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The kinetoplast DNA of the Australian trypanosome, *Trypanosoma copemani*, shares features with *Trypanosoma cruzi* and *Trypanosoma lewisi* ☆

Adriana Botero [a,*], Irit Kapeller [b], Crystal Cooper [c], Peta L. Clode [c,d], Joseph Shlomai [b], R.C Andrew Thompson [a]

[a] School of Veterinary and Life Sciences, Murdoch University, South Street, Murdoch, WA 6150, Australia
[b] Department of Microbiology and Molecular Genetics and the Kuvin Center for the Study of Infectious and Tropical Diseases, The Hebrew University- Hadassah Medical School, Jerusalem, Israel
[c] Centre for Microscopy, Characterisation and Analysis, University of Western Australia, Stirling Hwy, Crawley, WA 6009, Australia
[d] School of Biological Sciences, University of Western Australia, Stirling Hwy, Crawley, WA 6009, Australia

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ABSTRACT

Kinetoplast DNA (kDNA) is the mitochondrial genome of trypanosomatids. It consists of a few dozen maxicircles and several thousand minicircles, all catenated topologically to form a two-dimensional DNA network. Minicircles are heterogeneous in size and sequence among species. They present one or several conserved regions that contain three highly conserved sequence blocks. CSB-1 (10 bp sequence) and CSB-2 (8 bp sequence) present lower interspecies homology, while CSB-3 (12 bp sequence) or the Universal Minicircle Sequence is conserved within most trypanosomatids. The Universal Minicircle Sequence is located at the replication origin of the minicircles, and is the binding site for the UMS binding protein, a protein involved in trypanosomatid survival and virulence. Here, we describe the structure and organisation of the kDNA of *Trypanosoma copemani*, a parasite that has been shown to infect mammalian cells and has been associated with the drastic decline of the endangered Australian marsupial, the woylie (*Bettongia penicillata*). Deep genomic sequencing showed that *T. copemani* presents two classes of minicircles that share sequence identity and organisation in the conserved sequence blocks with those of *Trypanosoma cruzi* and *Trypanosoma lewisi*. A 19,257 bp partial region of the maxicircle of *T. copemani* that contained the entire coding region was obtained. Comparative analysis of the *T. copemani* entire maxicircle coding region with the coding regions of *T. cruzi* and *T. lewisi* showed they share 71.05% and 71.28% identity, respectively. The shared features in the maxicircle/minicircle organisation and sequence between *T. copemani* and *T. cruzi*/*T. lewisi* suggest similarities in their process of kDNA replication, and are of significance in understanding the evolution of Australian trypanosomes.

1. Introduction

The kinetoplast is a network of circular DNA (kDNA) inside a large mitochondrion. It contains the mitochondrial genome, which consists of thousands of interlocked DNA circles of two types, maxicircles and minicircles that form a complex network (Lukeš et al., 2002; Jensen and Euglund, 2012). Maxicircles comprise only a small portion of the kDNA network, with only a few dozen identical copies. Their size ranges from approximately 20–40 Kb, depending upon the species, and they encode mitochondrial gene products (Shlomai, 2004). Maxicircles are composed of two regions; the first region is a coding region that contains homologs of mitochondrial genes characteristic of other eukaryotes (Simpson et al., 1987) and a variable non-coding region, also known as the divergent region (DR). The coding region contains two rRNA genes (12S rRNA and 9S rRNA), 14 protein coding genes (ND8, ND9, ND7, COIII, Cyb, ATPase6, MURF1 now known to be ND2, ND1, COII, COI, ND4, ND3, RSP12 and ND5), four genes of unknown function (MURF2, MURF5, CR3 and CR4), and a few guide RNAs (gRNAs) (Blom et al., 1990; Kannan and Burger, 2008; Ruvalcaba-Trejo and Sturm, 2011). Minicircles comprise the major portion of the kDNA network and are present in several thousand

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers MG948554, MG948555, MG948556 and MG948557.

* Corresponding author. Fax: +61 8 93602466.
E-mail address: L.BoteroGomez@murdoch.edu.au (A. Botero).

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copies that differ in size and sequence between species (Ray, 1987). They encode gRNAs that contain the genetic information for editing of mitochondrial RNA transcripts (Aphasizhev and Aphasizheva, 2014).

A considerable diversity in kDNA structure and conformation has been demonstrated within different kinetoplastids, including parasitic trypanosomatids and free-living bodonids. A classical disk-shaped kDNA network has been reported in several species, including *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania tarentolae*, and *Crithidia fasciculata*. Minicircles within the network are catenated, and are released from the network through decatenation by a type II DNA topoisomerase (yet to be discovered) prior to their replication (Shapiro and Englund, 1995). In contrast, the minicircles in species of more primitive kinetoplastids such as *Bodo caudatus* and others from the family Bodonidae are not organised in a network. Instead, they are distributed in diverse forms across the mitochondrial matrix known as poly-kDNA, pan-kDNA, mega-kDNA, and pro-kDNA (Vickerman, 1990; Lukeš et al., 2002). Correlations between these diverse patterns of kDNA organisation and genetic analysis based on nuclear rRNA genes have contributed to a better understanding of the evolution of kDNA and have facilitated the establishment of phylogenetic relationships between kinetoplastids (Lukeš et al., 2002).

A common feature within minicircles is the presence of a conserved region that contains three highly conserved sequence blocks (CSBs). CSB-1 (10 bp sequence) and CSB-2 (8 bp sequence) present lower interspecies homology, while CSB-3 (12 bp sequence) or the Universal Minicircle Sequence (UMS) is conserved within most trypanosomatids and is part of the minicircle replication origin (Ray, 1989). However, the number of conserved regions and their location in each minicircle differ among species (Ponzi et al., 1984; Sugisaki and Ray, 1987; Degrave et al., 1988). The regions of the minicircles flanked by the conserved regions are heterogeneous in sequence and have been used to investigate intraspecific variations within species (Telleria et al., 2006). Due to the minicircle abundance and heterogeneity in sequence, they have been frequently used in the development of PCR-based sensitive and specific diagnostic molecular tools (Noyes et al., 1998; Botero et al., 2010; Ceccarelli et al., 2014). CSB-3 or UMS is the specific binding site for the UMS binding protein (UMSBP) (Tzfati et al., 1992, 1995; Abeliovich et al., 1993), a protein involved in kDNA replication and segregation (Milman et al., 2007). The UMSBP of *C. fasciculata* has been extensively studied. It is a single-stranded sequence-specific DNA binding protein that binds the UMS (12 mer) and a hexameric sequence (in the context of a 14 mer) (Abu-Elneel et al., 1999) that are conserved at the replication origins of *C. fasciculata* kDNA minicircles, as well as in the minicircles of other trypanosomatid species (Ray, 1989). Recent studies using antibodies raised against *C. fasciculata* UMSBP revealed the presence of *C. fasciculata* UMSBP orthologues in other trypanosomatids such as *T. cruzi* and *T. brucei* (Milman et al., 2007), as well as *Leishmania donovani* (Singh et al., 2016). The importance of UMSBP in the survival of trypanosomatids was demonstrated using RNA interference experiments. It was shown that simultaneous knockdown of the two UMSBP orthologous genes in *T. brucei* not only affects the initiation of minicircle replication, but also inhibits segregation of the daughter networks and blocks nuclear division (Milman et al., 2007). Moreover, a recent study showed that the deletion of *L. donovani* UMSBP induced kDNA loss, apoptosis, and regulated the virulence of the parasite in macrophages and mice (Singh et al., 2016).

Since the 1950s several species of Australian trypanosomes have been described from wildlife. These include *Trypanosoma thylacis*, *Trypanosoma irwini*, *Trypanosoma vegrandis*, *Trypanosoma gilletti*, *Trypanosoma binneyi*, *Trypanosoma copemani*, *Trypanosoma noyesi*, and *Trypanosoma teixeirae* (Mackerras, 1959; Noyes et al., 1998; Smith et al., 2008; Austen et al., 2009; Averis et al., 2009; McInnes et al., 2009, 2011; Paparini et al., 2011; Botero et al., 2013, 2016b; Thompson et al., 2013; Austen et al., 2011; Barbosa et al., 2016). Interestingly, *T. copemani* has been shown to invade cells, and has been associated with pathological changes in wildlife hosts that are similar to those seen with the human pathogen, *T. cruzi* (Botero et al., 2013, 2016a). Moreover, *T. copemani* has been found to infect numerous endangered and threatened species of wildlife, and has been associated with the drastic decline of the critically endangered woylie (*Bettongia penicillata*) (Austen et al., 2009; McInnes et al., 2011; Botero et al., 2013, 2016a). Despite the importance of the kDNA in trypanosomatid survival and regulation of virulence (Milman et al., 2007; Singh et al., 2016), there is a complete lack of knowledge about the kinetoplast and the organisation of the kDNA within this organelle in Australian trypanosomes.

The aim of this study was to investigate the ultrastructure and organisation of kDNA in the Australian wildlife trypanosome, *T. copemani*. In particular, the organisation of the maxicircles and minicircles, and the presence of the UMS element and the UMSBP were investigated.

2. Materials and methods

2.1. Parasites

Parasites were grown under optimal conditions for each individual species (Botero et al., 2016a). Epimastigotes of two genotypes of *T. copemani* (G1 and G2) were grown in 75 ml flasks containing Grace's medium with 10% Fetal Calf Serum (FCS) and penicillin–streptomycin. *Trypanosoma cruzi* epimastigotes were grown in 75 ml flasks containing RPMI 1640 medium with 10% FCS and penicillin–streptomycin. All strains were grown at 28 °C.

2.2. Isolation of the kDNA network

Ethidium bromide (EtBr)-cesium chloride (CsCl) stepwise gradients were used to isolate the kDNA of *T. copemani* (Saucier et al., 1981; Hajduk et al., 1984). Approximately 1×10^{10} *T. copemani* G1 and G2 cells from the stationary phase were harvested by centrifugation at 16,000*g* at 4 °C, for 5 min and then washed once with PBS and once with NET100 buffer (10 mM Tris–Cl pH 8, 100 mM NaCl, 100 mM EDTA, pH 8). The pellet, containing trypanosomes, was submitted to lysis by resuspending in NET100 buffer and 1 mg/ml of proteinase K. Sodium Sarkosinate (Sarcosyl) was added slowly (3% final concentration) and the suspension was gently mixed and incubated for 30 min at 4 °C. Then, 24 ml of CsCl (1.386 density) and 4 ml of CsCl (1.750 density – mixed previously with EtBr to final concentration of 20 µg/ml) were added to an ultra-clear SW28 rotor tube. Ten milliliters of sample lysate were then added to the tube and centrifuged in a SW28 rotor at 21,410*g* at 4 °C, for 15 min. The upper phase of the suspension was discarded and the middle phase (fluorescent band under ultraviolet light) was collected. The EtBr was removed by several extractions with 0.1× SSC-saturated isoamyl alcohol, and then dialyzed overnight against Tris EDTA buffer (TE) at pH 8.0 and 4 °C. The kDNA preparation was treated again with proteinase K (as above), followed by phenol–chloroform extraction and ethanol precipitation.

2.3. Deep sequencing, assembly, and annotation of minicircles and maxicircle

Approximately 100 µl of 100 ng/ml of genomic DNA from *T. copemani* G1 and G2 cells grown in culture was isolated using

the QIAamp blood and tissue DNA MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of DNA was confirmed using Nanodrop; samples tested negative for mycoplasma via PCR, and DNA was run on a 1% agarose gel to investigate signs of fragmentation prior to sequencing. Deep sequencing was performed at the Australian Genome Research Facility (AGRF, Melbourne, Australia) using an Illumina NextSeq500 platform with 300 cycles and Mid output kit. A paired end library preparation for *T. copemani* G1, and a mate pair preparation (gel free) for *T. copemani* G2 yielded fragments of 150 bp long reads. Data were generated with the Illumina bcl2fastq pipeline version 2.19 and the integrity of the files was established using TestFiles.exe on a windows platform before further analysis. Sequences were quality filtered and merged using CLC genomics v.10. Both libraries were de novo assembled into contigs using Geneious and Spades assemblers on Geneious 10.2. Maxicircle and minicircle contigs were identified by alignment with other *Trypanosoma* spp. on GenBank using BLAST. All contigs were aligned using MUSCLE (Edgar. 2004) and manually annotated by comparison with other sequences available on GenBank including maxicircle sequences from *T. cruzi* (GenBank: DQ343645), *Trypanosoma lewisi* (GenBank: KR072974), *Trypanosoma cruzi marinkellei* (GenBank: KC427240), and *Trypanosoma brucei brucei* (GenBank: M94286), and minicircle sequences from *T. cruzi* (GenBank: TCU07845), and *T. lewisi* (GenBank: M17995, M17996). Two full minicircle sequences and a partial sequence of the maxicircle of *T. copemani* G1 were deposited in GenBank under the accession numbers MG948555, MG948556, and MG948557, respectively. A partial sequence of the minicircles of *T. copemani* G2 was identified and was deposited in GenBank under the accession number MG948554.

2.4. Phylogenetic analysis

A phylogenetic tree was constructed using the full coding region from the maxicircles of *T. copemani* G1 (this study), *T. cruzi* CL Brener (GenBank: DQ343645), *Tryapnosoma cruzi esmeraldo* (GenBank: DQ343646), *T. cruzi marinkellei* B7 (GenBank: KC427240), *Trypanosoma rangeli* (GenBank: KJ803830), *T. lewisi* (GenBank: KR072974), *Trypanosoma vivax* MT1 (GenBank: KM386508), *T. vivax* Liem176 (GenBank: KM386509), *T. brucei brucei* (GenBank: M94286), *Trypanosoma equiperdum* STIB842 (GenBank: EU185800), and the maxicircle of *L. tarentolae* (GenBank: M10126) which was used as an outgroup. All sequences were aligned using MUSCLE (Edgar, 2004) and then manually refined using Geneious 10.2. Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian methods, implemented using MEGA 6 (Tamura et al., 2011) and Mr Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). JModelTest 2.1.1 was used to find the most appropriate nucleotide substitution model for ML and Bayesian analyses (Posada, 2008). The model of nucleotide substitution chosen was GTR + I + G (general time reversible gamma proportion of invariant sites). The Markov chain Monte Carlo (MCMC) was run for 10,000,000 generations, until the mean standard deviation of split frequencies was lower than 0.01, and the trees were sampled every 100th generation. The first 2500 trees were discarded as burn-in.

2.5. Western blot analysis

Whole cell protein extracts of *T. copemani* G1, *T. copemani* G2, and *T. cruzi* were obtained by centrifugation of 1×10^6 epimastigotes of each parasite at 5000*g* for 5 min, at room temperature. The pellet was washed twice with PBS and then resuspended in 40 µl of double-distilled water. Then, 10 µl of 10% SDS were added and samples were sonicated for 5 min. After sonication, cell lysates were solubilized in cracking buffer containing final concentrations of 50 mM Tris–HCl, pH 6.8, 4% SDS, 3.5% β-mercaptoethanol, 10% glycerol and 10 mM EDTA. The solution was then centrifuged at 10,000*g* at 4 °C, for 30 min in order to remove cell debris. The supernatant was recovered and the protein concentration was determined using the Direct Detect Assay-free cards (EMD Millipore corporation, United States). Protein extract (30 and 40 µg) was heated at 70 °C for 10 min and loaded onto a NuPag10 4–12% Bis-Tris gel (Invitrogen, United States). Recombinant *C. fasciculata* UMSBP was used as a positive control of hybridization. Protein bands on the gel were transferred onto nitrocellulose membranes using the trans-blot turbo-transfer system (Bio-Rad, United States). Then, the membrane was blocked by incubation in 5% skim dry milk (Difco, United States) diluted in Tris buffered saline with tween (50 mM Tris–Cl, 0.1% Tween-20) (TBST) pH 7.5 for 30 min with constant shaking, and probed with a 1:4000 dilution of *C. fasciculata* anti-UMSBP antibodies overnight at 4 °C. The membrane was then washed three times with TBST and then incubated with a 1:10,000 dilution of enhanced chemiluminescent (ECL) peroxidase labelled conjugated goat anti-rabbit secondary antibody (Jackson Immuno Research Laboratories, Inc. United Sates) for 2 h, followed by ECL detection as recommended by the manufacturer (Amersham Pharmacia Biotech, United Kingdom). The preparation of *C. fasciculata* recombinant UMSBP was performed as reported previously (Tzfati et al., 1995; Sela and Shlomai, 2009).

2.6. DNA topoisomerase II assay

Decatenation of *T. copemani* kDNA was conducted in a 10 µl reaction mixture containing four units of human topoisomerase II α (TopoGen Inc, Port Orange, Florida, USA), 50 mM Tris–Cl pH 8, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM of DTT, 0.5 mM of ATP and 30 µg/ml of BSA (Topo II reaction buffer TG4040), and 0.1 µg of kDNA. Reactions were incubated for 60 min at 37 °C, and then stopped by the addition of 0.1 vol of stopping buffer (5% Sarcosyl, 0.025% bromophenol blue, 50% glycerol). Samples were loaded onto 1% agarose gel containing 1 µg/ml of ethidium bromide (Life Technologies, United States) and electrophoresed at 100 V for 30 min. Topo II decatenated *C. fasciculata* kDNA and *C. fasciculata* linear kDNA markers (TopoGen Inc, United States) were used as controls in the assay.

2.7. Transmission electron microscopy

Trypanosoma copemani G1 and G2 epimastigotes were fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS:cell culture medium (pH 7.2) resulting in a working glutaraldehyde concentration of 2.5%. All subsequent processing was performed in a PELCO Biowave microwave, where samples were post-fixed in 1% OsO_4 in PBS, followed by progressive dehydration in ethanol/acetone, before being infiltrated and embedded in Procure-Araldite epoxy resin. Sections (120 nm thick) were cut with a diamond knife and mounted on copper grids. Digital images were collected from unstained sections at 120 kV on a JEOL 2100 TEM (Japan) fitted with a Gatan ORIUS1000 camera. The thickness and diameter of the kinetoplast were measured in trypanosome sections where the basal body of the flagellum was seen and where most of the DNA fibres within the kinetoplast were continuously distributed from side to side (indicating that the kinetoplast disk was cut parallel to its long axis and through its central region, respectively).

3. Results

3.1. Kinetoplast morphology and ultrastructure

The kinetoplast of both *T. copemani* strains exhibited the classical disk-shaped conformation, present in other trypanosomatids

such as *T. cruzi*, *T. brucei*, *Leishmania* and *C. fasciculata* (Shapiro and Englund, 1995). The *T. copemani* kinetoplast was positioned adjacent to the basal body of the flagellum (fbb) (Fig. 1). Within the kinetoplast, the kDNA network was organised in the form of a condensed disk-like structure that measured 0.73 ± 0.25 μm (mean ± S.E.; $n = 11$) × 0.19 ± 0.04 μm (mean ± S.E.; $n = 11$) (Fig. 1).

3.2. Trypanosoma copemani minicircle sequence and organisation

After removal of low quality reads from raw data, we obtained 7,000,006 clean reads from *T. copemani* G1 and 1,085,177 reads for *T. copemani* G2. These data were finally assembled into 367,392 and 310,743 contigs, respectively, using the Geneious and Spades assemblers on Geneious 10.2. Two different fully circularised minicircle contigs from *T. copemani* G1 were identified by BLAST search against sequences of the minicircles of other *Trypanosoma* spp. deposited in GenBank. Both *T. copemani* G1 minicircle classes presented a size of 2048 bp. The first class (which we named G1M1) contained two conserved regions present as direct repeats located approximately 180° apart, and two hypervariable regions flanked by the two conserved regions (Fig. 2A). The second class (which we named G1M2) contained four conserved regions present as direct repeats located approximately 90° apart, and four hypervariable regions flanked by the four conserved regions (Fig. 2B). Several partial sequences of the minicircles of *T. copemani* G2 were also found, and all contained one conserved region.

Each conserved region in both *T. copemani* G1 and G2 minicircles (approximately 130 bp long) contained the three CSBs previously reported in most trypanosomatids. Although all CSBs were the same in the two classes of minicircles from *T. copemani* G1, CSB-1 and CSB-2 were different between both *T. copemani* G1 and G2. Alignments of *T. copemani* G1 (G1M1 and G1M2) and *T. copemani* G2 conserved regions with the minicircle conserved regions of *T. cruzi* (GenBank: TCU07845), and *T. lewisi* (GenBank: M17996) revealed similarities with both species (Table 1). The first sequence block (CSB-1), which has been shown to differ between species, was the same in *T. cruzi* and *T. copemani* G2. The CSB-1 of both classes of minicircles of *T. copemani* G1 differed in one nucleotide from those of *T. copemani* G2/*T. cruzi*. The second sequence block (CSB-2), which has also been shown to differ between species, was the same in *T. cruzi* and *T. copemani* G1, and in *T. copemani* G2 and *T. lewisi*, with only one nucleotide difference between *T. copemani* G1/*T. cruzi* and *T. copemani* G2/*T. lewisi*. The third sequence block CSB-3 or UMS (minicircle origin of replication), was exactly the same as the one reported in several species of trypanosomatids (Table 1).

3.3. Trypanosoma copemani maxicircle partial sequence

A long partial sequence from the maxicircle of *T. copemani* G1 (19,257 bp), which included a short fragment of the divergent region and all the coding region beginning at the 12S rRNA gene, and ending after the 3′ end of the ND5 gene, was also found. The data obtained indicated that the *T. copemani* maxicircle encodes 20 firmly clustered genes, with the same gene order as *T. cruzi*, *T. lewisi*, *T. cruzi marinkellei*, and *T. brucei* maxicircles (Table 2).

Comparative analysis of the full coding region of the maxicircle of *T. copemani* G1 with that of *T. cruzi* and *T. lewisi* showed they share 70.8% and 71.2% identity, respectively. Three *T. copemani* G1 genes exhibited the same length as those of *T. lewisi* (MURF2, ND7, and ND5), and two as those of *T. cruzi* (12S rRNA, and CR4). However, genes CytB, COII, COI, and ND4 exhibited the same length in all three species (Table 2). Most of these sequences are from genes where the transcripts either undergo minor or do not undergo RNA editing in *T. cruzi* and *T. lewisi*. Genes with transcripts that are subject to extensive editing generally exhibited a lower pairwise percentage of identity (Table 3).

3.4. Phylogenetic analysis based on the maxicircle coding region

Phylogenetic analysis was performed using the entire coding region (20 genes) from *T. copemani* G1 and the maxicircle coding region of 10 species of trypanosomatids that are available in GenBank. Both ML and Mr Bayes phylogenies showed that *T. copemani* G1 clusters with *T. lewisi* and with trypanosomes within the *T. cruzi* clade (including *T. rangeli* and *T. cruzi marinkellei*) with 100% support (Fig. 3). However, *T. copemani* G1 is more closely related to *T. lewisi* than to *T. cruzi* and allied species. The tree topology obtained in this study supports previous phylogenetic analyses using either 18S rDNA and glycosomal glyceraldehyde 3-phosphate dehydrogenase (g*GAPDH*) genes (McInnes et al., 2011).

3.5. Trypanosoma copemani contains the UMSBP

Considering that the UMS element is present in *C. fasciculata* minicircles, and that it was also found in *T. copemani* minicircles, we investigated the presence of a UMSBP in *T. copemani* using antibodies raised against *C. fasciculata* UMSBP (Tzfati et al., 1995). These anti CfUMSBP antibodies have been previously shown to detect UMSBP in other species such as *T. brucei* (Milman et al., 2007), *T. cruzi* (Coelho et al., 2003), and *L. donovani* (Singh et al., 2016). Western blot analysis, using anti-CfUMSBP antibodies, detected two peptides of approximately 16.4 kDa and 25.7 kDa in

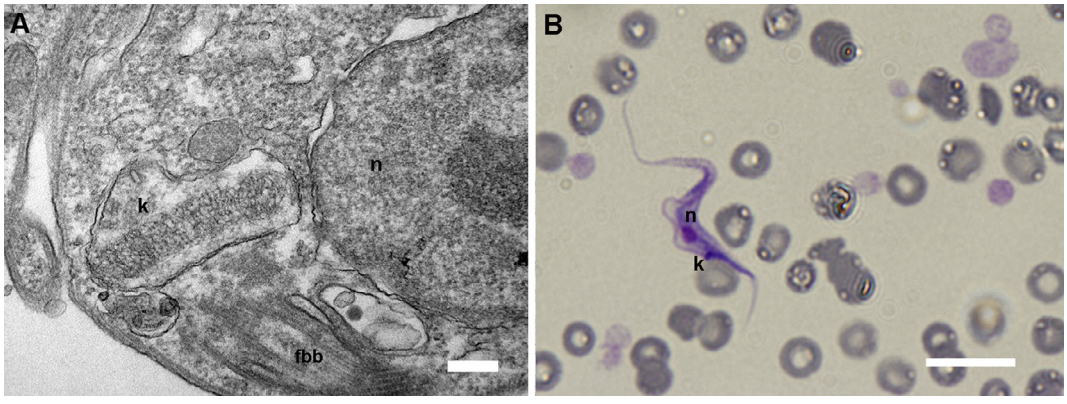

Fig. 1. Morphology and ultrastructure of *Trypanosoma copemani*. (A) Transmission electron micrograph showing a cross-section through a *T. copemani* epimastigote. (B) Diff Quick staining (Siemens Healthcare Ltd, Canada) of a bloodstream form of *T. copemani*. k, kinetoplast; n, nucleus; fbb, flagellum basal body. Scale bars: (A) 0.2 μm, and (B) 10 μm.

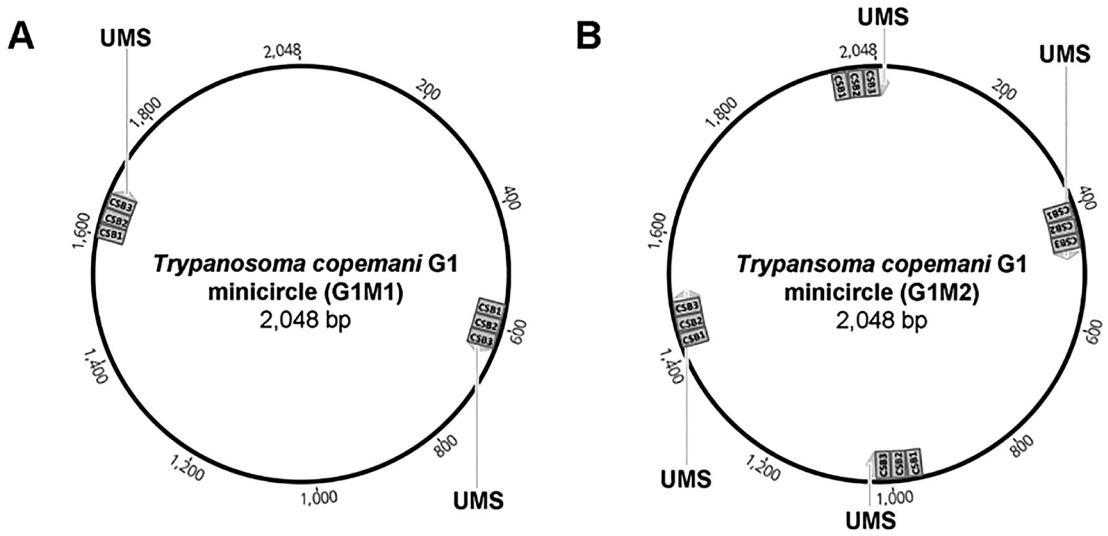

Fig. 2. Schematic representation of the two classes of minicircles of *Trypanosoma copemani* genotype 1 (G1). (A) First class of minicircle or G1M1. (B) Second class of minicircle or G1M2. CSB1, first conserved sequence block; CSB2, second conserved sequence block; CSB3 or UMS, third conserved sequence block or universal minicircle sequence. This image was created by Biomatters on Geneious version 10.2.

Table 1
Conserved sequence blocks 1, 2 and 3 or Universal Minicircle Sequence of different *Trypanosoma* spp. and *Trypanosoma copemani* genotypes (G) 1 and 2.

Organism	CSB-1[a]	D1-2	CSB-2[a]	D2-3	CSB-3 or UMS[a]	UMS per minicircle	Sequence reference
T. cruzi Y strain	AGGGGCGTTC	30	CCCCGTAC	47	GGGGTTGGTGTA	4	TCU07845[b]
T. copemani G1 G1M1 class	AGGGGCGTGC	28	CCCCGTAC	46	GGGGTTGGTGTA	2	This study MG948556[b]
T. copemani G1 G1M2 class	AGGGGCGTGC	28	CCCCGTAC	46	GGGGTTGGTGTA	4	This study MG948555[b]
T. copemani G2	AGGGGCGTTC	29	CCCCGTAT	47	GGGGTTGGTGTA	Unknown	This study MG948554[b]
T. lewisi	GAGGGCGTTC	32	CCCCGTAT	46	GGGGTTGGTGTA	2	M17996[b]
T. congolense	AAGGGCGTTC	29	TCCCGTAC	47	GGGGTTGGTGTA	1	Nasir et al. (1987)
T. equiperdum	ATGGGCGTGC	21	TCACGTGC	38	GGGGTTGGTGTA	1	Barrois et al. (1981)
T. brucei	ATGGGCGTGC	20	TCCCGTGC	41	GGGGTTGGTGTA	1	Jasmer and Stuart (1986)

D1-2, average distance (in bp) between CSB-1 and CSB-2; D2-3: average distance (in bp) between CSB-2 and CSB-3 or UMS.
[a] Nucleotides in larger font represent residues at the indicated position which differ from those of *T. cruzi*.
[b] GenBank accession numbers.

Table 2
Gene lengths and order in *Trypanosoma copemani* genotype 1 (G1) maxicircle and comparison with the gene length of other *Trypanosoma* spp.

Gene	*T. copemani* position[b]	*T. copemani* length[c,d]	*T. cruzi* length[c,d]	*T. lewisi* length[c,d]	*T. cruzi marinkellei* length[d]	*T. brucei* length[d]
12S rRNA	1–1161	**1161**	**1161**	1168	1170	1149
9S rRNA	1206–1810	605	609	608	605	611
ND8	1843–2125	283	279	285	278	266
DN9[a]	2190–2538	349	338	350	346	321
MURF5[a]	2574–2718	145	148	241	145	234
ND7	2872–3642	**771**	755	**771**	758	702
COIII	3693–4112	420	423	414	425	439
Cyb	4200–5279	**1080**	**1080**	**1080**	1080	1080
ATPase6	5316–5631	316	336	304	338	369
MURF1/(ND2)[a]	5680–7019	1340	1341	1341	1341	1237
CR3[C]	7006–7126	~121	~119	~123	~111	~164
ND1[a]	7122–8066	944	942	942	942	957
COII	8076–8704	**629**	**629**	**629**	629	626
MURF2	8735–9787	**1053**	1056	**1053**	1048	1041
COI[a]	9778–11,427	**1650**	**1650**	**1650**	1650	1734
CR4[a]	11,480–11,686	**207**	**207**	212	210	185
ND4	11,790–13,103	**1314**	**1314**	**1314**	1314	1311
ND3[a]	13,095–13,267	173	193	187	197	256
RPS12	13,344–13,524	179	191	190	189	172
ND5	13,545–15,317	**1773**	1770	**1773**	1770	1770

[a] Genes that are encoded by the reverse strand.
[b] Gene positions are given relative to the start of the 12S rRNA gene.
[c] The gene lengths in bold are similar to either *Trypanosoma cruzi* or *Trypanosoma lewisi*.
[d] Maxicircle gene lengths of *T. lewisi*, *T. cruzi*, *Trypanosoma cruzi marinkellei*, and *Trypanosoma brucei* that are based on data from GenBank (accession numbers: KR072974, DQ343645, KC427240, and M94286 respectively).

Table 3
Pairwise percentage of identity between *Trypanosoma copemani* maxicircle genes and the maxicircle genes of *Trypanosoma cruzi* and *Trypanosoma lewisi*.

T. copemani genes	RNA editing[a]	*T. cruzi*[b] (%)	*T. lewisi*[b] (%)
12S Rrna	–	84	83.2
9S Rrna	–	85	86.6
ND8	Extensive	75.5	76.8
DN9	Extensive	67.7	72.1
MURF5	None	76.4	71.6
ND7	Extensive	79.2	78.3
COIII	Extensive	77.8	74.2
Cyb	Minor	82.4	84.9
ATPase6	Extensive	76.7	78.4
MURF1/(ND2)	None	75.2	75.5
CR3	Extensive	74	70.5
ND1	None	74.4	73.7
COII	Minor	80.8	78.1
MURF2	Minor	76.8	76.9
COI	None	80.9	80.8
CR4	Extensive	66.7	67.7
ND4	None	76.7	77
ND3	Extensive	63.5	62.8
RPS12	Extensive	77.3	79.8
ND5	None	77.6	76.8

[a] RNA editing patterns for *T. cruzi*, and *T. lewisi*.
[b] Maxicircle gene sequences of *T. cruzi*, and *T. lewisi* are based on data from GenBank (accession numbers: DQ343645, and KR072974 respectively).

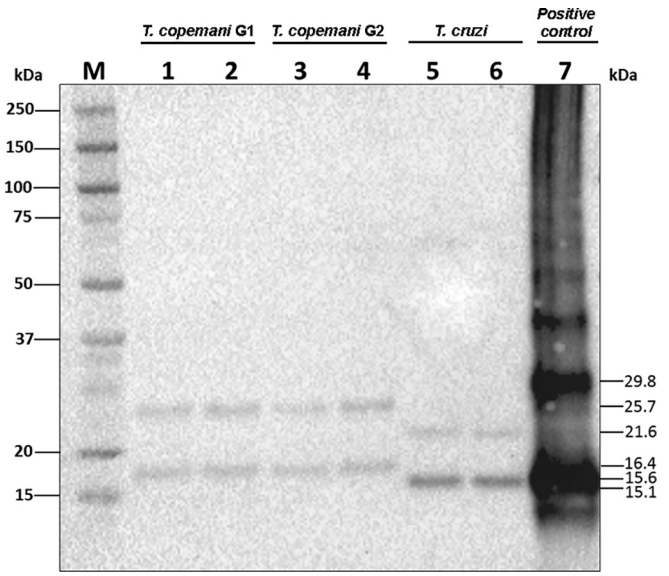

Fig. 4. Western blot analysis using antibodies raised to *Crithidia fasciculata* Universal Minicircle Sequence Binding Protein. Whole cell extract proteins from epimastigotes of *Trypanosoma copemani* genotype 1 (G1), *T. copemani* G2, and *Trypanosoma cruzi* were used. Lane 1: *T. copemani* G1 (30 μg of proteins); lane 2: *T. copemani* G1 (40 μg of proteins); lane 3: *T. copemani* G2 (30 μg of proteins); lane 4: *T. copemani* G2 (40 μg of proteins); lane 5: *T. cruzi* (30 μg of proteins); lane 6: *T. cruzi* (40 μg of proteins); lane 7: *C. fasciculata* recombinant UMSBP (positive control); M, marker. Apparent molecular masses of standard proteins are indicated in kiloDaltons.

both *T. copemani* G1 and G2 protein extracts (Fig. 4). When *T. cruzi* protein extracts were used, the antibody recognised two peptides of approximately 15.6 kDa and 21.6 kDa (Fig. 4). When *C. fasciculata* recombinant UMSBP was used, the antibody recognised a peptide of approximately 15.1 kDa and a series of higher UMSBP oligomeric forms, which could be the product of partial and not full reduction of the *C. fasciculata* recombinant UMSBP.

3.6. Trypanosoma copemani kDNA network is a topological catenane

In attempts to investigate whether the minicircles of *T. copemani* were catenated into the kDNA network, the enzyme DNA

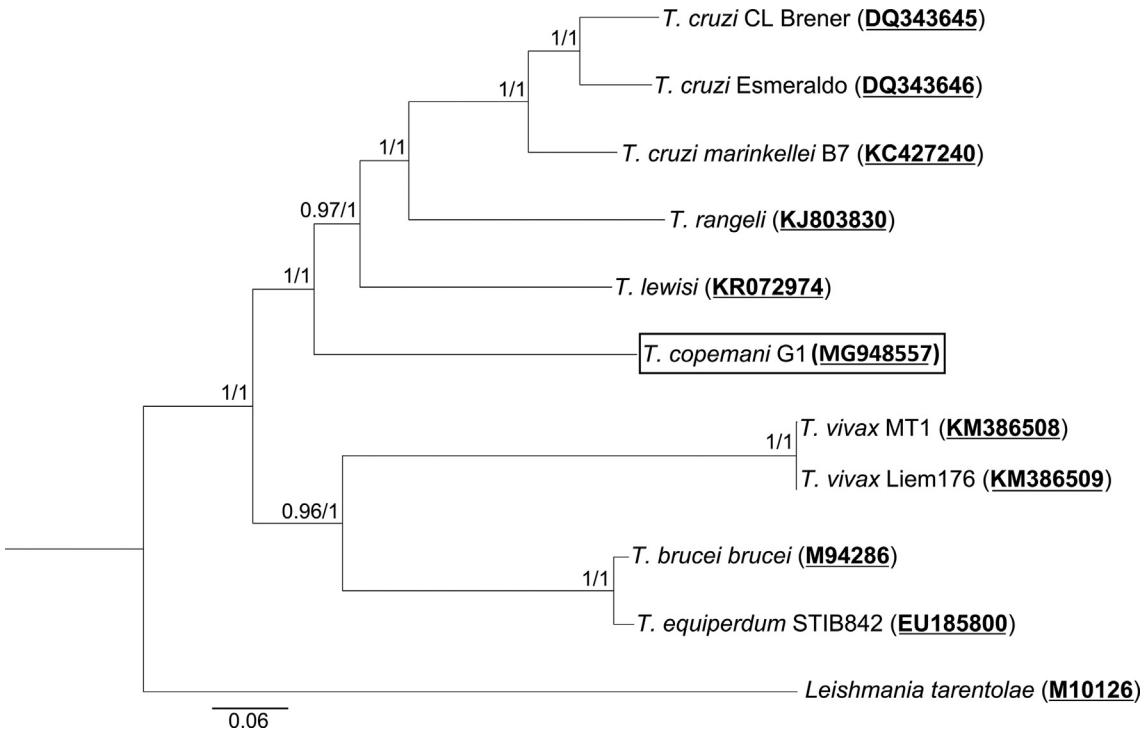

Fig. 3. Phylogenetic relationships between *Trypanosoma copemani* genotype 1 (G1) and other species of *Trypanosoma* based on the full maxicircle coding region. This tree topology was generated using Maximum Likelihood and Mr Bayes. Numbers adjacent to isolate names indicate GenBank accession numbers. The sequence obtained in this study is bound in a box. Bootstrap support for 1000 replicates for Maximum Likelihood and Bayesian posterior probabilities, respectively, are shown at nodes. *Leishmania tarentolae* was used as an outgroup. Scale bar indicates substitutions per site.

topoisomerase II was used. Incubation of purified kDNA with DNA topoisomerase II, under decatenation conditions, revealed that the kDNA minicircles of both *T. copemani* G1 and G2 are topologically interlocked to form a catenane network. Minicircles, which were decatenated by topoisomerase II, were analysed by electrophoresis in agarose gels. Untreated kDNA networks failed to enter the agarose gel, while partially decatenated networks of *T. copemani* yielded monomeric minicircles bands, corresponding to nicked, linear and covalently closed minicircles (Fig. 5).

4. Discussion

The organisation of minicircles and maxicircles within kDNA differs within species and these differences have been used to cluster them into various groups (Lukeš et al., 2002). The most complex type of kDNA organisation is the kDNA network structure present in pathogenic trypanosomatids such as *T. cruzi*, *T. brucei* and *Leishmania*, and in the insect trypanosomatid *C. fasciculata* – all late diverging kinetoplastids (Lukeš et al., 2002). The kDNA network is condensed in the mitochondrial matrix into a disk-shaped structure, where minicircles are topologically relaxed, and each is catenated on average to three neighbours (Chen et al., 1995), and aligned side by side (Rauch et al., 1993). Pro-kDNA is the second most organised kDNA structure and is present in the late diverging free-living bodonid *Bodo saltans*. The majority of minicircles in pro-kDNA are covalently closed, topologically relaxed and organised in a single bundle-like structure with only a few catenanes (Blom et al., 1990). In contrast, all other types of kDNA structure, poly-kDNA, pan-kDNA and mega-kDNA, are present in early diverging kinetoplastids such as *Dimastigella trypaniformis*, *Bodo caudatus*, *Cryptobia helicis* and *Trypanoplasma borreli* (Lukeš et al., 2002). The kDNA fills most of the kinetoplast as with pan-kDNA, or fills various separate foci throughout the kinetoplast as with poly-kDNA, all of which lack the highly ordered kDNA packaging seen in trypanosomatids (Lukeš et al., 2002). Interestingly, the kinetoplast of *T. copemani* presented a classical kDNA network structure,

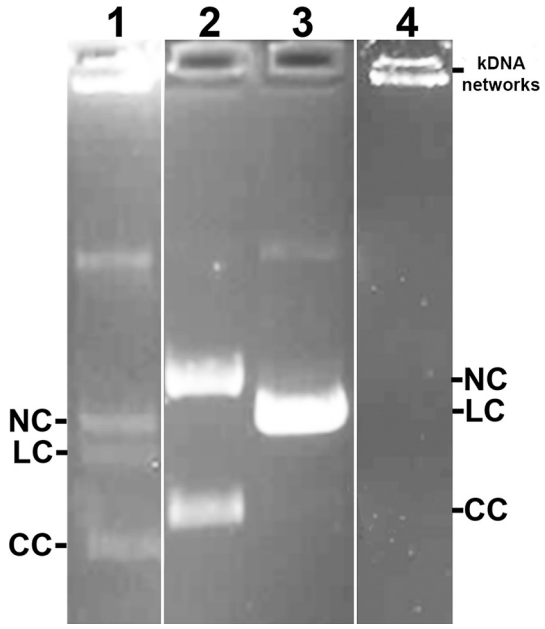

Fig. 5. DNA topoisomerase II products in *Trypanosoma copemani* kinetoplast DNA (kDNA). Lane 1: pure *T. copemani* kDNA in presence of topoisomerase II (decatenation). Lane 2: marker of decatenated *Crithidia fasciculata* kDNA. Lane 3: marker of linear *C. fasciculata* kDNA. Lane 4: pure, untreated *T. copemani* kDNA. NC, decatenated nicked circular kDNA minicircles; LC, linearized kDNA minicircles; CC, decatenated covalently-closed circular minicircles.

similar to that seen in late diverging kinetoplastids such as *T. cruzi* and *T. brucei*. Furthermore, the products of decatenation by topoisomerase II indicated that the minicircles of *T. copemani* are catenated, confirming that the arrangement of kDNA is in the form of a catenated network as seen in *T. cruzi* and *T. brucei*. The shared features in the kinetoplast ultrastructure between late diverging kinetoplastids and *T. copemani* will be of value in understanding the evolution of Australian trypanosomes, although further studies are needed to better understand these relationships.

Not only does the kDNA organisation differ between species, several studies have also reported differences in the size of minicircles, and in the number, position and sequence of its conserved regions. The size of the minicircles of *T. lewisi*, for example, are approximately 1 kb and contain two conserved regions located 180° apart (Ponzi et al., 1984). In contrast, *T. cruzi* minicircles are approximately 1.4 kb in size, and contain four conserved regions located at 0°, 90°, 180° and 270° apart (Degrave et al., 1988). Furthermore, *T. brucei* and *L. tarentolae* minicircles are approximately 1 kb in size and contain only one conserved region (Kidane et al., 1984; Jasmer and Stuart, 1986; Hines and Ray, 2011). Additionally, a species of *Trypanosoma* from South America, *T. rangeli*, presents two classes of minicircles with different size and molecular organisation. The first class presents a single conserved region and a size of 1764 bp, while the second class presents two conserved regions located as direct repeats 180° apart and a size of 1587 bp (Vallejo et al., 1994). The results of this study have shown that *T. copemani* G1 presents two distinct classes of minicircles; the first one contains two conserved regions located 180° apart, similar to *T. lewisi* minicircles and *T. rangeli* second class minicircles; and the second one presents four conserved regions located 90° apart, similar to *T. cruzi* minicircles. Unfortunately, only partial sequences from *T. copemani* G2 minicircles were obtained but all contained the full conserved region including the three conserved sequence blocks CSB-1, CSB-2 and CSB-3 (origin of replication). Interestingly, when the full conserved regions of the minicircles of *T. copemani* G1 and G2 were individually compared with other trypanosomatids in GenBank, the most similar sequences were *T. lewisi* and *T. cruzi* with 81.0% and 78.5% identity, respectively, with *T. copemani* G1, and 76.9% and 80.8% identity, respectively, with *T. copemani* G2. *Trypanosoma copemani* G1 and G2 have previously been shown to exhibit considerable differences in their biological behaviour such as growth requirements, susceptibility to drugs, and capability to infect cells *in vitro* (Botero et al., 2016a, 2017). Furthermore, *T. copemani* G2 has been found in blood and tissues of marsupials with pathological changes similar to those seen during *T. cruzi* infections, while *T. copemani* G1 has only been found in blood (Botero et al., 2013). Adding to the differences between *T. copemani* G1 and G2, this study found that the conserved sequence blocks (CBS-1 and CBS-2), which have been shown to be species-specific, were different in both strains. Taking into account the differences in the biology, lifestyle and sequence of the minicircle conserved region, *T. copemani* G1 and G2 could actually be different species. Phylogenetic studies based on the 18S rDNA and g*GAPDH* genes have also shown that although both *T. copemani* G1 and G2 are closely related, *T. copemani* G1 forms a subclade with other genotypes from Australian wildlife such as Charlton, AAP, AAI, H26, Q3, Q10, GP63 and GP94, while *T. copemani* G2 sits by itself in a separate subclade (Noyes et al., 1999; Austen et al., 2009; McInnes et al., 2011; Botero et al., 2013). Identifying the full coding region of the maxicircles of *T. copemani* G2 could further assist in separating *T. copemani* G1 and G2 as separate species.

When the aligned minicircle conserved regions of *T. copemani* G1, G2, *T. cruzi*, and *T. lewisi* were analysed more carefully, similarities in the sequence and spacing between the three CSBs were found. These three CSBs have been reported previously in other species of trypanosomatids including *C. fasciculata*, *L. tarentolae*,

T. brucei, *Trypanosoma congolense* and *T. equiperdum* (Barrois et al., 1981; Kidane et al., 1984; Ponzi et al., 1984; Jasmer and Stuart, 1986; Nasir et al., 1987; Sugisaki and Ray, 1987). However, differences in the sequence of both first and second blocks (CSB-1 and CSB-2) between different species of trypanosomatids have been reported (Ray, 1989). Interestingly, *T. copemani* G1 CSB-2 was identical to the CSB-2 of *T. cruzi* minicircles, and the CSB-1 differed only in one nucleotide, while for *T. copemani* G2, the CSB-2 was identical to the CSB-2 of *T. lewisi*, and the CSB-1 was identical to the CSB-1 of *T. cruzi*. As expected, both *T. copemani* G1 and G2 presented the conserved CSB-3 or UMS element, which has been shown to be conserved in all trypanosomatids studied. The similarities in the number, position, and sequence of the minicircle conserved regions of *T. copemani* G1, *T. copemani* G2, *T. cruzi*, and *T. lewisi*, support the theory that the UMS and the CSB-1 are involved in the initiation of minicircle L-strand and H-strand replication (reviewed in Shapiro and Englund, 1995). This suggests that *T. copemani* may have a common mechanism of minicircle replication with these two *Trypanosoma* spp. However, the significance of these genetic similarities needs to be further investigated.

In addition to the similarities in the kDNA of *T. copemani* with those of *T. cruzi* and *T. lewisi*, comparative analysis of the maxicircle coding region of the three species revealed a high level of sequence similarity. Our phylogenetic analysis also confirmed the well-established close relatedness of *T. copemani* with *T. cruzi and T. lewisi*, with *T. lewisi* being more closely related. The *T. copemani* G1 maxicircle coding region contained the same genes and gene order seen in other trypanosomatids such as *T. cruzi*, *T. lewisi*, *T. brucei*, *T. rangeli* and *Leishmania tarentolae* (El-Sayed et al., 2005). Most of these maxicircle genes encode proteins that are involved in oxidative metabolism, and which are necessary in the vector-host transmission cycle. *Trypanosoma lewisi* and *T. cruzi* are transmitted by fleas and reduviid bugs, respectively. Both parasites belong to the stercorarian group in which metacyclic trypomastigotes are located in the epithelium of the rectum of the insect, and transmission occurs when the infective forms are released in faeces near a bite site after feeding on a mammalian host (Hoare, 1972). Interestingly, *T. copemani* has been found to belong to the stercorarian group with the tick *Ixodes australiensis* acting as a vector (Austen et al., 2011). Considering that the kinetoplast plays an important role in the life cycle of trypanosomes, it could be possible that *T. copemani* shares more life cycle pathways with those of *T. cruzi* and *T. lewisi*, but this need to be further investigated. *Trypanosoma copemani* G2, for example, has been shown to be able to infect cells *in vitro* such as *T. cruzi* (Botero et al., 2013, 2016a). Some of the maxicircle genes also encode proteins that are involved in virulence and pathogenicity. Interestingly, *T. copemani* has been shown to be pathogenic as well as *T. cruzi*, and *T. lewisi*. *Trypanosoma cruzi* is the agent of Chagas disease in humans, while *T. lewisi* has been implicated in the extinction of two native rats in Australia (Wyatt et al., 2008), and has recently been shown to atypically infect humans (Truc et al., 2013; de Sousa, 2014). Interestingly, *T. copemani* has been implicated in the drastic decline of the marsupial *Bettongia penicillata* or woylie. This close genetic relationship in the maxicircle coding region between *T. copemani*, *T. lewisi* and *T. cruzi* warrants further investigations, especially considering the mounting evidence that the two first mentioned trypanosomes have been implicated in wildlife extinctions or declines.

The UMSBP of *C. fasciculata* has been extensively studied. This protein is a single-stranded sequence-specific DNA binding protein that binds the UMS elements in kDNA minicircles (Tzfati et al., 1992, 1995). In T. *brucei*, the two UMSBP orthologues have been suggested to act in minicircle replication initiation and kDNA segregation (Milman et al., 2007). *Crithidia fasciculata* UMSBP is a protein of approximately 13.7 kDa (Tzfati et al., 1992, 1995; Onn et al., 2004) and the mass of the recombinant *C. fasciculata* UMSBP tagged protein used here was approximately 1 kDa larger. It has been shown that antibodies raised against the UMSBP of *C. fasciculata* can cross-react with UMSBPs of other trypanosomatids such as *T. cruzi*, *L. donovani*, and *T. brucei* (Coelho et al., 2003; Milman et al., 2007; Singh et al., 2016). *Trypanosoma cruzi* UMSBP was previously shown to be approximately 14 kDa and to bind the dodecamer UMS element (Coelho et al., 2003). The present study confirmed these previous results and showed that antibodies raised against *C. fasciculata* UMSBP also recognise *T. cruzi* UMSBP. However, there was a difference in the size of the protein (approximately 15.1 kDa in this study). In addition, this study found a second *T. cruzi* protein of 21.6 kDa that reacted with the anti UMSBP antibodies, which may be the product of oligomerization, which was seen with *C. fasciculata* recombinant UMSBP (used as a control). This study reports for the first known time the presence of the UMSBP in *T. copemani.* Antibodies raised against the UMSBP of *C. fasciculata* detected a UMS protein in *T. copemani* cell extracts of approximately 16.4 kDa, and a second protein of approximately 27.7 kDa that could be the product of oligomerization or a UMSBP orthologue. Many other trypanosomatids such as *Leishmania major*, *Leishmania infantum*, *Leishmania braziliensis*, *T. brucei*, *T. vivax*, and *T. congolense* have been shown to contain two UMSBP orthologous genes (Milman et al., 2007). Our suggestion that the 27.7 kDa protein could be a UMS orthologue in *T. copemani* requires further investigation. The identification of *T. copemani* UMSBP, a protein that has been involved in the process of kDNA replication and more recently has been implicated in the regulation of virulence in other trypanosomatids, is the first step towards understanding the mechanism of kDNA replication and virulence in this parasite.

The current study has not only demonstrated strong similarities in the organisation and structure of kDNA minicircles and maxicircles between *T. copemani* and late emerging trypanosomatids such as *T. lewisi* and *T. cruzi*, but has also provided preliminary information and a foundation to better understanding the role of kDNA in the evolution of diversity, transmission and pathogenicity within kinetoplastids.

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