotide polymorphism differentiating TdV from the hybrids. b Electropherogram confirms the presence of a T (thymine) in TcV in the same position, showing a C (cytosine) in TcV and TcVI sequences. (TIFF 724 kb)

Additional file 2: Figure S2. Comparison between Td, Tcbat and TcIII nucleotide sequences generated with GPI. a Sequence alignment shows one single nucleotide polymorphism differentiating Tc from Tcbat and one polymorphism separating Tc from TcIII sequences. b Electropherogram confirms the presence of A (adenine) in TcIII sequences in the same position, showing a G (guanine) in Tc sequences. The Tcbat sequence was retrieved from GenBank and the electropherogram is not publicly available. (TIFF 527 kb)

Additional file 3: Table S1. Number of haplotypes, nucleotide diversity and haplotype diversity of sequences generated with cox1 and GPI. (DOCX 12 kb)

Additional file 4: Table S2. Inter- and intraspecific genetic distance based on cox1 sequences. (DOCX 14 kb)

Additional file 5: Table S3. Inter- and intraspecific genetic distance based on GPI sequences. (DOCX 15 kb)

Additional file 6: Table S4. Cox1 sequences partition into groups inferred with ABGD, based on Kimura 2-parameters. (DOCX 14 kb)

Additional file 7: Table S5. GPI sequences partition into groups inferred with ABGD, based on Kimura 2-parameters. (DOCX 14 kb)

Abbreviations

ABGD: Automatic Barcode Gap Discovery; BI: Bayesian inference; BrBOL: Brazilian Barcode of Life; COLPROT: Protozoa Collection; COLTRYP: Trypanosomatid collection from wild and domestic mammals and vectors; cox1: cytochrome c oxidase subunit 1 gene; cox2-nad1: cytochrome c oxidase subunit 2-NADH dehydrogenase subunit 1 gene region; cyt b: cytochrome b; DTU: Discrete typing unit; gGAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPI: Glucose-6-phosphate isomerase; ITS: Internal transcribed spacer; K2P: Kimura 2-parameter; LIT: Liver infunction tryptoscope; ML: Maximum likelihood; MLST: Multilocus sequencing typing; MP: Maximum parsimony; mPPT: multi-rate Poisson Tree Processes; NJ: Neighbor-joining; NNN: Novy-McNeal-Nicolle medium; PPT: single-rate Poisson Tree Processes; SSU rDNA: Small subunit ribosomal DNA; V7 V8: Variable regions 7 and 8

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Availability of data and materials

All sequences analyzed are available in GenBank under the accession numbers within the article.

Authors’ contributions

MSR, KAM and AMJ conceived and designed the experiments. MSR performed and analyzed the molecular characterization. KAM provided additional suggestions on content. MSR and AMJ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable. No special permission was required for the present study. We used DNA extracted from the cultures obtained from animals collected during previous field expeditions conducted by our group. The field expeditions were endorsed by the Ethics Committee of FIOCRUZ (Oswaldo Cruz Foundation, Brazil) (CEUA L-015/04; CEUA P-292-06).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Blaxter M. Imagining Sisyphus happy: DNA barcoding and the unnamed majority. Philos Trans R Soc Lond Ser B Biol Sci. 2016;371(1702):1–5.
2. The Internet Classics Archive. The History of Animals by Aristotle. Translated by D’Arcy Wentworth Thompson. http://classics.mit.edu/Aristotle/history_anim.shtml. Accessed 20 Feb 2017.
3. Linnaeus C. Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. 1.758. http://www.biodiversitylibrary.org/item/10277/page/3/mode/1up. Accessed 20 Feb 2017.
4. Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc Biol Sci. 2003;270(1365):1–21.
5. Trivedi S, Aloufi AA, Ansari AA, Ghosh SK. Role of DNA barcoding in marine biodiversity assessment and conservation: an update. Saudi J Biol Sci. 2016; 23:161–71.
6. Hebert PDN, Hollingsworth PM, Hajibabaei M, Hebert PDN. From writing to reading the encyclopaedia of life. Philos Trans R Soc Lond B Biol Sci. 2016; 371:1–9.
7. Strüder-Kypke MC, Lynn DH. Comparative analysis of the mitochondrial cytochrome c oxidase subunit I (COI) gene in ciliates (Alveolata, Ciliophora) and evaluation of its suitability as a biodiversity marker. Syst Biodivers. 2010; 8:131–48.
8. Lin X, Stur E, Bresk T. Exploring genetic divergence in a species-rich insect genus using 2790 DNA barcodes. PLoS One. 2015;10:e0138993.
9. Tavares ES, Baker AJ. Single mitochondrial gene barcodes reliably identify species in diverse clades of birds. BMC Evol Biol. 2008;8:81.
10. Oba Y, Chihara H, Murase Y, Moriyama A, Kumazawa YDNA. Barcoding of Japanese click beetles (Coleoptera, Elateridae). PLoS One. 2015;10:e016612.
11. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, et al. From the cover: nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci USA. 2012;109:6241–6.
12. de Boer HJ, Duangthid A, Martin G, Abbad A, Kool ADNA: Barcoding reveals limited accuracy of identifications based on folk taxonomy. PLoS One. 2014; 9(1):e84291.
13. Stern RF, Horak A, Andrew RL, Coffroth MA, Andersen RA, Küpper FC, et al. Environmental barcoding reveals massive dinoflagellate diversity in marine environments. PloS One. 2010;5(1):e13991.
14. Zhao Y, Genteleski E, Yi Z, Lin X. Genetic differentiation of the mitochondrial cytochrome c oxidase subunit I gene in genus Paramecium (Protista, Ciliophora). PLoS One. 2013;8:e77044.
15. Sundberg P, Kivist S, Strand M. Evaluating the utility of single-locus DNA barcoding for the identification of ribbon worms (phylum Nemertea). PLoS One. 2016;11:1–17.
16. Meyer CP, Pauly G. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 2005;3:e422.
17. Cupedo L, Zijfledt G, Seuren H, Beliet SM. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of Leishmania. Mol Biochem Parasitol. 1995;63(1):145–55.
18. Fernandes Q, Sturm NR, Derré R, Campbell DA. The mini-exon gene: a genetic marker for zymodeme III of Trypanosoma cruzi. Mol Biochem Parasitol. 1998;95:129–33.
19. Lewis MD, Ma J, Yeo M, Carrasco HI, Llewellyn MS, Miles MA. Genotyping of Trypanosoma cruzi: systematic selection of assays allowing rapid and accurate discrimination of all known lineages. Am J Trop Med Hyg. 2009;81:1041–9.
20. Brenakre SF, Allaga C, Waleczek E, Buitrago R, Salas R, Barnabé C, et al. Genetic characterization of Trypanosoma cruzi DTUs in wild Triatoma infestans from Bolivia: predominance of TcI. PLoS Negl Trop Dis. 2012;6: e1650.
21. Perez E, Monje M, Chang B, Buitrago R, Parrado R, Barnabé C, et al. Predominance of hybrid discrete typing units of Trypanosoma cruzi in domestic Triatoma infestans from the Bolivian Gran Chaco region. Infect Genet Evol. 2013;13:1116–23.
22. Cavazzana M, Marcili A, Lima L, da Silva FM, Junqueira ACV, Veludo HH, et al. Phylogeographical, ecological and biological patterns shown by nuclear (ssrRNA and gGAPDH) and mitochondrial (Cyt b) genes of trypanosomes of the subgenus Schizotrypanum parasitic in Brazilian bats. Int J Parasitol. 2010; 40:3345–55.
23. Ramírez JD, Duque MC, Montilla M, Cucunubá Z, Guhl F. Natural and emergent Trypanosoma cruzi genotypes revealed by mitochondrial (Cyt b) and nuclear (SSU rDNA) genetic markers. Exp Parasitol. 2012;132:487–94.
24. Dloszcz P, Tomasi M, Latournier JL, Messerlin LA, Monge Rumi MW, Ragone PG, et al. Optimized multilocus sequence typing (MLST) scheme for Trypanosoma cruzi. PLoS Negl Trop Dis. 2014;8(8):e3117.
25. Marcili A, Lima L, Cavazzana M, Junqueira ACV, Veludo HH, Maia Da Silva F, et al. A new genotype of Trypanosoma cruzi associated with bats evidenced by phylogenetic analyses using SSU rDNA, cytochrome b and Histone H2B genes and genotyping based on ITS1 rDNA. Parasitology. 2009;136:641–55.
26. Lima L, Espinosa-Alvarez O, Ortiz PA, Trejo-Várón JA, Canavana JC, Pinto CM, et al. Genetic diversity of Trypanosoma cruzi in bats, and multilocus phylogenetic and phylogeographical analyses supporting TcB as an independent DTU (discrete typing unit). Acta Trop. 2015;151:166–77.
27. Botero A, Cooper C, Thompson CK, Clode PL, Rose K, Thompson RA. Morphological and phylogenetic description of Trypanosoma noyesi sp. nov.: an Australian wildlife trypanosome within the T. cruzi clade. Protist. 2016; 167:425–39.
28. Lewis MD, Llewellyn MS, Yeo M, Acosta N, Gaunt MW, Miles MA. Recent, independent and anthropogenic origins of Trypanosoma cruzi hybrids. PLoS Negl Trop Dis. 2011;5:e1363.
29. Zingales B, Miles MA, Campbell DA, Tibayrenc M, Macedo AM, Teixeira MWG, et al. The revised Trypanosoma cruzi subspecific nomenclature: rationale, epidemiological relevance and research applications. Infect Genet Evol. 2012;12:490–53.
30. Chagas C. Nova tripanoziomae humana: estudos sobre a morfologia e o ciclo evolutivo do Schizotrypanum cruzi n. gen., n. sp., ajente etiológico de nova entidade morbida do homem. Mem Inst Oswaldo Cruz. 1909;1:159–218.
31. Miles MA, Toye PJ, Oswald SC, Godfrey DG. The identification by isoenzyme patterns of two distinct strain-groups of Trypanosoma cruzi circulating independently in a rural area of Brazil. Trans R Soc Trop Med Hyg. 1977; 71(3):217–25.
32. Tibayrenc M, Ward P, Moya A, Ayala FJ. Natural populations of Trypanosoma cruzi, the agent of Chagas disease, have a complex multiclonal structure. Proc Natl Acad Sci USA. 1986;83:115–9.
33. Tibayrenc M, Ayala FJ. The population genetics of Trypanosoma cruzi revisited in the light of the predominant clonal evolution model. Acta Trop. 2015;151:156–65.
34. Brisse S, Henriksson J, Barnabé C, Douzeny EJP, Berkvens D, Serrano M, et al. Evidence for genetic exchange and hybridization in Trypanosoma cruzi based on nucleotide sequences and molecular karyotype. Infect Genet Evol. 2003;2:173–83.
35. Machado CA, Ayala FJ. Nucleotide sequences provide evidence of genetic exchange among distantly related lineages of Trypanosoma cruzi. Proc Natl Acad Sci USA. 2001;98(13):7396–401.
36. Barnabé C, Brienlé SF. Scarce events of mitochondrial introgression in Trypanosoma cruzi: new case with a Bolivian strain. Infect Genet Evol. 2012;12:879–83.
37. Messerlin LA, Miles MA. Evidence and importance of genetic exchange among field populations of Trypanosoma cruzi. Acta Trop. 2015;151:150–5.
38. Andrade SG, Magalhães JB. Biodemes and zymodemes of Trypanosoma cruzi: new case with a Bolivian strain. Infect Genet Evol. 2012;12:879–83.
39. Moore CL, Chiarle E, Carmona EP, Mattel ED, Romana AJ, Simpson L. Strains and clones of Trypanosoma cruzi can be characterized by pattern of restriction endonuclease products of kinetoplast DNA minicircles. Proc Natl Acad Sci USA. 1980;77:6810–4.
40. Zingales B, Andrade SG, Britóns MRS, Campbell DA, Chiarle E, Fernandes O, et al. A new consensus for Trypanosoma cruzi intraspecific nomenclature.
second revision meeting recommends Tcl to TcVI. Mem Inst Oswaldo Cruz. 2009;104:1051–4.

42. Tibayrenc M, Ayala FJ. Isozyme variability in Trypanosoma cruzi, the agent of Chagas disease: genetical, taxonomical, and epidemiological significance. Evolution. 1988;42:277–92.

43. Tibayrenc M, Ayala FJ. Towards a population genetics of microorganisms: the clonal theory of parasitic protozoa. Parasitol Today. 1991;7:228–32.

44. Tibayrenc M. Population genetics of parasitic protozoa and other microorganisms. Adv Parasitol. 1995;36:48–115.

45. Souto RP, Fernandes O, Macedo AM, Campbell DA, Zingales B. DNA markers define two major phylogenetic lineages of Trypanosoma cruzi. Mol Biochem Parasitol. 1996;82:141–52.

46. Tibayrenc M. Genetic epidemiology of parasitic protozoa and other infectious agents: the need for an integrated approach. Int J Parasitol. 1998;28:85–104.

47. Brisse S, Barnabé C, Tibayrenc M. Identification of six Trypanosoma cruzi phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int J Parasitol. 2000;30:35–44.

48. Kawashita SY, Sanson GF, Fernandes O, Zingales B, Briones MR. Maximum-likelihood divergence date estimates based on RNA gene sequences suggest two scenarios of Trypanosoma cruzi intraspecific evolution. Mol Biol Evol. 2001;18:2250–9.

49. Mendonça MBA, Nehme NS, Santos SS, Cupollo E, Vargas N, Junqueira A, et al. Two main clusters within Trypanosoma cruzi zymodeme 3 are defined by distinct regions of the ribosomal RNA cistron. Paratropism. 2002;124:177–84.

50. De Freitas JM, Augusto-Pinto L, Pimenta JR, Bastos-Rodrigues L, Gonçalves VF, Teixeira SMR, et al. Ancestral genomes, sex, and the population structure of Trypanosoma cruzi. PLoS Pathog. 2006;2:e24.

51. Barnabé C, Mobarac HI, Jurado MR, Cortez JA, Brenière SF. Reconsideration of the seven discrete typing units within the species Trypanosoma cruzi, a new proposal of three reliable mitochondrial clades. Infect Genet Evol. 2016;43:3917–36.

52. Ramirez JD, Llewellyn MS. Reproductive clonality in protozoan pathogens - truth or artefact? Mol Ecol. 2014;23:4195–202.

53. Gutièrrez GH, Auwerdaehe A, Ramirez JD. From ancient to contemporary E. coli genome phylo-epidemiology of Chagas disease in the Americas. Int J Parasitol. 2014;44(9):605–12.

54. Maia da Silva F, Marcili A, Lima L, Cavazzana M, Ortiz PA, Campaner M, et al. Trypanosoma rangeli isolates of bats from Central Brazil genotyping and phylogenetic analysis enable description of a new lineage using spliced-leader gene sequences. Acta Trop. 2014;139:138–44.

55. Rocha FL, Roque ALR, Arrais RC, Santos JP, Lima VOS, Xavier SCDC, et al. Identification and phylogeography of Trypanosoma rangeli and Rhodius (Hemiptera: Reduviidae) supports a long coexistence of parasite lineages and their sympatric vectors. Mol Ecol. 2007;16:3361–73.

56. Briones MR, Souto RP, Stoff BS, Zingales B. The evolution of two Trypanosoma cruzi subgroups inferred from rRNA genes can be correlated with the interchange of American mammalian faunas in the Cenozoic and has implications to pathogenicity and host specificity. Mol Biochem Parasitol. 1999;104:219–32.

57. Flores-López CA, Machado CA. Analyses of 32 loci clarify phylogenetic relationships among Trypanosoma cruzi lineages and support a single hybridization prior to human contact. PLoS Negl Trop Dis. 2011;5(8):e1272.

58. Hamilton PB, Lewis MD, Cruckshank C, Gaunt MW, Yeo M, Llewellyn MS, et al. Identification and lineage genotyping of south American trypanosomes using fluorescent fragment length barcoding. Infect Genet Evol. 2011;11:444–51.

59. García L, Ortiz S, Osorio G, Torrico MC, Torrico F, Solari A. Phylogenetic analysis of Bolivian bat trypanosomes of the subgenus Schizotrypanum based on cytochrome b sequence and minicircle analyses. PLoS One. 2012;7:1–7.

60. Westenberger SJ, Barnabé C, Campbell DA, Sturm NR. Two hybridization events define the population structure of Trypanosoma cruzi. Genetics. 2005;171:527–43.

61. Tomasi N, Diosque P. Evolution of Trypanosoma cruzi: clarifying hybridisations, mitochondrial introgressions and phylogenetic relationships between major lineages. Mem Inst Oswaldo Cruz. 2015;110(3):403–13.

62. da Costa AP, Nunes PH, Leite BHS, Ferreira JG, Tomhosalio R, da Rosa AR, et al. Diversity of bats trypanosomes in hydroelectric area of Belo Monte in Brazilian Amazonia. Acta Trop. 2016;164:185–93.

63. Lauthier JJ, Tomasi N, Barnabé C, Rumi MMM, D’Amato AMA, Ragone PG, et al. Candidate targets for multilocus sequence typing of Trypanosoma cruzi: validation using parasite stocks from the Chaco region and a set of reference strains. Infect Genet Evol. 2012;12:350–8.

64. Jansen AM, Xavier SC, Roque ALR. The multiple and complex evolutionary origins of an enigmatic Trypanosoma cruzi domestic genotype. Parasit Vectors. 2012;5:226.

65. Barnabé C, Brisse S, Tibayrenc M. Phylogenetic diversity of bat trypanosomes of subgenus Schizotrypanum based on multilocus enzyme electrophoresis, random amplified polymorphic DNA, and cytochrome b nucleotide sequence analyses. Infect Genet Evol. 2003;3:201–8.

66. Pinto CM, Ocáñavar-Maigorría S, Tapia EE, Lobos SE, Zurita AP, Aguirre-Villacís F, et al. Phylogenetic relationships of bat trypanosomes in Ecuador: new insights into the diversity, transmission, and origins of Trypanosoma cruzi and Chagas disease. PLoS One. 2013;10(10):e0139999.

67. Marcili A, da Costa AP, Soares HS, Acosta IDCL, de Lima JTR, Minervino AHH, et al. Isolation and phylogenetic relationships of bat trypanosomes from different biomes in Mato Grosso, Brazil. J Parasitol. 2013;99:1071–6.

68. Messenger LA, Llewellyn MS, Bhattacharya T, Frantzén O, Lewis MD, Ramirez JD, et al. Multiple mitochondrial introgression events and heteroplasmasy in
Trypanosoma cruzi revealed by maxicircle MLST and next generation sequencing. PLoS Negl Trop Dis. 2012e15846.

86. Lemey P, Salemi M, Vandamme AM, editors. The Phylogenetic handbook. A practical approach to Phylogenetic analysis and hypothesis testing. 2nd ed. USA: Cambridge University Press; 2009.

87. Hamilton PB, Teixeira MMG, Stevens IR. The evolution of Trypanosoma cruzi: the “bat seeding” hypothesis. Trends Parasitol. 2012;28:136–41.