Novel insertions in the mitochondrial maxicircle of *Trypanosoma musculi,* a mouse trypanosome

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Abstract

Trypanosoma musculi is a, globally distributed, mouse-specific haemoflagellate, of the family Trypanosomatidae, which shares similar characteristics in morphology with *Trypanosoma lewisi*. The kinetoplast (mitochondrial) DNA of Trypanosomatidae flagellates is comprised of catenated maxicircles and minicircles. However, genetic information on the *T. musculi* kinetoplast remains largely unknown. In this study, the *T. musculi* maxicircle genome was completely assembled, with PacBio and Illumina sequencing, and the size was confirmed at 34,606 bp. It consisted of two distinct parts: the coding region and the divergent regions (DRs, DRI and II). In comparison with other trypanosome maxicircles (*Trypanosoma brucei*, *Trypanosoma cruzi* and *T. lewisi*), the *T. musculi* maxicircle has a syntenic distribution of genes and shares 73.9%, 78.0% and 92.7% sequence identity, respectively, over the whole coding region. Moreover, novel insertions in *MURF2* (630 bp) and in *ND5* (1,278 bp) were found, respectively, which are homologous to minicircles. These findings support an evolutionary scenario similar to the one proposed for insertions in *Trypanosoma cruzi*, the pathogen of American trypanosomiasis. These novel insertions, together with a deletion (281 bp) in *ND4*, questions the role of Complex I in *T. musculi*. A detailed analysis of DRII indicated that it contains numerous repeat motifs and palindromes, the latter of which are highly conservative and contain A_5C elements.

The comprehensively annotated kinetoplast maxicircle of *T. musculi* reveals a high degree of similarity between this parasite and the maxicircle of *T. lewisi* and suggests that the DRII could be a valuable marker for distinguishing these evolutionarily related species.

Key words: *Trypanosoma musculi*, Maxicircle, Indels, Minicircle, Palindrome, Divergent regions, *Trypanosoma lewisi*

Key Findings

- The *Trypanosoma musculi* maxicircle is comprehensively annotated.
- The insertions in *T. musculi* genes may be due to a gene translocation.
- Divergent region (DR) II may be a good bio-barcode for trypanosomes.

Introduction

Trypanosomes are protozoan parasites that are distributed globally which infect humans, vertebrate animals and intermediate invertebrate hosts. Among them, members of the subgenus *Herpetosoma*, such as *Trypanosoma lewisi* and *Trypanosoma musculi*, are commonly found in rodents (Hoare, 1972; Kostygov *et al.*, 2021). These two trypanosomes cannot be easily distinguished due to their high degree of similarity in morphological characteristics and genetic markers such as the SSU rDNA sequences (Hong *et al.*, 2017). However, they do significantly differ in many aspects. In particular, *T. lewisi* infects only rats and sometimes humans (Sarataphan *et al.*, 2007; Verma *et al.*, 2011), while *T. musculi* infects only mice and is unlikely to be pathogenic to humans (Zhang *et al.*, 2018). Some research has indicated that both *T. musculi* and *T. lewisi* can modulate the host immune response in coinfections with various other infectious agents, to potentially cause more harm to the hosts, by altering the infection kinetics and increasing the duration of colonization in the host (Gao *et al.*, 2021; Lowry *et al.*, 2014; Nzoumbou-Boko *et al.*, 2017; Vaux *et al.*, 2016). To gain a better understanding of the biological characteristics, the kinetoplast DNA (kDNA) of *T. lewisi* has been comprehensively analyzed (Li *et al.*, 2020; Lin *et al.*, 2015). However, little is known of the details of the kDNA in *T. musculi*.

Trypanosomes are members of the kinetoplastea group of protozoa, named due to the presence of the kDNA. Trypanosome kDNA is a specific network structure of interlocking mitochondrial DNA circles, which consists of thousands of minicircles with dozens of maxicircles (Lukes *et al.*, 2002). Previous research in *Trypanosoma brucei* has shown that kDNA comprises at least 5% of the total cellular DNA, while most other eukaryotic mitochondrial DNA accounts for no more than 1% (Lukeš *et al.*, 2018). In general, kDNA maxicircles encode functional homologues of mitochondrial genes which are flanked by non-coding regions that diverge significantly in sequence and size amongst trypanosome species (Simpson *et al.*, 1987; Sloof *et al.*, 1992; Westenberger *et al.*, 2006). One of unusual features of the kinetoplast is that most of the maxicircle gene transcripts are not mature and do not encode functional open reading frames. These encrypted transcripts become translatable only after post-transcriptional processing, namely RNA editing, that inserts and deletes uridine residues (Stuart *et al.*, 1997; Stuart *et al.*, 2005). RNA editing was first discovered in cytochrome oxidase subunit 2 (*COII*) gene of *Trypanosoma brucei* and *Crithidia fasciculata*, whose mRNA transcripts have four uridine insertions (Benne *et al.*, 1986). The minicircles, recognized by a conserved motif of twelve nucleotides (GGGGTTGGTGTA) (Ray, 1989), encode guide RNA (gRNA) molecules that accurately position the editing machinery to ensure correct maxicircle transcripts are produced (Blum & Simpson, 1990).

Here, using PacBio and Illumina sequencing reads, the complete maxicircle sequence of *T. musculi* was assembled and annotated, including the repetitive non-coding variable region. Comparative analyses indicate that the gene organization and distribution in *T. musculi* maxicircles are highly conserved with *T. brucei*, *T. cruzi* and *T. lewisi*. The maxicircle kDNA gene organization of *T. musculi* and comparison with its species relatives were also presented. In addition, the genetic information on the divergent region (DR) II reveals that it may provide a good marker for molecular diagnosis and molecular epidemiological investigation of trypanosomes.

Materials and methods

Parasites, ultrastructure, kDNA extraction and restriction endonuclease digestion

Trypanosoma musculi Partinico II strain was gifted by Prof. Philippe Vincendeau of Université de Bordeaux, France, which was originally obtained from the London School of Hygiene and Tropical Medicine (Krampitz, 1969). *T. musculi* Particino 2, Lincicome and CDC strains were purchased from American Type Culture Collection (ATCC). Trypanosomes were harvested from the blood of infected mice and cultured in RPMI-1640 medium at 37°C supplemented with 10% fetal bovine serum (FBS) and a feeder layer of mouse macrophages as modified from Behr (Behr *et al.*, 1990). Protocols for the use of mice were approved by the Institutional Review Board for Animal Care at Sun Yat-Sen University under license of 31672276. For transmission electron microscopy, trypanosome specimens were prepared according to the method of Bozzola (Bozzola, 2014), and observed by using the JEM-100CX-II microscope system. For *T. musculi* DNA preparations, total DNA was purified using a phenol-chloroform method and kDNA was extracted by sucrose gradient ultracentrifugation according to previously published methods (Pérez-Morga & Englund, 1993). The isolated kDNA network was visualized on a 1% agarose gel and analyzed with restriction enzymes

HindIII, *EcoRI*, *BamHI*, *RsaI*, *HaeIII* and *TaqI* (New England Biolabs, USA). A computer-simulated restriction enzyme digestion map of *T. musculi* maxicircle was generated using the Dnaman 9.0 software (Lynnon Corporation, Quebec, Canada) based on the sequence assembled in this study.

Immunofluorescence assay

Trypanosome cells (1×10^7 cells/mL) were centrifuged for 5 minutes at 3,000 × g and washed twice in PBS. The cells were then transferred onto clean slides, which were left to air-dry in a fume hood, following fixation by methanol for 10 minutes. Dried slides were rehydrated and washed twice in PBS for 5 minutes at room temperature. The slides were then incubated with primary mAb-anti-L8C4 (1:800) followed by incubation with Cy3-Conjugated goat anti-mouse IgG (A10521, Thermo Fisher) (1:400) followed by a counterstaining consisting of 1× PBS with 3 μg/mL 4,6-diamidino-2-phenylindole (DAPI) (KOHL *et al.*, 1999). They were then photographed using a Leica fluorescence microscope.

Deep sequencing, sequence assembly and PCR verification

In order to generate a high quality maxicircle assembly, a kDNA Illumina library was constructed and sequenced using Illumina HiSeq2000 technology commercially (Novogene, China). Also, a PacBio Sequel library was constructed using total DNA and sequenced commercially (Annoroad, China). The Illumina reads were checked for quality and trimmed using fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Trimmomatic (Bolger *et al.*, 2014).

Canu 2.0 software was used in *de novo* assembly of the *T. musculi* genome with parameter "genomeSize=30m minReadLength=600 minOverlapLength=300 corOutCoverage=100 corMinCoverage=2 correctedErrorRate=0.035" using the PacBio reads (Koren *et al.*, 2017). Then, the genome contigs, assembled from PacBio reads, were polished using the Illumina reads by pilon software to improve genome assembly (Walker *et al.*, 2014). Finally, the assembly sequences were aligned, with BLAST, to a previously obtained *T. lewisi* maxicircle assembly (KR072974) and redundant overlap deleted by MEGA 7.0 to yield the complete maxicircle sequence (Camacho *et al.*, 2009; Kumar *et al.*, 2016). To obtain *T. b. rhodesiense*, *Trypanosoma grayi* and *T. lewisi* complete maxicircle genomes, processed reads were assembled from WGS data (SRX3199071, SRX620256 and SRR11918574, respectively) freely available on NCBI using SPAdes 3.12.0 with parameter "--plasmid --careful -t 16 -m 200" (Antipov *et al.*, 2016). Then, alignment and trimming were also carried out as completed for *T. musculi*. The sequences of the *T. musculi* maxicircle coding region were also corrected by PCR verification using 12 pairs of primers. Meanwhile, *MURF2*, *ND4* and *ND5* genes were also amplified in three additional *T. musculi* strains (*T. musculi* Particino 2, Lincicome and CDC). Primers used are summarized in Table S1.

Gene annotation

Annotation of *T. musculi* maxicircle coding regions was performed by comparison with *T. brucei* (EATRO 427, M94286.1), *T. cruzi* (CL, DQ343645.1) and *T. lewisi* (CPO02, KR072974.1) manually using BLAST. Patterns of RNA editing of *T. musculi* maxicircle genes were predicted according to GC percent and RNA editing pattern of *T. lewisi* (Li *et al.*, 2020).

Data analysis

Dotplot graphs of the *T. musculi* maxicircle sequence plotted against itself and four other Trypanosomatidae species were generated by YASS software with default parameter (allow 10% indels, 25% mutations and e-value $< 1 \times 10^{-5}$ in alignment) (Noé & Kucherov, 2005). GC percentage, assembly coverage and homology search algorithms were drawn using Circos v0.69 (Krzywinski *et al.*, 2009). Regions (>300 bp) with sufficient sequence identity were plotted as colored ribbons, denoting the percentage of sequence identity. BioEdit software was used to create alignments and calculate nucleotide percentage identity matrices among different Trypanosomatidae species (Hall, 1999). Curation of the palindromes and inserted sequence homology analysis was performed using BLAST. MEME software was used to identify motifs and generate LOGO diagrams in the DRI region (Bailey *et al.*, 2015).

Phylogenetic analysis

The entire coding region of the kinetoplast maxicircles were aligned using ClustalO 1.2.4 (Sievers *et al.*, 2011) and the alignment was trimmed using Gblocks 0.91b with option "-t=d -b4=5 -b5=h" (Talavera & Castresana, 2007). Maximum likelihood trees were generated by RAxML 8.2.12 with 1,000 bootstrap replicates (Stamatakis, 2014). Neighbor joining and Minimum evolution trees were performed using MEGA 7.0 including 1000 bootstrap pseudo-replicates. Maxicircle genome sequences used in this work are summarized in Table S2.

Results

Morphology, ultrastructure, kDNA isolation and restriction enzyme digestion

In culture, *T. musculi* cells tend to attach to each other and form a rosette-like pattern (Fig. 1A), via their flagella, as determined using specific antibodies against the paraflagellar rod (Fig. 1C). At this stage, *T. musculi* is at the epimastigote stage in which the kinetoplast lies closely beside the nucleus. Ultrastructural analysis showed that the kinetoplast DNA disk was 660 ± 99 nm in length and 152 ± 26 nm in width ($n = 50$) (Fig. 1B), which is similar to closely related species such as *T. lewisi* (Lin *et al.*, 2015).

A total of 10^{10} *T. musculi* were harvested and high quality kDNA was obtained with a 260/280 absorbance ratio of 1.80. Kinetoplast DNA was found to be intact and free from contamination with nuclear or host DNAs as judged by agarose electrophoresis (Fig. S1A). Meanwhile, kDNA was incubated with endonucleases of *HindIII*, *EcoRI*, *BamHI*, *RsaI*, *HaeIII* and *TaqI* and a computer-simulated restriction enzyme digestion map was generated based on the maxicircle assembly which is described later (Fig. S1B-C). Some bands smaller than 4.0 kb did not correspond to the computed simulated patterns of *T. musculi* maxicircle, which implies the presence of a high number of possible heterogeneous minicircles in the kDNA of *T. musculi*. The bands consistently observed in Fig. S1B at ~1.3 kb suggest the presence of minicircles of a similar size as reported in *T.*

lewisi (Li *et al.*, 2020). Patterns that are free of kDNA in the wells were achieved using *HaeIII* and *TaqI* and indicated a high frequency of cleavage of kDNA minicircles. Most likely, the bands with molecular sizes of >4 kb correlated with the computer simulated patterns (Fig. S1C), are derived from kDNA maxicircles, except a band (~5 kb) in *RsaI* lane, a potential result of incomplete digestion. Moreover, the presence of four large molecular weight bands, in the *EcoRI* digestion, with sizes of >10 kb, ~7 kb ~6 kb and ~4 kb, indicated that the full-size kDNA maxicircle is larger than the sum of 27 kb.

Assembly and annotation of the kDNA maxicircle

Genomic DNA from the *T. musculi* Partinico II strain was sequenced on PacBio Sequel and Illumina platforms and contigs were assembled with the long-read assembler Canu 2.0 and corrected with the Illumina reads in Pilon. Then, a contig in length of 38,603 bp was identified in a BLAST search against the *T. lewisi* maxicircle (KR072974). This had two overlapping regions of 4,002 bp (covering positions from -3,341 or 31,266 to 661 bp) at each end (Fig. S2) confirming completion of the circle. Meanwhile, the maxicircle sequence has also been confirmed using five overlapping raw reads from the PacBio library (Fig. S2) and the maxicircle coding region sequences were also further refined using 12 pairs of primers and Sanger sequencing validation (Fig. S3). Finally, a 34,606 bp-long complete *T. musculi* maxicircle sequence was obtained, with an average coverage of 13.2X from Illumina reads and 268X from PacBio reads, including the coding regions (16,975 bp) and the DRs

(17,631 bp). The overall GC content of the maxicircle was 23.7%, with 27.5% in coding regions and 20.1% in DRs (Fig. 2).

Twenty genes were annotated in the *T. musculi* maxicircle by comparison with known Trypanosomatidae species (*T. brucei*, *T. cruzi* and *T. lewisi*), as listed in Table 1. All genes were found to be syntenic with the maxicircles of the comparator Trypanosomatidae species *T. brucei*, *T. cruzi* and *T. lewisi* (Fig. 2, S4). A sequence homology analysis (Fig. S5), showed that the *T. musculi* maxicircle has 92.7% identity to *T. lewisi* (blue ribbons). The ribbons change to yellow when compared with *T. cruzi* (78.0% identity) and *T. brucei* (73.9% identity), largely due to the low similarity in extensively-edited genes (Table 2). Moreover, three breaks shown as discontinuations of lines or ribbons (Fig. S4, S5) appear in *T. musculi* genes *MURF2*, *ND5* and *ND4*, with two sections (630 bp and 1,278 bp) inserted and one section deleted (281 bp), respectively (Fig. 3A and Table 1).

The confirmation of mutations in *MURF2*, *ND5* and *ND4* were performed with three other strains (*T. musculi* Particino 2, Lincicome and CDC). PCR results showed that the insertion in *MURF2* is specific to *T. musculi* Partinico II strain and not present in other three strains, while the insertion in *ND5* and the deletion in *ND4* exists in all tested strains (Fig. 3B). Furthermore, these insertions and the deletion have also been confirmed by inspecting alignments of the raw reads mapped back to the maxicircle assembly. Alignment analysis of insertions showed that a fragment (150 bp) at the 5’ end region of *MURF2* insertion sequence is homologous to both the 5’ end and middle regions of *ND5* insertion sequence (Fig. 3C). Moreover, those sequences in *MURF2* and *ND5* respectively share 95.3%, 96% and 95.3% identity with conserved regions of *T. lewisi* minicircles (MN447336.1, MN447339.1 and MN447386.1), and these 150 bp homologous regions cover three conserved sequence blocks (CSBs) of minicircles, indicating a minicircle origin of both insertions in *MURF2* and *ND5*. Together with the data shown in Fig. S1 and Fig. 3C, it seems that, unsurprisingly, *T. musculi* has a similar size and structure to minicircles reported for *T. lewisi* (Li *et al.*, 2020), i.e. ~1.3-kb category I minicircles that have two conserved regions with CSB1-3 motifs (and perhaps also ~1.5 kb category II minicircles with only one conserved region; such a band is also apparent in Fig. S1B). It therefore appears that the ~1.3 kb *ND5* insertion corresponds to a (degenerated) category I minicircle and the 630 bp *MURF2* insertion corresponds to half a category I minicircle.

RNA editing patterns of the maxicircle have been well studied in *T. brucei* and *T. lewisi* (Gerasimov *et al.*, 2018; Li *et al.*, 2020) and they are well correlated with GC%. The GC% pattern in *T. musculi* is fairly similar to *T. brucei* and *T. lewisi.* However, unexpectedly high GC contents were noticed in *MURF2* and *ND5* (Fig. S6), and they are precisely attributed to the insertions in both genes. In another region, *COII* and its cis-acting gRNA were identified (Table 1).

The whole coding region of the maxicircle is considered as a valuable marker for phylogenetic relationships of Trypanosomatidae species (Kaufer *et al.*, 2019). In order to further confirm the evolutionary relationship of *T. musculi* and other Trypanosomatidae species, sequences corresponding to whole coding region of *T. musculi* were aligned with the sequences from other Trypanosomatidae species to infer phylogenetic relationships. In the tree, *T. musculi* and *T. lewisi* are identified as belonging to the same subgenus *Herpetosoma*, clustered with the sister groups of *Schizotrypanum* and *Aneza* (Fig. 4).

Sequence analysis of the maxicircle divergent regions

A common theme of the maxicircle DR is the presence of various repeat arrays, which is also the case for *T. musculi*. The full map of DR of *T. musculi* was built by the YASS and Circos packages to identify homologous regions and to show global patterns of DR organization (Fig. 5A, 5B). Dotplot analyses of the DR showed two typical sections (I, II), flanking either the 12S rRNA or *ND5*. DRI is in a length of about 1.6 kb which is composed of short and highly repetitive units of about 107 bp, with two motifs being found (Fig. 5C). While DRII is in a length of about 14 kb, it consists of a series of tandem elements, namely α, β, γ, σ, and short version α', β', γ' (Fig. S7).

Palindromes are a typical structure already found in *T. cruzi*, *T. lewisi* and *T. rangeli*. Based on identifying homologues using BLAST, four AT-rich conserved palindromes were shown up in the DRII (Fig. 5B, 5D). Palindromes I and IV have the same perfect palindrome structure, 34 bp long, and are located at 19,898 bp and 28,055 bp. While palindromes II and III have one T-to-A substitution, they are located at 23,648 bp and 26,061 bp. A further BLAST analysis with the maxicircles of *T. b. brucei* (Lister 427, MN904526.1), *T. b. equiperdum* (STIB 818, EU185799.1), *Trypanosoma congolense* (IL3000, GCA_003013265.1) and *Trypanosoma vivax* (Y486, MT090068.1) enabled the identification of similar palindromes in these species (Fig. 5D), only one of each species is shown for illustrative purposes. These palindromes are highly conserved and contain an A_5C element.

Unlike highly conserved coding regions, DRs show species specificity among trypanosomes (Fig. S5). It displays about 70% sequence identity in DRI between the *T. musculi* and *T. lewisi* maxicircle, while there are only some similar sequences (~400 bp) in DRII. Moreover, there are no other homologous sequences between *T. musculi* and the other two species (*T. cruzi* and *T. brucei*) in the DRs (Fig. S5). Therefore, these results suggest that DRII is highly divergent among trypanosomes, which may have the potential to be a good molecular marker for distinguishing *T. musculi* from related species.

Discussion

In this study, a sequence of the 34,606 bp kDNA maxicircle genome from *T. musculi* was reported and an in-depth investigation of *T. musculi* maxicircle sequences and comparative analysis with other Trypanosomatidae species were also undertaken. The size of the total coding region of the *T. musculi* kDNA maxicircle is 16,975 bp with two pronounced insertions in *T. musculi MURF2* (630 bp), *ND5* (1,278 bp) and one deleted fragment of *ND4* (281 bp) (Fig. 3A). It is different from *T. brucei*, *T. cruzi* and *T. lewisi*, in which their sizes are around 15,000 bp length. The two insertions in *T. musculi* maxicircle genes correspond to either to a partial minicircle (630 bp) containing the one of the CSBs or to a complete minicircle (1,278 bp) containing two CSBs. Such insertions have not been observed in other Trypanosomatidae species except *Leishmania donovani* (1S LdBob strain) and *T. cruzi* (TcV strain) where the insertions were considered to be derived from minicircles due to the conserved CSBs. Therefore, the insertions were also thought to be a consequence of gene translocation, from minicircles to maxicircles (Berná *et al.*, 2021; Nebohácová *et al.*, 2009). Mostly gRNA genes are encoded in minicircles, but some gRNA genes, such as gMurf2 (30-79) and gNd7 (216-252)

(Koslowsky *et al.*, 2014; Li *et al.*, 2020), were reported to be encoding in maxicircles in *T. brucei* and *T. lewisi*, respectively. Moreover, 7 maxicircle-encoded gRNAs were identical in *L. tarentolae* LEM125 and UC strains, which mediate the editing of *Cyb*, *MURF2*, *A6* and *ND7* transcripts (Simpson *et al.*, 2015). It can be assumed that the insertions deriving from minicircles may also possibly encode gRNA genes for RNA editing, therefore these may be an intermediate stage indicating that maxicircle encoding gRNA genes have originated from minicircles.

Maxicircle gene deletions are only rarely found in Trypanosomatidae species, such as the similar deletions seen in *ND4* of the *T. cruzi* Esmeraldo strain (Westenberger *et al.*, 2006), and *ND7* gene from asymptomatic *T. cruzi* isolates (Baptista *et al.*, 2006). The effect of these insertions and deletions on the parasite life cycle is still unclear. *ND5* and *ND4* are known as non-edited genes in other known Trypanosomatidae species and it is inconceivable that these large insertions/deletions could be corrected by U-insertion/deletion editing of the mRNAs. Nevertheless, all of the above insertions/deletions are found in *ND4*, *ND5* as well as *ND7* genes, and these genes all encode subunits of the mitochondrial respiratory chain NADH-dehydrogenase (Complex I). Since the presence of a functional Complex I in Trypanosomatidae species has long been debated (Duarte & Tomás, 2014; Opperdoes & Michels, 2008). Deletions in kDNA encoding Complex I subunits were identified in some strains of *T. cruzi* that seems no impact in mitochondrial bioenergetics, ROS production or redox state in this parasite (César Carranza *et al.*, 2009). Although the presence of Complex I and its involvement in respiration has been clearly demonstrated in *T. brucei*, it appears to be non-essential for procyclic forms (Beattie & Howton, 1996; Surve *et al.*, 2012; Verner *et al.*, 2011). The lack of editing in several Complex I subunits in *L. tarentolae* UC strain also suggests that it may not be essential (Simpson *et al.*, 2015). Therefore, the possibility that the role of Complex I subunits is less important in *T. musculi* was favored with the presence of insertions/deletions in *ND5* and *ND4*. In addition, another insertion occurs in *MURF2*, whose function remains uncertain but hypothesis could be risen. *MURF2* might be a new component in Complex I. The insertion in *MURF2* may be a recent event as it only found in Partinico II strain, but not other three strains of *T. musculi*. The loss of conservation in *MURF2* could probably be attributed to the loss of function of Complex I components and consequently on selection pressures on the gene. To verify this hypothesis, a highly sensitive and accurate identification of the functioning of Complex I in Trypanosomatidae species would be interesting to investigate.

The DR of the kinetoplast maxicircle was initially described as a variable and non-coding region and the DR structure seemed to be drastically different in various species (Borst *et al.*, 1980; Borst *et al.*, 1982; Maslov *et al.*, 1984; Muhich *et al.*, 1983; Stuart & Gelvin, 1982). Therefore, the function of the DRs remains as an enigma. Studies on *T. brucei*, *Crithidia oncopelti*, *Leptomonas collosoma* and *Leishmania seymouri* revealed some CSBs-like sequences in their maxicircle DRs. As CSBs are essential for minicircle replication (Ryan *et al.*, 1988), CSBs-like sequences may play a similar role in maxicircle replication (Flegontov *et al.*, 2006; Gorbat *et al.*, 1990; Myler *et al.*, 1993; Sloof *et al.*, 1992). However, CSB-I or III-like regions were not identified in *T. musculi* DRs, instead, only a CSB-II-like region (CCCGTGT) is located at 19,817 bp. CSB-I or III-like regions were also not found in DRs of the closely related *T. lewisi*, suggesting a CSB-independent maxicircle replication mechanism exists in these species. Therefore, although CSB-like sequences were present in the insertions of *T. musculi MURF2* and *ND5*, it is not clear whether they are also involved in maxicircle replication.

It has been demonstrated that hairpins or cruciform structures (palindromes) are frequently associated with promoters and may also act as protein-binding sites (Wadkins, 2000). Palindromes with an A_5C-element in DRs are suggested as recognition sites for binding of transcription factors or transcription initiation (Flegontov *et al.*, 2006; Vasil'eva *et al.*, 2004). Some palindromes also have been identified in *T. musculi* as well as in a variety of other Trypanosomatidae species, which each consists of one A_5C-element. It may be speculated that these palindromes play a significant role in Trypanosomatidae species maxicircles, judged by their high degree of sequence conservation in the evolution of Kinetoplastida species.

The trypanosome maxicircle presents itself as a complex evolutionary system, and it may be an excellent taxonomic marker in phylogenetic analysis. The coding region of the maxicircle in phylogenetic analyses provides a robust evolutionary insight into the relationships within Trypanosomatidae species (Kaufer *et al.*, 2019; Kay *et al.*, 2020; Lin *et al.*, 2015). A close affinity between *T. musculi* and *T. lewisi* in *Herpetosoma* was also supported, which clustered with the sister groups of *Schizotrypanum* and *Aneza*. Unlike the highly conserved coding region, the DRs of maxicircle, especially DRII sequence, was found to be significantly divergent and species-specific (Kay *et al.*, 2020). The homologies in DRII between closely related species, e.g., *T. musculi* and *T. lewisi*, phylogenetic clades of *T. cruzi*, are limited (Fig. S5, S8). Such a characteristic of DRII provides an opportunity for developing a valuable molecular marker for distinguishing closely related species and subspecies. Actually, a preliminary test on three *T. musculi* strains and six *T. lewisi* strains revealed a consistent amplification of DRII fragments, which could enable them to be distinguished from each other and 13 strains of other trypanosomes (Hong *et al.*, 2017).

In general, this study reports the first detailed description and analysis of the kDNA maxicircle genome of *T. musculi* and reveals a relatively high overall conservation of gene content and synteny with other trypanosome species. Furthermore, the divergence of DRII suggests its potential as a valuable marker for distinguishing these evolutionarily related species.

Supplementary materials

Data

Nucleotide sequence data reported in this paper are available in GenBank databases under accession numbers: *Trypanosoma musculi* maxicircle sequence (OM000218), *Trypanosoma lewisi* maxicircle sequence (OM000219), *Trypanosoma grayi* maxicircle sequence (OM049542), and *Trypanosoma brucei rhodesiense* maxicircle sequence (OM049543). PacBio and Illumina sequencing data have been deposited in NCBI's Sequence Read Archive (SRA) with BioProject ID PRJNA792722.

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Authors contributions

JF Wang, RH Lin, DH Lai and ZR Lun designed the study. JF Wang, RH Lin and X Zhang conducted data gathering and performed statistical analyses. JF Wang, RH Lin and DH Lai drafted the manuscript and undertook data extraction and screening. G Hide, ZR Lun and DH Lai critically reviewed the paper. All authors approved the final version and agree to be accountable for all aspects of the work.

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Conflict of interest

The authors declare there are no conflicts of interest.

Ethical standards

The animal ethical approval of sample collection was obtained from the Institutional Review Board of Animal Care at Sun Yat-Sen University (License no. 31672276).

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Tables

Table 1. Gene positions and lengths in the maxicircle of *T. musculi*.

Gene	**RNA editing**	***T. musculi*** **position**	***T. musculi*** **length**	***T. lewisi*** **length**	***T. cruzi*** **length**	***T. brucei*** **length**
12S rRNA	-	1-1164	1164	1168	1161	1149
9S rRNA	-	1210-1818	609	608	608	611
ND8	extensive	1859-2130	272	285	279	266
ND9#	extensive	2195-2536	342	350	338	321
uS3m#	none	2575-2823	249	241	264	234
ND7	extensive	2874-3647	774	771	755	702
COIII	extensive	3721-4135	415	414	424	439
Cyb	minor	4222-5301	1080	1080	1080	1080
ATPase6	extensive	5342-5644	303	304	336	369
ND2	none	5687-7038	1352	1341	1341	1237
CR3*	extensive	6979-7111	~133	~123	~119	/
ND1 **#**	none	7129-8112	984	942	942	957
COII	minor	8111-8739	629	629	629	626
COII **gRNA**	-	8746-8758	-	-	-	-
MURF2	minor	8767-10439	1673	1053	1056	1041
(Insertion)	-	8897-9526	630	-	-	-
COI **#**	none	10429-12081	1653	1650	1650	1734
CR4#	extensive	12102-12319	218	212	207	185
ND4 Δ	none	12428-13464	1037	1314	1314	1311
ND3 **#**	extensive	13453-13636	184	187	193	256
RPS12	extensive	13711-13908	198	190	191	172
ND5	none	13929-16975	3047	1773	1770	1770
(Insertion)	-	14923-16200	1278	-	-	-

Gene positions are shown relative to the start of the *12S rRNA*

These genes are encoded on the reverse strand

**CR3* two end positions from *T. musculi*, *T. lewisi*, *T. cruzi* and *T. brucei* are uncertain

Δ A fragment deletion is found in the *T. musculi ND4* gene

Table 2. Comparison of the average percentage identities of *T. musculi* kDNA maxicircle with those from the other three Trypanosomatidae species.

Comparison of *T. musculi*	Whole coding region	5’-edited genes	Extensively-edited genes	rRNAs	Non-edited genes
vs. *T. lewisi*	92.7%	95.8%	92.4%	95.4%	92.2%
vs. *T. cruzi*	78.0%	84.5%	73.7%	84.0%	78.4%
vs. *T. brucei*	73.9%	84.4%	60.2%	80.5%	74.8%

Entire coding region: start from 5’end of *12S rRNA* to 3’ end of *ND5*

5’-edited genes: *Cyb*, *COII*

Extensively-edited genes: *ND8*, *ND9*, *ND7*, *COIII*, *ATPase6*, *CR3*, *CR4*, *ND3*, *RPS12*

Non-edited genes: *uS3m*, *ND2*, *ND1*, *COI*

MURF2, *ND4* and *ND5* genes are not calculated in *T. musculi* (5’-edited genes or non-edited genes) due to insertions/deletion

Legends to Figures

Fig. 1. Morphology and ultrastructure of *T. musculi*. (A) Giemsa staining of the epimastigote form of *T. musculi* from *in vitro* culture (scale bar 5 μm). Flagellum (F), nucleus (N) and kinetoplast (K) are indicated. (B) Electron micrograph of the *T. musculi* trypomastigote form (scale bar 200 nm). Basal body (BB), flagellum (F), flagellar pocket (FP) and kinetoplast (K) are indicated. (C) Immunofluorescence analysis (IFA) shows epimastigote-like forms of *T. musculi* from *in vitro* cultivation with antibody L8C4 detecting paraflagellar rods (green) and DAPI detecting kinetoplast DNA and nuclear DNA (blue) (scale bars 5 μm).

Fig. 2. Circos plot of the *T. musculi* maxicircle. The outer track indicates the gene arrangements and gene distribution; the middle track represents the GC content (orange for above-average and green for below-average) and the inner track is a histogram of assembly coverage.

Fig. 3. Analysis of insertions or deletions in the *T. musculi* maxicircle genes *MURF2*, *ND5* and *ND4*. (A) A diagram of insertions or deletions in the *T. musculi* maxicircle genes *MURF2* (630 bp insertion), *ND5* (1278 bp insertion) and *ND4* (281 bp deletion). Insertion regions are labeled as grey boxes. (B) PCR amplification of the *T. musculi MURF2*, *ND5* and *ND4* genes from *T. musculi* Partinico II (1), *T. musculi* Particino 2 (2), *T. musculi* Lincicome (3), *T. musculi* CDC (4), and fragments are analyzed on a 1.0 % agarose gel. M, DL2000 marker (Takara, China). N, negative control. The positions of primers are showed in (A) and Table S1. (C) Alignment of

the conserved regions from the *T. lewisi* minicircle (MN447335.1) and insertion sequences of *MURF2* and *ND5*. Conserved sequence blocks (CSB-I, II, III) are highlighted in grey.

Fig. 4. Phylogenetic analysis of the Trypanosomatidae species using the maxicircle coding regions. Phylogenetic tree is performed based on Maximum likelihood /Neighbor joining/Minimum evolution methods with 1,000 bootstrap replicates with the respective bootstrap confidences indicated at branch points. Branch lengths are indicated by the black line and the scale bar represents the number of nucleotide substitutions per site.

Fig. 5. Sequence analyses of the divergent region of the *T. musculi* maxicircle. (A) Dot plot analysis of the *T. musculi* maxicircle, the main diagonal line represents the sequence's alignment with itself and the lines about the main diagonal represent repetitive patterns within the maxicircle sequence. (B) Circos plot of the divergent region of *T. musculi* maxicircle, the outer track indicates gene arrangement and gene distribution. Ribbons inside the circle connect homologous regions, color represents percent of sequence identity in range [70%; 100%] in the order yellow, green, and blue. Four palindromes are showed with red arrows. (C) The repetitive sequences from DR I are aligned and two motifs predicted by MEME are indicated with black line. LOGO diagrams show nucleotides at a given position of each motif and relative frequency represented by height. (D) The palindromes from the divergent region of the Trypanosomatidae species are shown with the palindromic bases highlighted.

Legends to Supplementary Figures

Fig. S1. Restriction endonuclease analysis of *T. musculi* kDNA. (A) Kinetoplast DNA from *T. musculi* is resolved on a 1.0% agarose gel. (B) Restriction endonuclease digestion of *T. musculi* kDNA as analyzed on a 1.0% agarose gel. (C) Computer simulated virtual restriction patterns derived from the complete *T. musculi* maxicircle with the same set of restriction enzymes from (B). Asterisks indicate >4.0 kb-long bands identified both in (B) and (C), and a triangle indicates the band (~5 kb) only present in (B). The marker is DL10000 (Takara, China).

Fig. S2. The assembly contigs in *T. musculi.* The maxicircle is drawn in a linear form, starting from the *12S rRNA* gene. Regions covered by raw reads and the assembly contigs are shown below in green and overlaps at each end are colored in blue.

Fig. S3. PCR amplification of *T. musculi* maxicircle DNA using twelve pairs of primers, fragments are analyzed on a 1.0 % agarose gel. The marker is DL10000 (Takara, China). The information on the primers has been summarized in Table S1.

Fig. S4. Dot plot comparative analysis of the coding region of *T. musculi* compared with the coding region of *T. lewisi* (A), *T. cruzi* (B) and *T. brucei* (C), respectively. Diagonal lines indicate that the DNA sequences of two compared species are homologous in the corresponding regions (allow 10% indels, 25% mutations and e-value $< 1 \times 10^{-5}$ in alignment). The break regions in seen in the *T. musculi* vs. other Trypanosomatidae species are due to the insertions and deletions in *T. musculi* which are, respectively, marked by blue and orange ellipses.

Fig. S5. Circos plots that compare the maxicircles of two species, (A) *T. musculi* (left sequence) and *T. lewisi* (right sequence); (B) *T. musculi* (left sequence) and *T. cruzi* (right sequence); (C) *T. musculi* (left sequence) and *T. brucei* (right sequence). Ribbons inside the circle connect homologous regions (>300 bp), and the colors represent the percent of sequence identity in the range [70%; 100%] in the order yellow, green, and blue. Sequence identity was calculated using BLAST. Recently developed primers (TM1-2 and TL1-3) for distinguishing *T. musculi* and *T. lewisi* were indicated (Hong *et al.*, 2017).

Fig. S6. GC percentage graphs of the maxicircle coding regions of *T. brucei* (A) and *T. musculi* (B). The regions where percentage GC content value lie above the dashed lines may likely have RNA editing. Window size is 100 bp.

Fig. S7. Diagram of the repeated elements (α, α’, β, β’, γ, γ’, σ) identified by dot plot analysis of DRII.

Fig. S8. Circos plots that compare the maxicircles from three *T. cruzi* strains. (A) *T. cruzi* TCC (left sequence) and *T. cruzi* Dm28c (right sequence); (B) *T. cruzi* TCC (left sequence) and *T. cruzi* Y (right sequence); (C) *T. cruzi* Dm28c (left sequence) and *T. cruzi* Y (right sequence).

Ribbons inside the circle connect homologous regions (>300 bp), and the colors represent the percent of sequence identity in the range [70%; 100%] in the order yellow, green, and blue. Sequence identity was calculated using BLAST.

Figures

Figure 1

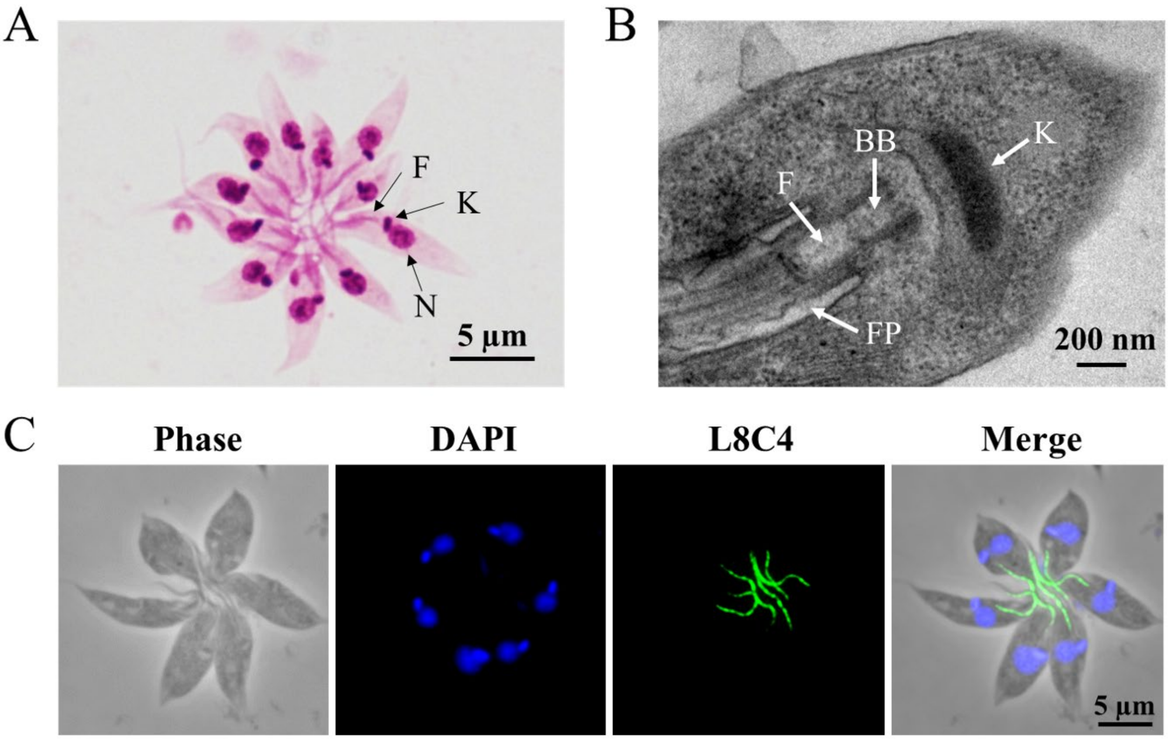
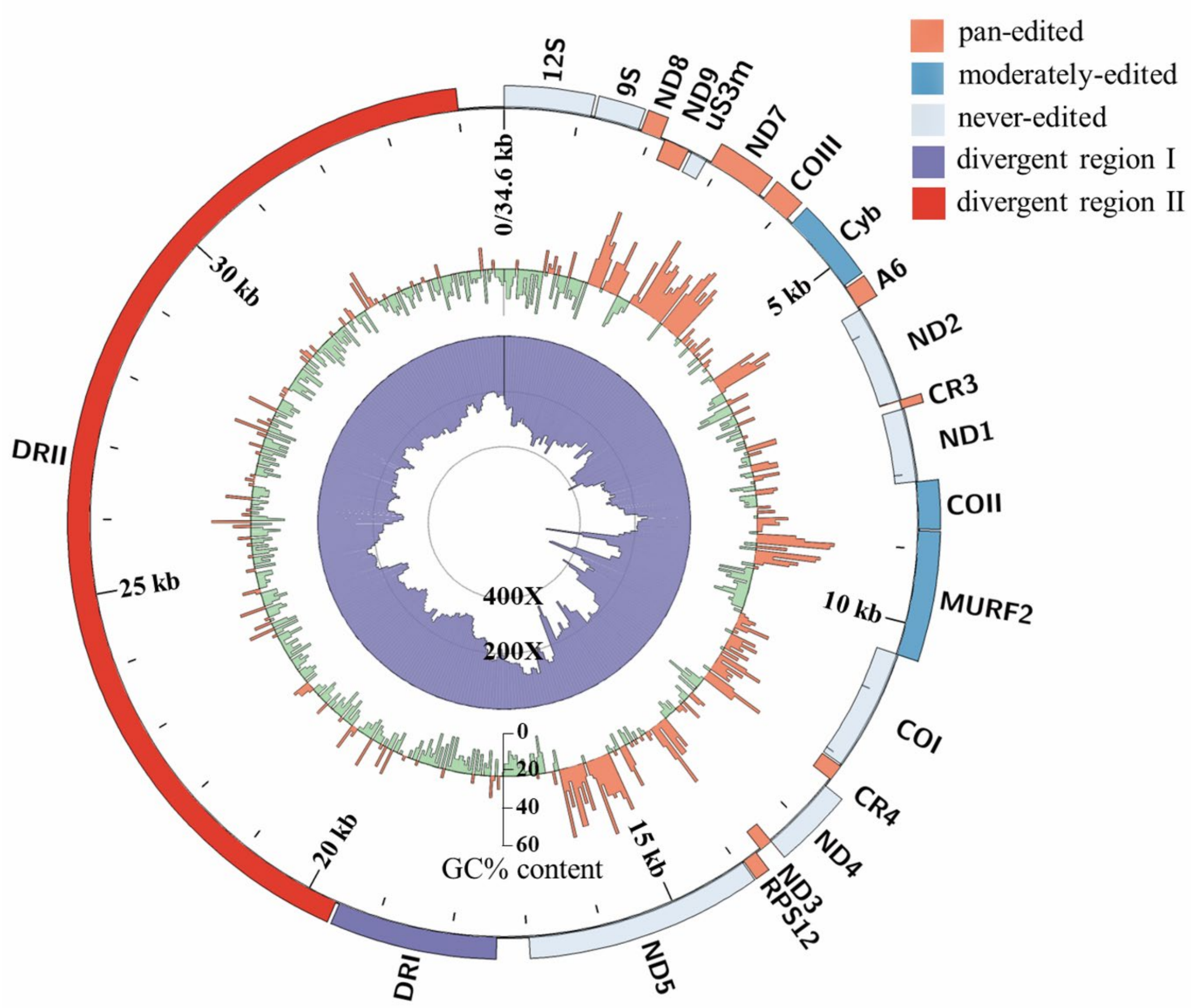


Figure 2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divergent region II
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40
60
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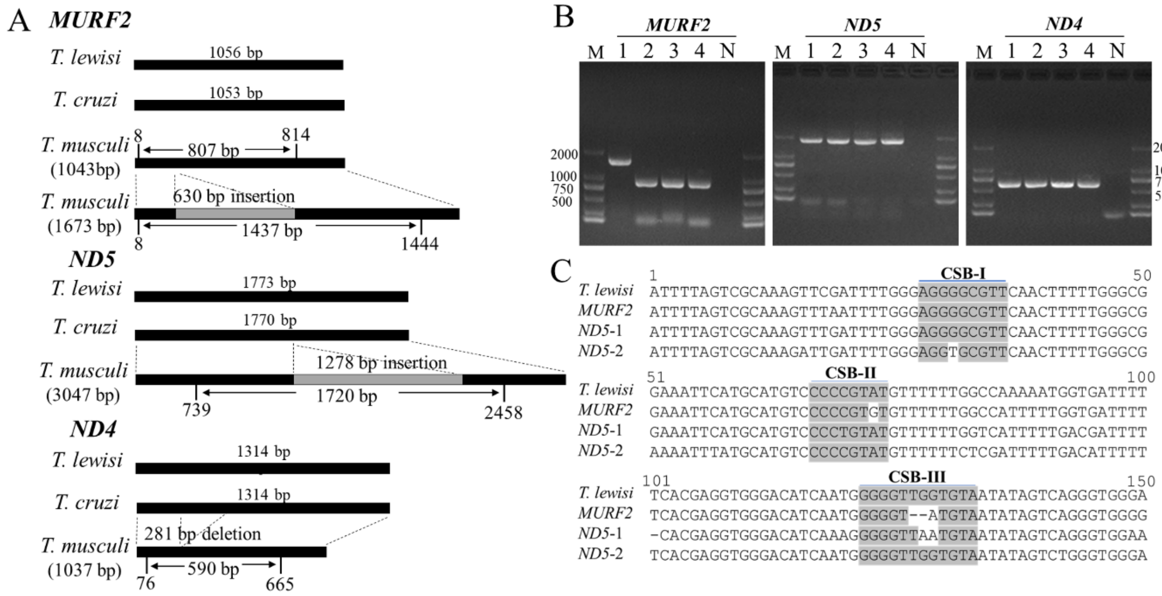
Figure 3



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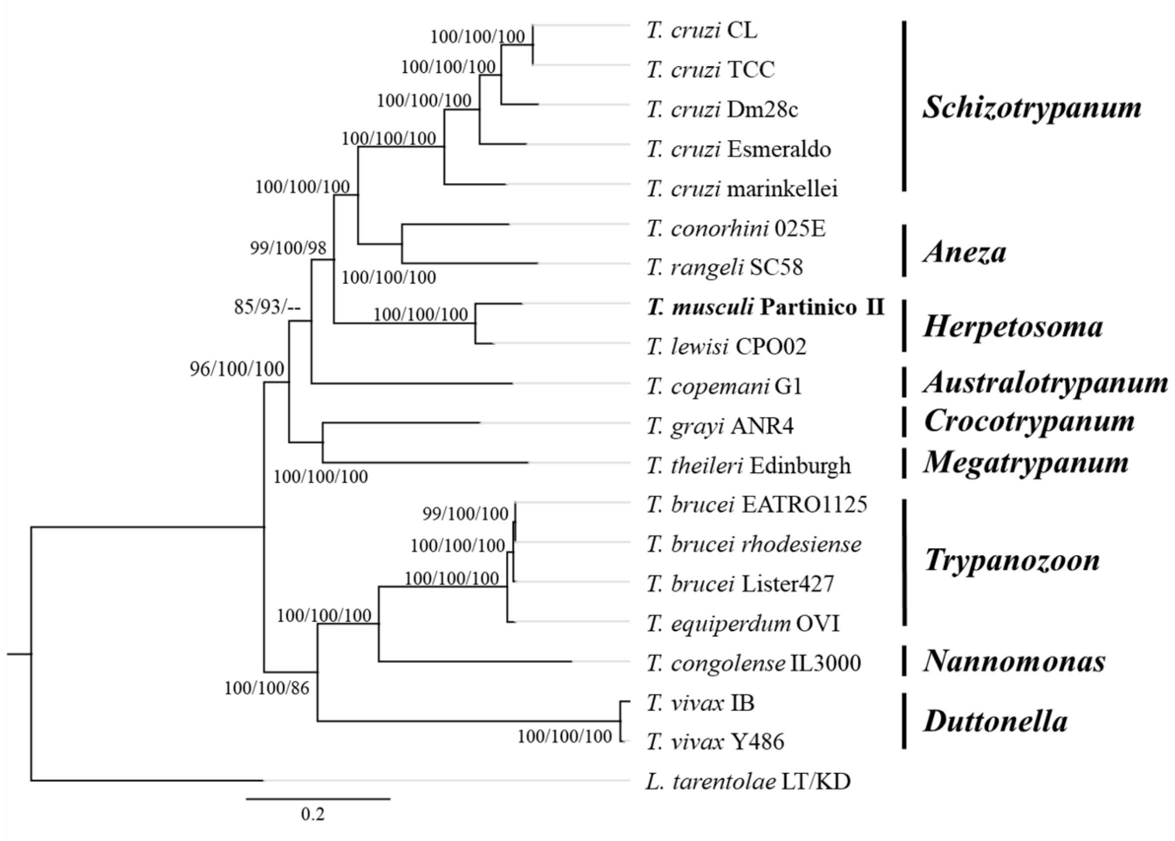

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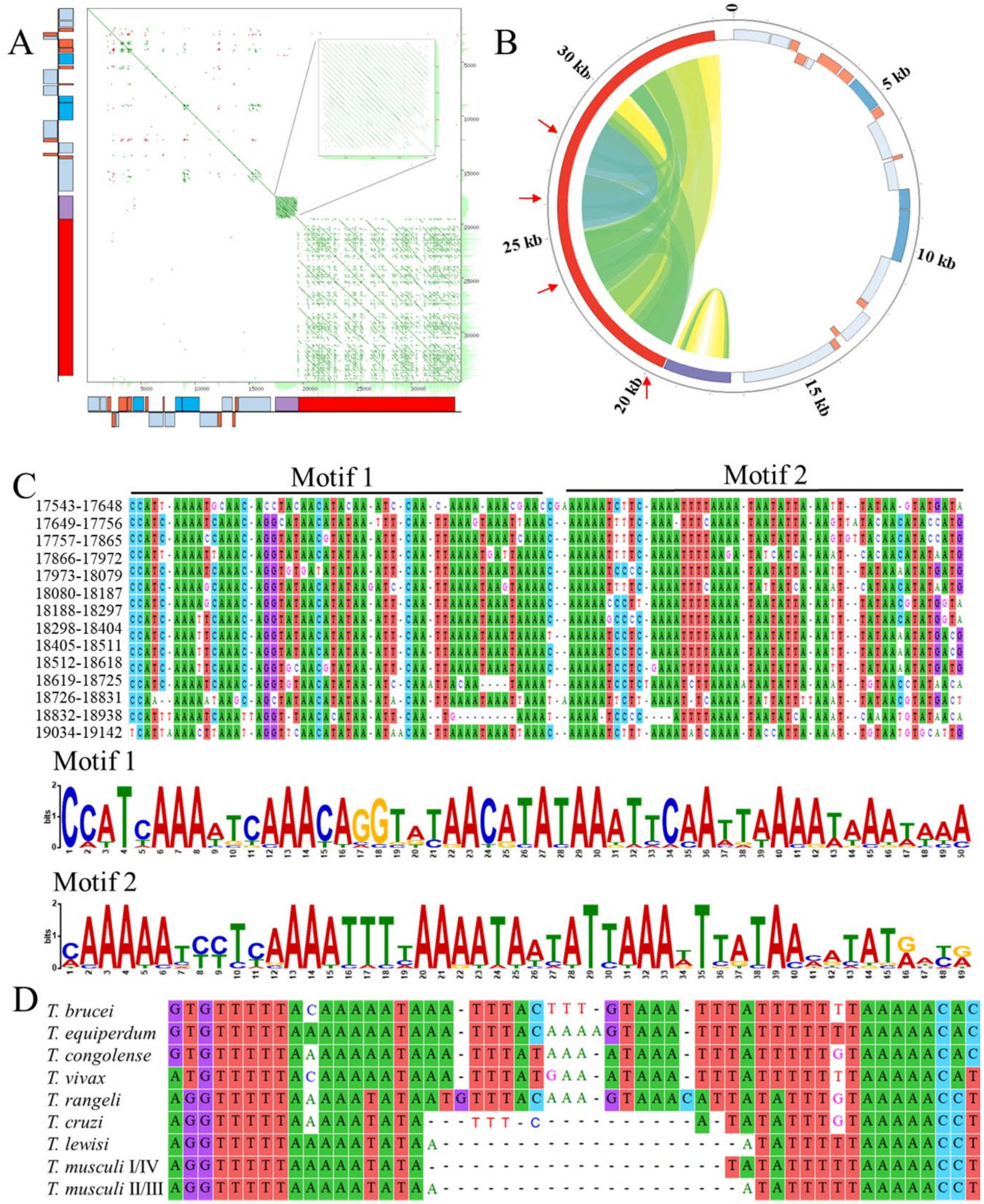

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T. vivax ATGTTTTTACAAAAATAAA-TTTATGAA-ATAAA-TTTATTTTTTTAAAAACAT
T. rangeli AGGTTTTTAAAAATATAATGTTTACAAA-GTAAACATTATATTTGTAAAAACCT
T. cruzi AGGTTTTTAAAAATATA---TTT-C----------A-TATATTTGTAAAAACCT
T. lewisi AGGTTTTTAAAAATATAA--------------------ATATTTTTAAAAACCT
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Figure S1

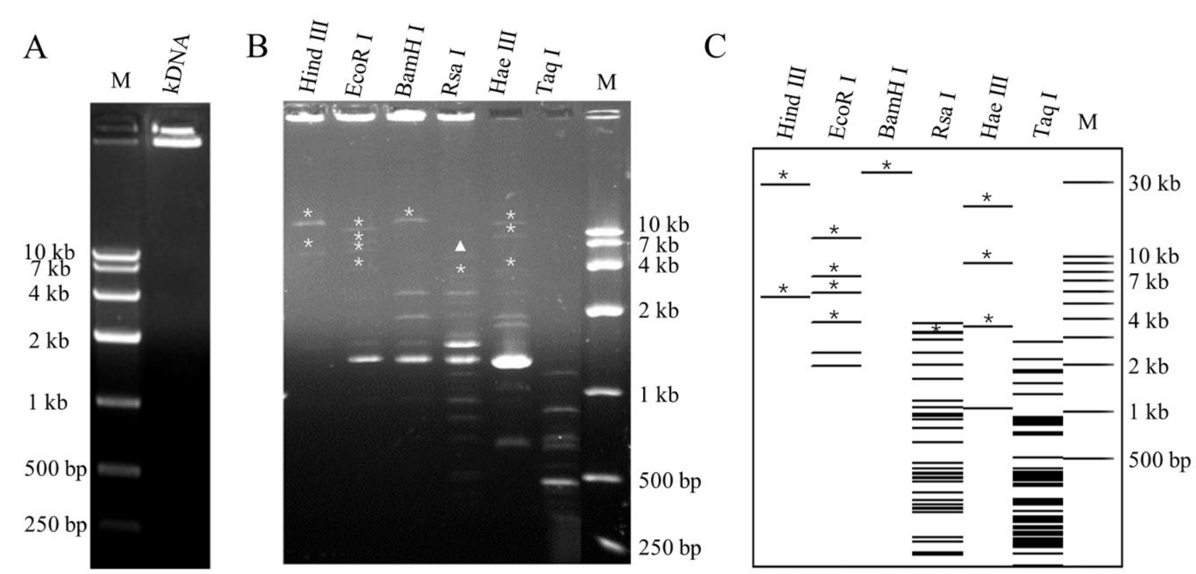
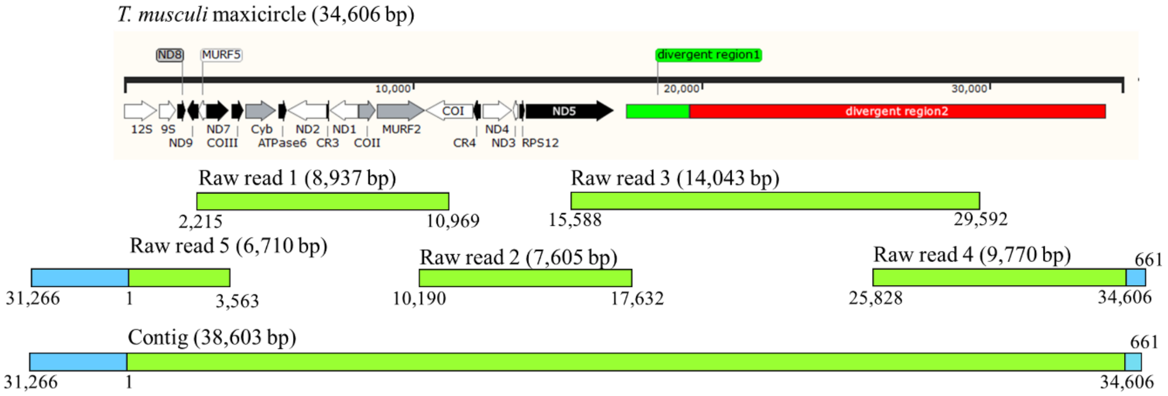


Figure S2



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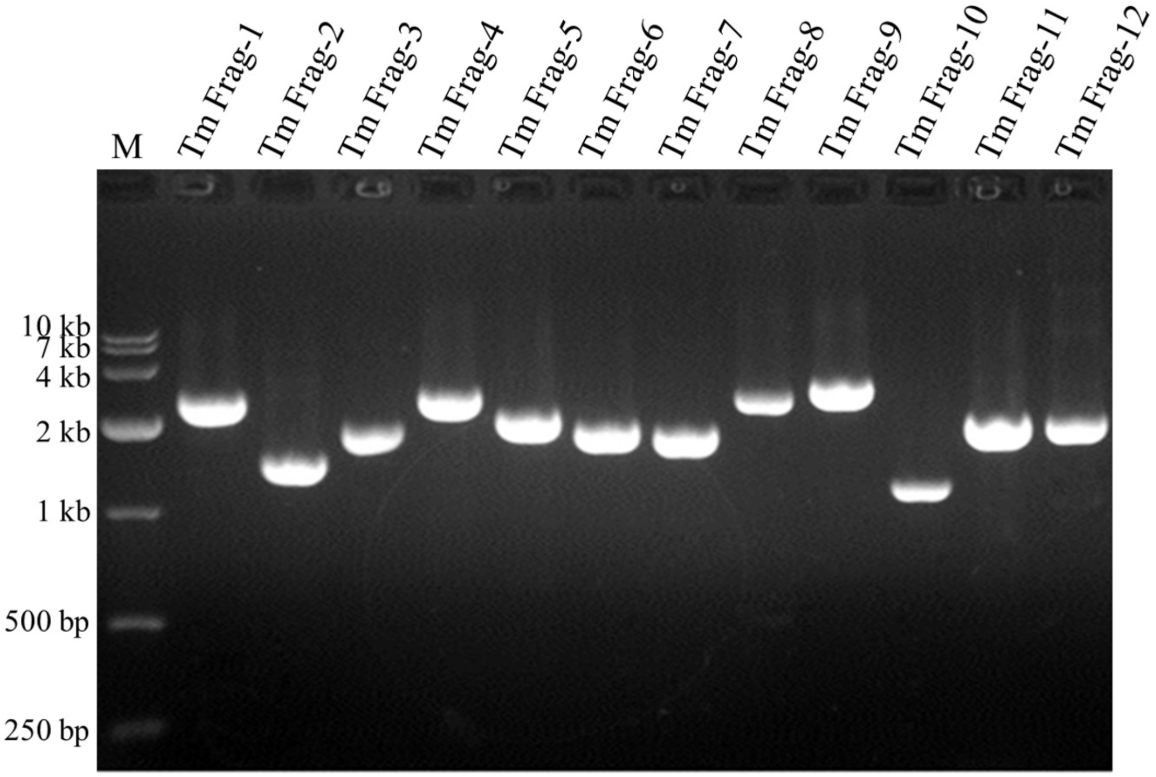
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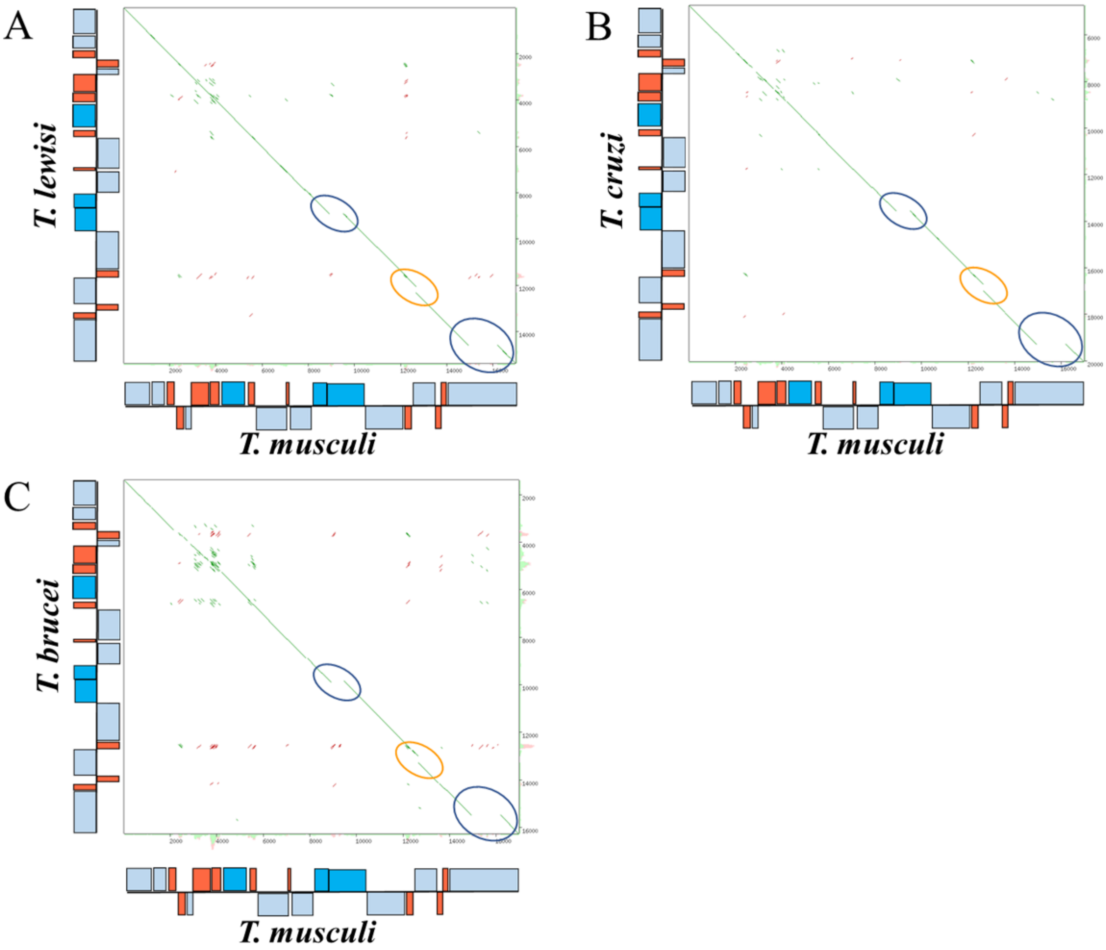

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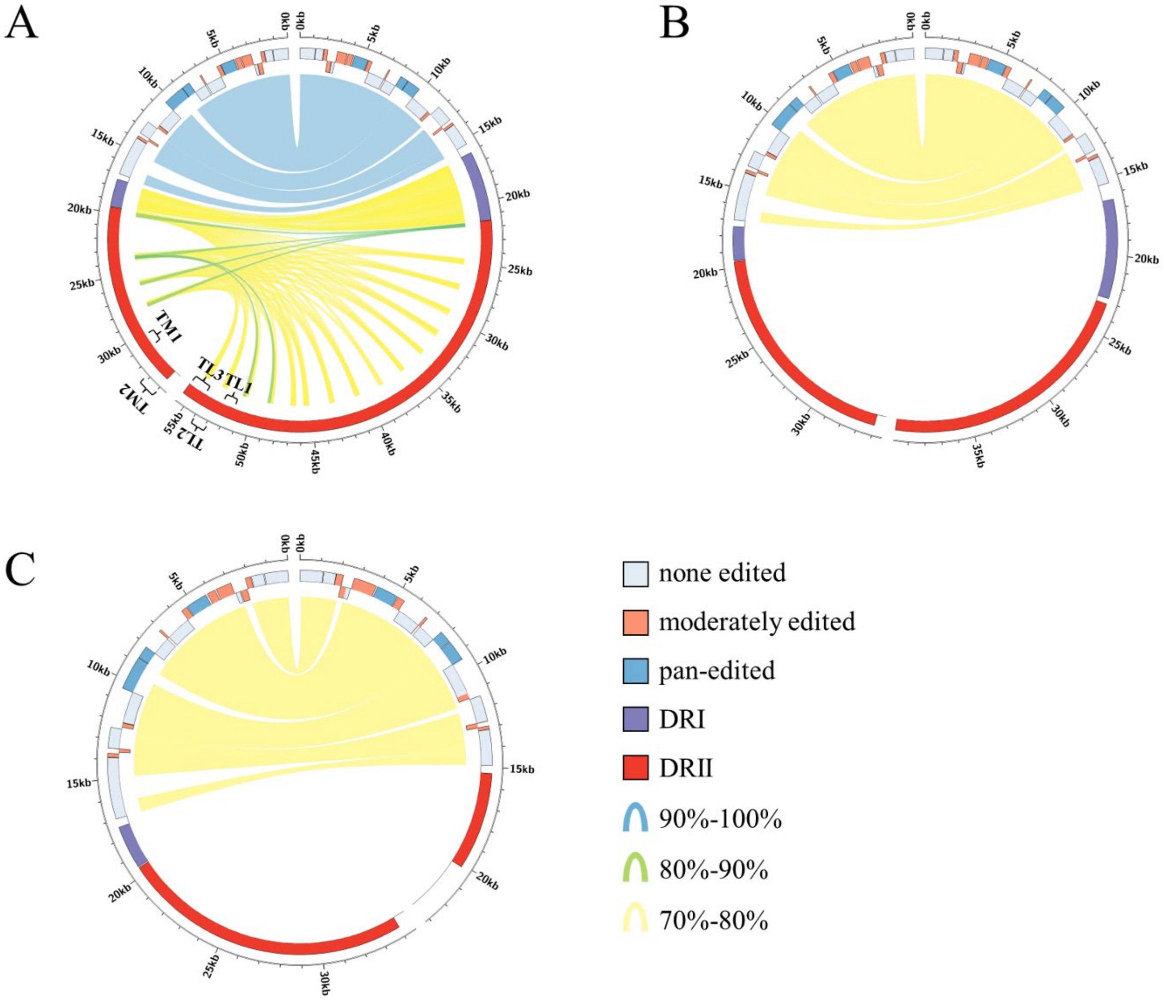
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TM2
TL1
TL2
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DRII
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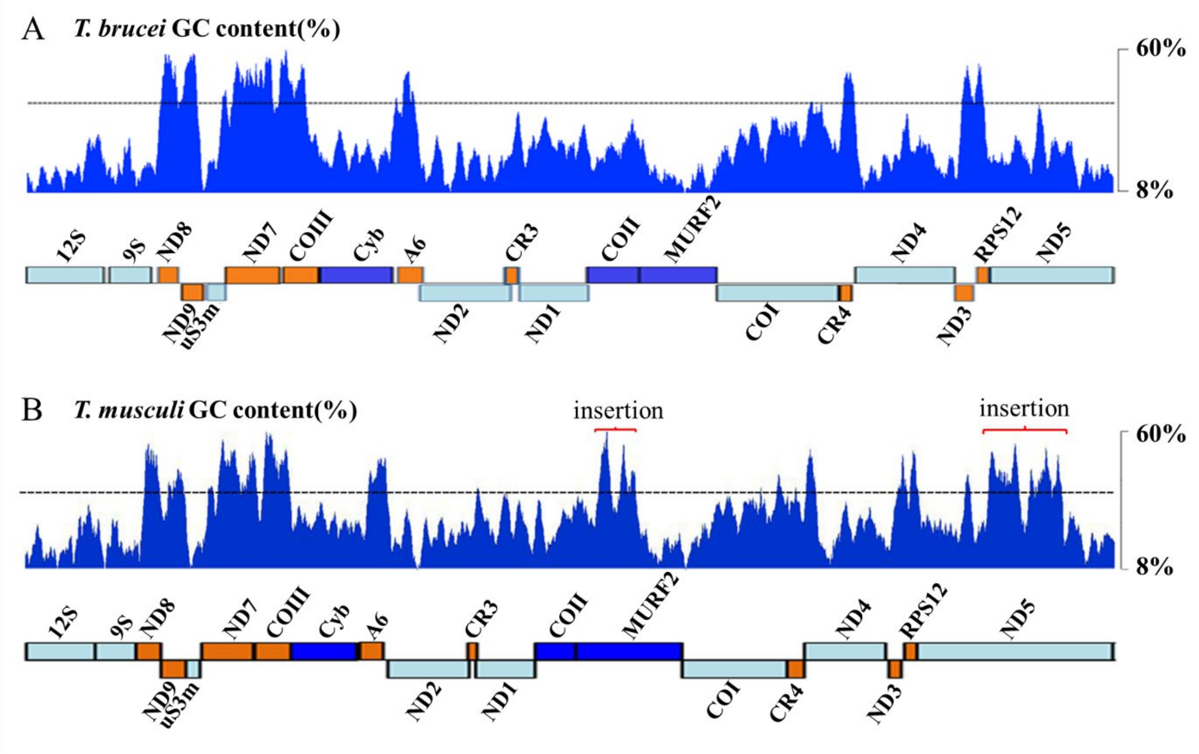
80%-90%
70%-80%

Figure S6

Figure S7



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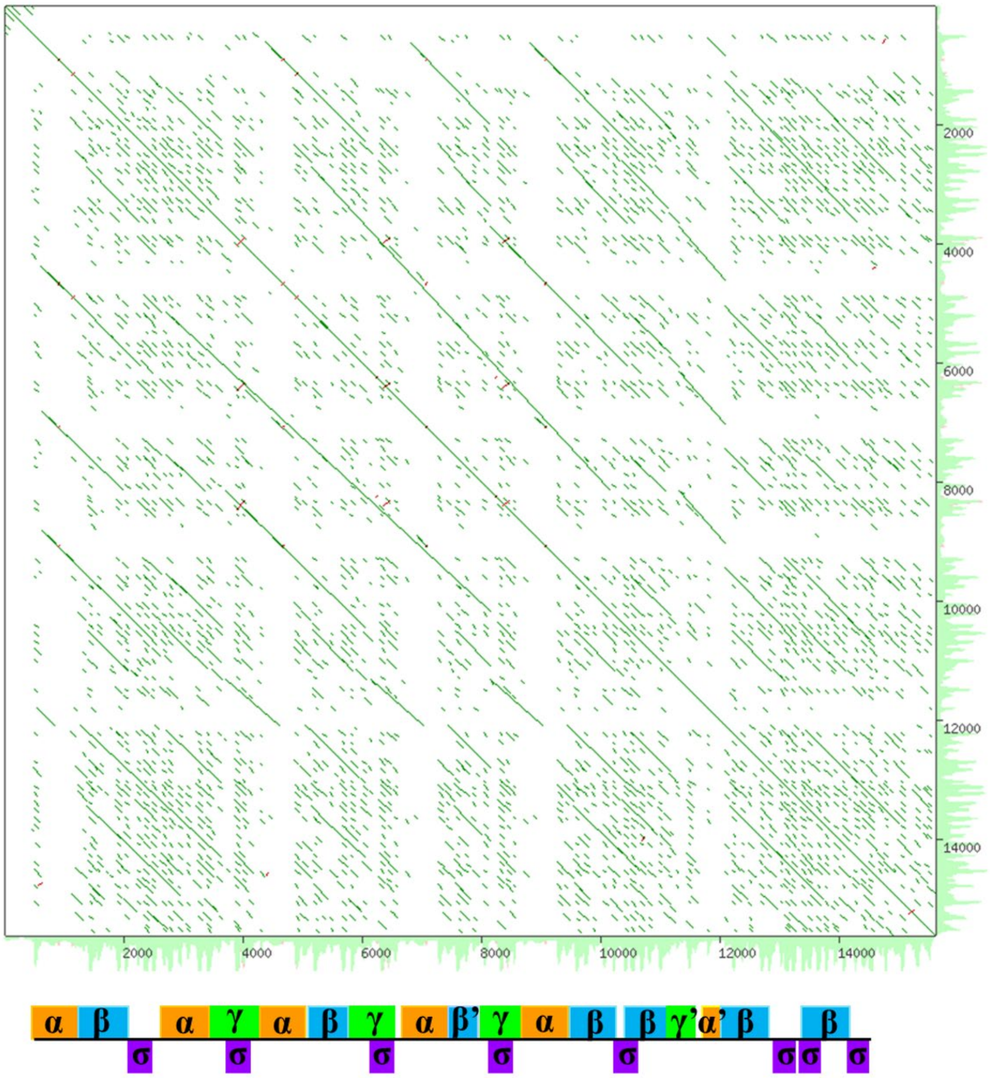
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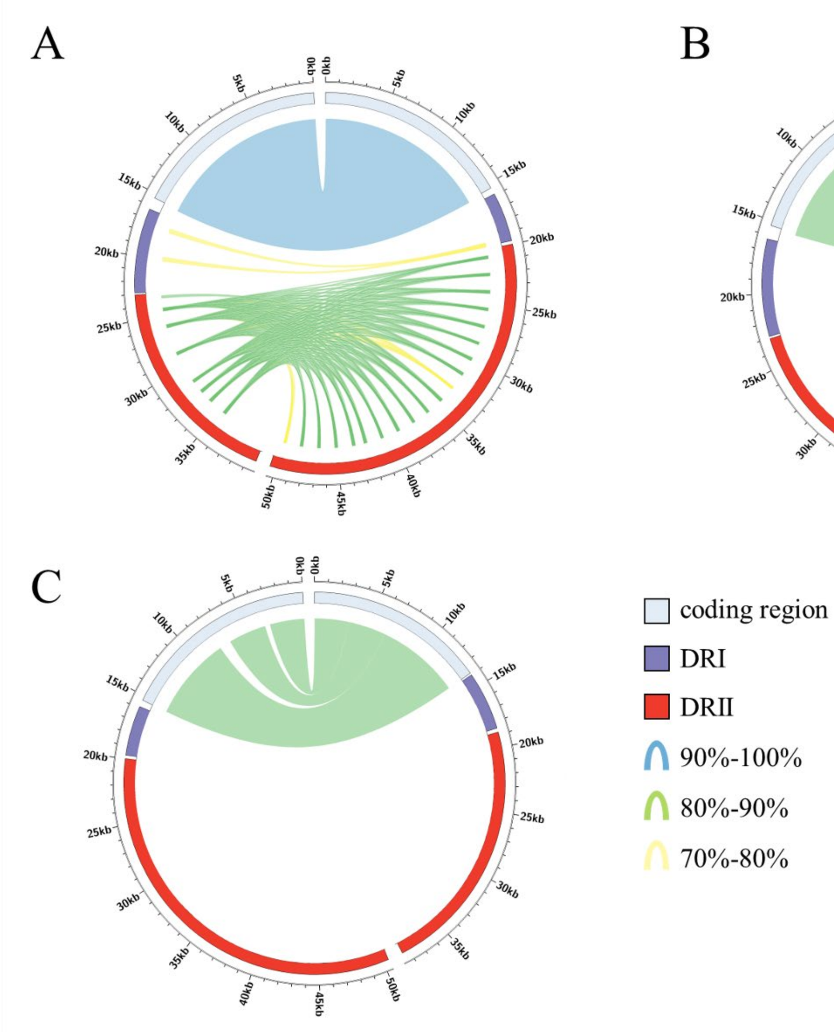
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80%-90%
70%-80%

Supplementary Table S1. Primers for PCR amplification of *T. musculi* (Tm) maxicircle

Primer name	Forward Primer (5’ →3’)	Reserve Primer (5’ →3’)	Region	Length (bp)
Tm Frag-1	AAACTATTCTTCCCATCTTACAA	CTGGCATCCATTTCTGACTA	33470-1438	2575
Tm Frag-2	AGTACAATTCGTTAGTTGGGTT	GGGGTAGAGAAGGAGGAGAT	1060-2493	1434
Tm Frag-3	AGGTTGCCCTCTTGTCGTCA	TCCCGTGTTCCTCCTTTCG	2216-4089	1874
Tm Frag-4	TTACCCGTTTCAGCACAGG	TCCTACTCAATGGGATGTCTTC	2909-5561	2653
Tm Frag-5	TGGACCAGAGGAGGTTGAAG	AGCGTTATGTTAGCATGTTTGT	3892-5999	2108
Tm Frag-6	GAAGACATCCCATTGAGTAGG	GTGAGAGTGAATTAGTTGCAGG	5540-7436	1897
Tm Frag-7	AAAGGTGAGGAAACCCATAAC	TCCCACCTATTTCACATACACC	7171-8986	1816
Tm Frag-8	CCAGGAAGGTGTAATGAGGT	ACTGAAGAGGGAATGGGTGT	8620-11718	3099
Tm Frag-9	GGTAGGAGGGTGATGTATTATTG	GATTTGTTATCTGTTAGTGGCG	9046-12028	2983
Tm Frag-10	GCACACCGCATCCTACTAAC	TGTAGCCTATGTATGGTTATGAA	11931-13093	1163
Tm Frag-11	TGTTCGCAATAAAGTATCCG	ACTACTAAGGTAGCCGCATGT	12694-14685	1992
Tm Frag-12	ACAGCATACTGCGGTTGAG	AGTGGATTCATACACCCATAAC	14327-16247	1921
MURF2	TAAGAAGTTTTGATTGAGTCG	CAGATGGCATTAAAACGAAAC	8775-10211 (8-1444)	1437
ND5	TGCGGCTACCTTAGTAGTGT	CAAGTCTTTACAATAAAACCCA	14668-16387 (739-2458)	1720
ND4	GTTTTTGTTTTGCGATTGAT	TGTAGCCTATGTATGGTTATGAA	12504-13093 (76-665)	590

Supplementary Table S2. Accession numbers of trypanosomatidae species sequences used in the work.

Species (Strain)	Accession numbers
Trypanosoma cruzi (CL)	DQ343645.1
Trypanosoma cruzi (TCC)	MN904528.1
Trypanosoma cruzi (Dm28c)	MN904527.1
Trypanosoma cruzi (Esmeraldo)	DQ343646.1
Trypanosoma cruzi (marinkellei)	KC427240.1
Trypanosoma cruzi (Y)	MW421591.1
Trypanosoma conorhini (025E)	MKKU01000412.1
Trypanosoma rangeli (SC58)	KJ803830.1
Trypanosoma musculi (Partinico II)	OM000218
Trypanosoma lewisi (CPO02)	KR072974, OM000219
Trypanosoma copemani (G1)	MG948557.1
Trypanosoma grayi (ANR4)	OM049542
Trypanosoma theileri (Edinburgh)	GCF_002087225.1
Trypanosoma brucei (EATRO 1125)	MK584625.1
Trypanosoma brucei (EATRO 427)	M94286.1
Trypanosoma brucei rhodesiense	OM049543
Trypanosoma brucei (Lister 427)	MN904526.1
Trypanosoma brucei equiperdum (OVI)	CZPT02000280.1
Trypanosoma brucei equiperdum (STIB	EU185799.1
Trypanosoma congolense (IL3000)	GCA_003013265.1
Trypanosoma vivax (IB)	MT090069.1
Trypanosoma vivax (Y486)	MT090068.1
Leishmania tarentolae (LT/KD)	MK514114.1

LOCUS Seq1 34606 bp DNA circular INV 22-DEC-2021

DEFINITION Trypanosoma musculi strain Partinico II maxicircle kinetoplast, complete sequence.

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VERSION
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              Trypanosomatida; Trypanosomatidae; Trypanosoma.
REFERENCE    1    (bases 1 to 34606)
  AUTHORS     Wang,J.F., Lin,R.H., Zhang,X., Zheng,L.L., Hide,G., Lun,Z.R. and
              Lai,D.H.
  TITLE         Mitochondrial maxicircle of Trypanosoma musculi, a mouse
              trypanosome
  JOURNAL     unpublished
REFERENCE    2    (bases 1 to 34606)
  AUTHORS     Wang,J.F., Lin,R.H., Zhang,X., Zheng,L.L., Hide,G., Lun,Z.R. and
              Lai,D.H.
  TITLE         Direct Submission
  JOURNAL     Submitted (22-DEC-2021) School of Life Sciences, Sun Yat-Sen
              University, No. 135, Xingang Xi Road, Guangzhou, Guangdong 510275,
              China
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1466