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Identification of novel trypanosome genotypes in native Australian marsupials

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ABSTRACT

In the present study, the occurrence and molecular phylogeny of trypanosome parasites were studied in both wild and captive marsupials from Western Australia and Queensland. Blood samples were screened by PCR at the 18S rDNA locus, and the glycosomal glyceraldehyde phosphate dehydrogenase gene.

Overall, 5.3% of the blood samples were positive at the 18S rDNA locus. All positives belonged to wild-captured Western Australian individuals, where trypanosome-specific DNA was detected in 9.8% of the screened samples from wild marsupials, in common brushtail possums, and woylies. The detection rate of trypanosome DNA in these two host species was 12.5% and 20%, respectively.

Phylogenetic analyses based on two loci, indicated that the possum-derived trypanosome isolates were genetically distinct, and most closely related to the Australian marsupial trypanosomes H25 from a kangaroo, and BRA2 from a bush rat. This is the first study to genetically characterise trypanosome isolates from possums.

The analysis of the woylie-derived isolates demonstrated that this marsupial host can harbour multiple genotypes within the same geographical location and furthermore multiple genotypes within the same host, indicative of mixed infections. All the woylie-derived genotypes grouped with trypanosomes found in Australian marsupials, suggesting that they are more likely to belong to an endemic or Australasian trypanosome species.

This is the first study to genetically characterise trypanosome isolates from possums (*Trichosurus vulpecula*).Although the clinical significance of these infections is currently unknown, the identification of these novel sequences may support future investigations on transmission, threats to endangered wildlife, and evolutionary history of the genus *Trypanosoma*.

Keywords: Trypanosomes, possum, woylie, phylogeny, marsupial.

1. Introduction

Trypanosomes are parasitic haemoprotozoa usually transmitted by arthropod or leech vectors that infect all classes of vertebrates, and are the etiological agents of severe diseases accompanied by a range of clinical signs including fatigue, fever, anaemia, and death, in both animals and humans (Hamilton et al., 2004). Little is known about the prevalence and pathogenesis of trypanosomes in Australian marsupials, and few genetic characterisation studies have been conducted.

To date, 5 named trypanosome species and >8 genotypes have been identified in native Australian mammals, including eutherians, marsupials, and monotremes. *Trypanosoma thylacis* was identified in the northern brown bandicoot (*Isoodon macrourus*) (Mackerras, 1959; Mackerras and Mackerras, 1960), *T. binneyi* in the platypus (*Ornithorhynchus anatinus*) (McMillian and Bancroft, 1974), *T. copemani* in the Gilbert's potoroo (*Potorous gilbertii*) (Austen et al., 2009), the quokka (*Setonix brachyurus*) (Austen et al., 2009), the common wombat (*Vombatus ursinus*) (Noyes et al., 1999) and the koala (*Phascolarctos cinereus*) (McInnes et al., 2011), and *T. irwini* and *T. gilletti* from the koala (McInnes et al., 2009; McInnes et al., 2011).

Novel trypanosome sequences have been obtained from a wallaby (*Macropodidae*) (ABF) (Hamilton et al., 2004), a kangaroo (*Macropodidae*) (H25) (Noyes et al., 1999), the woylie (*Bettongia penicillata*) (WYA), the chuditch (*Dasyurus geoffroii*) (CHA), the boodie (*Bettongia lesueur*) (BDA), the golden bandicoot (*Isoodon auratus*) (GBA), the Shark Bay mouse (*Pseudomys fieldi*) (SMA), the bushrat (*Rattus fuscipes*) (BRA), the ash-grey mouse (*Pseudomys albocinereus*) (AMA), the dibbler (*Parantechinus apicalis*) (DBA), and the common planigale (*Planigale maculata*) (MMA) (Smith et al., 2008; Averis et al., 2009).

In the present study, we examined the occurrence and molecular phylogeny of trypanosome parasites found in both wild and captive Australian marsupials in Western Australia (WA) and Queensland (Qld).

2. Materials and methods

2.1 Animal sources

A total of 113 blood samples were collected from wild and captive marsupials, and screened by molecular methods and microscopy for trypanosome presence (Table 1).

Wild animals were trapped using standard procedures in a study approved by Murdoch University Animal Ethics Committee (Permit W2284/09). Net weight, body condition, presence and condition of joeys in the pouch, and evidence of illness or injury, were recorded. Of the 113 collected samples, 61 originated from wild Australian marsupials trapped in 2009, in the Jarrah Forest region, south-east of Dwellingup, WA (32°42'51.91"S; 116° 3'43.07"E), comprising: 18 chuditchs (*Dasyurus geoffroii*), 24 common brushtail possums (*Trichosurus vulpecula*), 4 southern brown bandicoots (*Isoodon obesulus*), and 15 woylies (*Bettongia penicillata*).

Blood samples (n=33) were also collected from various marsupials sheltered at the Kanyana Wildlife Rehabilitation Centre in Perth, WA, including: 3 boodies (*Bettongia lesueur*), 5 juvenile western red kangaroos (*Macropus Rufus*), 4 juvenile western grey kangaroos (*Macropus fuliginosus*), 2 wallaroos (*Macropodidae*), 8 woylies 7 bilbies (*Macrotis lagotis*), 3 western barred bandicoots (*Perameles bougainville*), and 1 chuditch.

Additional blood samples were collected from wild northern quolls (*Dasyurus hallucatus*) (n=6) captured in regions surrounding Cairns, Qld, and from wild western ringtail possums (*Pseudocheirus occidentalis*) (n=13), trapped in the Locke Estate, east of Busselton, WA (33°38'59.80"S; 115°20'40.60"E). Blood samples were collected in potassium EDTA-treated microtubes (Sarstedt, Germany), and stored frozen at -20°C, until processing.

2.2 Ectoparasites

A small number of ectoparasites (fleas and ticks) (n ≈10) were collected at the same time as the blood samples from 4 animals from Dwellingup (one each of chuditch, common brushtail possum, southern brown bandicoot, and woylie), and screened by molecular methods, for trypanosome presence, as described below.

2.3 Microscopy

A single drop of peripheral blood was used to make thin blood films which were stained with a modified Wright's stain using an Ames Hema-Tek slide stainer (Bayer, Germany). Stained blood films were systematically examined using a BX50 microscope (Olympus, Japan) with screen views generated by a DP Controller (version 3.2.1.276, Olympus, Japan). At least 500 fields of the dense region, central monolayer and the feather of each blood film were examined at x 400 magnification for trypanosomes and recorded as positive or negative. If parasites were found, these were digitally photographed at x1000 magnification and further characterized; key morphological features were described and measurements were made using Image Pro Express version 5.1 (Media Cybernetics Inc., USA) as described by Austen et al., (2011).

2.4 Molecular analyses

DNA was isolated from 100 µL of blood, using the MasterPure Purification Kit (Epicentre Biotechnologies, USA), and from finely chopped ectoparasites using the Blood and Tissue Kit (QIAGEN, Germany), according to the manufacturer's instructions. Mock extractions were carried out from an equal volume of sterile molecular-grade water, to exclude DNA contamination in reagents and consumables. A nested PCR protocol, targeting a variable region of the trypanosome 18S rDNA using specific oligonucleotides, was performed for initial sample screening, as previously described (Maslov et al., 1996; McInnes et al., 2011). DNA isolates found positive at the 18S locus were also amplified using a second hemi-nested PCR of the glycosomal glyceraldehyde

phosphate dehydrogenase (gGAPDH) gene, as previously described (McInnes et al., 2011). All amplifications performed included negative and positive controls, consisting of sterile molecular-grade water, and genomic DNA preparations from trypanosomatid-infected animals identified (and sequenced) during previous analyses, respectively.

PCR products were run on a 1% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). PCR products corresponding to the expected length were excised, purified using a MO BIO UltraClean DNA purification kit (MOBIO Laboratories, USA), and sequenced using an ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, USA), on an Applied Biosystem 3730 DNA Analyzer.

2.5 Cloning

Gel-purified PCR products which provided mixed or low-quality DNA sequencing chromatograms were cloned in the pGEM-T Easy Vector System II (Promega, USA). After transformation of JM109 competent cells, plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, Germany) from cultured clones grown overnight, and sequenced as described above, or using SP6 and T7 promoter primers (Promega, USA).

2.6 Phylogenetic analysis

Phylogenetic analyses were conducted on the sequences obtained during the present study and additional sequences retrieved from GenBank (Benson et al., 2010) (Table 2). Sequencing chromatogram files were analysed by FinchTV 1.4 (http://www.geospiza.com/Products/finchtv.shtml), and imported into Bioedit Sequence Alignment Editor (Hall, 1999), and MEGA 5 (Tamura et al., 2007) for manipulations and alignments. Alignments obtained by MUSCLE (Edgar, 2004) or CLUSTAL W (Larkin et al., 2007) were

compared and processed by Gblocks (Castresana, 2000), using the Phylogeny.fr platform (Dereeper et al., 2008). After selecting the most appropriate evolutionary model by jModeltest 0.1.1 (Posada, 2008) or MEGA 5 (Tamura et al., 2007), maximum likelihood (ML) based on the Kimura 2-parameter model (Kimura, 1980), maximum parsimony (MP), and neighbor-joining (NJ) trees were constructed using MEGA 5 (Tamura et al., 2007). Models with the lowest BIC scores (Bayesian Information Criterion) were considered. Estimates of evolutionary divergence between sequences were calculated using MEGA 5 (Tamura et al., 2007) with the Jukes-Cantor model (Jukes and Cantor, 1969), as the number of base substitutions per site from between sequences. The rate variation among sites was modelled with a gamma distribution (number of categories = 6). All positions containing gaps and missing data were eliminated.

The relationship between trypanosome 18S rDNA sequences identified in the present study and shorter 18S sequences (including the V7–V8 region) reported in previous papers (Smith et al., 2008; Averis et al., 2009) was also investigated. The analysis was only based on a short region (~500 bp) of 18S rDNA sequence, because only short 18S rDNA fragments are presently available on GenBank for these Australian marsupial trypanosome isolates.

3. Results

3.1 Detection rate of trypanosomes in marsupials

Overall, 5.3% (6/113) of the blood samples screened by PCR were positive for trypanosome 18S rDNA (Table 1). All positives samples belonged to the Dwellingup group, where trypanosomatid-specific DNA was detected in 9.8% of the screened samples, in common brushtail possums (n = 3) and woylies (n = 3). Respectively, 12.5% (3/24) and 20% (3/15) of the screened individuals from these two species were infected (Table 1). Trypanosomatid infection in the

possums was also confirmed by molecular results at the gGAPDH locus (Table 1). None of the DNA samples from the ectoparasites was positive for trypanosomatid DNA.

3.2 Microscopy

A total of 53 blood films from 5 host species were examined microscopically (Table 1). A total of five large trypomastigotes were observed in the blood film from a single common brushtail possum (*Trichosurus vulpecula*) (Figure 1); an individual that was also positive for trypanosomatid 18S rDNA. All five trypomastigotes were very similar in appearance; elongate in shape, tapered at both ends, with an undulating membrane extending over 85% of their length, ending in an anterior free flagellum. A pale staining nucleus was located approximately one fifth of the length from the posterior to anterior ends (nearest the posterior end) and a small, magenta-staining kinetoplast was observed at the organism's posterior end (Figure 1). Various granules and organelles were visible within in a pale basophilic staining cytoplasm. The average measurements for 5 organisms were as follows; total length = 35.6µm, breadth = 5.5µm, posterior to kinetoplast = 3.6µm, kinetoplast to nucleus = 3.2µm, nucleus to anterior = 20µm, and free flagellum = 8.8µm.

3.3 Sequence analysis and phylogenetic analysis

Three partial 18S rDNA sequences, inclusive of the V7-V8 region and which were 100% identical, were obtained from each of the 3 common brushtail possums: D15 (1,429 bp) D17 (1,370 bp) and D64 (1,478 bp). Based on the comparison of a 1,353 bp-long region, they differed at 4 sites from a kangaroo trypanosome (genotype H25) (Noyes et al., 1999).

Eleven partial 18S rDNA sequences (1,184 bp to 1,814 bp) were obtained from cloned PCR products, amplified from the 3 positive woylies (D4, D27 and S28). Genetically distinct sequences were obtained from the cloned PCR product from individual infected woylies, indicative of animals

harbouring mixed infections. Three different genotypes were obtained from woylie D4, and four from each of the two additional infected individuals, D27 and D28.

Genetic distance values of woylie genotypes, calculated from a processed 18S rDNA final dataset with 1008 positions, ranged between 0.8% (D28 clone 2), and 1.2% (D27 clone 3, and D28 clone 8), from phylogenetically closest trypanosome species *T. gilletti* from the koala trypanosome *T. gilletti* (McInnes et al., 2011).

Two separate analyses were conducted for the possum and the woylie clades to clarify the phylogenetic relationship of the trypanosomes identified in the present study, with Australian marsupial trypanosome isolates for which only short regions (~250-501 bp) of 18S rDNA sequences were available (Averis et al., 2009).

Phylogenetic analyses of the longer 18S rDNA sequence data (ML, MP, and NJ), produced similar tree topology and revealed that the three possum-derived isolates were genetically distinct but closely related to the kangaroo trypanosome H25 (Noyes et al., 1999) (Figure 2). This marsupial-derived clade, including possum and kangaroo-derived genotypes, was strongly supported by bootstrap values >85%. The trypanosome 18S rDNA sequences isolated from the woylies appear to be the longest fragments published for this locus from this host, thus far. Based on a final dataset containing over 1,000 bp, these genotypes formed a distinct clade (bootstrap values >91%) also including *T. gilletti* from the koala (McInnes et al., 2011) (Figure 2).

The shorter alignment of the 18S rDNA sequences (445 positions) used to estimate possum-derived trypanosomes indicated that the possum isolates identified in the present study were 0.2% distant (3 polymorphisms) from the bush rat-derived genotype BRA2 (Averis et al., 2009) and from the kangaroo *T.* sp. H25 (Noyes et al., 1999) (Figure 2a).

The shorter dataset used to estimate evolutionary divergence of woylie sequences (250 positions) revealed that they grouped closely with four isolates from woylie (WYA1, WYA2,

TRY1, and TRY2), and one from chuditch (CHA1) (Smith et al., 2008; Averis et al., 2009) (Figure 2b). The most closely related named species were *T. gilletti* from the koala (McInnes et al., 2011), and *T. copemani* from a range of marsupial hosts (Austen et al., 2009; McInnes et al., 2011). D4 clone 6 displayed 0.4% genetic distance from trypanosomes CHA1 (chuditch) and WYA2 (woylie) (Averis et al., 2009). D27 clone 2, and D28 clone 11 showed 0.4% and 0.8% genetic distance respectively, from TRY1 and TRY2 (Smith et al., 2008). Genetic distance between the woylie isolates and the named species *T. gilletti* (McInnes et al., 2011) ranged between 2.0 and 3.3%.

Partial fragments of the gGAPDH gene (~815 bp) were amplified and successfully sequenced from the three possums, but not from the woylies. The three possum-derived isolates were 100% identical to each other at this locus, and exhibited 23 polymorphisms from isolate H25 (gGAPDH sequences were not available for isolate BRA2), with 2.9% genetic distance (802 positions in the final dataset).

A consensus phylogenetic tree of the gGAPDH sequence data displayed a topology comparable with the 18S rDNA tree, and confirmed the close relationship between the possum-derived trypanosomes, the H25 isolate, and, secondarily, *T. cruzi* (Figure 3). The novelty of the gGAPDH genotypes found in our study was strongly supported by bootstrap values of 100% in the consensus tree (Figure 3).

4. Discussion

In the present study, the overall detection rate of trypanosome-infected animals from 12 marsupial species was 5.3% (6/113). A previous study, which examined the prevalence of trypanosomes in animals from 19 marsupial species in Western Australia, reported an overall prevalence of 8.6% (32/371) (Averis et al., 2009). In our study 16.7% of the marsupial species (2/12) were positive for trypanosomatids by PCR, while in a previous study, PCR positivity was detected in 47.4% of the marsupial species examined (9/19) (Averis et al., 2009). Amongst woylies,

a previous survey reported prevalences of 14-35% from different locations in WA (Smith et al., 2008). This range is in accordance with the present results, where trypanosome detection rate amongst woylies was 20%. Trypanosomes have previously been identified in brushtail possums from different locations within WA, with an overall prevalence of 18% (11/61) (Averis et al., 2009). This however, is the first study to genetically characterise trypanosome isolates from possums.

Phylogenetic analyses indicated that the possum-derived trypanosome isolates were genetically distinct and most closely related to the Australian marsupial trypanosome H25 from a kangaroo (Noyes et al., 1999) (18SrDNA and gGAPDH), and isolate BRA2 from a bush rat (Averis et al., 2009) (18SrDNA). The overall topology of the 18S rDNA and gGAPDH ML trees produced in this study in order to determine the evolutionary position of the possum-derived isolates amongst the trypanosomes (Figures 2 and 3), was comparable with trees previously published (Hamilton et al., 2007; McInnes et al., 2009; Viola et al., 2009).

Accurate morphological measurements of the possum-derived trypanosome isolates were made on 5 trypomastigotes, observed in the blood smear of just one individual of this host species. Definitive morphological features must await the discovery of more trypanomastigotes. Although the importance of trypomastigote morphology should not be discounted, delineating trypanosome species based on morphometrics alone is not reliable, as pleomorphism of bloodstream trypomastigotes of numerous *T.* spp. has been documented (Hoare, 1972; Ziccardi and Lourenço-de-Oliveira, 1999; Zintl et al., 2000; Lainson et al., 2008; Austen et al., 2009; McInnes et al., 2011).

It is generally accepted that the complex life cycles and inherent pleomorphism of trypanosomes necessitate significant reliance on genetic characterisation. In order to confidently identify species, reliance on 18S rDNA data alone is inappropriate due to intra-species variation at this locus, occasionally exceeding inter-species variation within some trypanosome clades (McInnes et al., 2011). A recent study proposed that significant portions of the 18S rDNA (inclusive of the

V7-V8 region) and gGAPDH genes (>65%) should be used for species delimitation in trypanosomes, and that the gGAPDH gene divergence to the most closely related trypanosome species be no less than 3.75% (a conservative measure which allows for a 50% buffer on the largest intra-species variation noted in this study) (McInnes et al., 2011). These observations are in line with those of Hamilton and Stevens (2011) who also state that accurate phylogenetic placement requires longer 18S rDNA sequences and/or sequences from other genes, together with careful taxon and out-group selection. By these criteria, the possum-derived trypanosome isolates are not a separate species, as the gGAPDH gene divergence to H25 was only 2.9%. However, the clade containing the possum-derived isolates and H25 as a group, is clearly a separate species from all other trypanosome species by this definition, as they are 9.7 and 9.0% divergent respectively from *T. cruzi marinkellei* and *T. dionisii*, at the gGAPDH locus.

Analysis of the longer region of the 18S rDNA gene (Figure 2) also indicates that the possum-derived isolates and H25 clade are most closely related to *T. cruzi* and not *T. lewisi*, and highlights the potential deficiencies of phylogenetic trees constructed from shorter fragments of the 18S rDNA. This was also recently noted by other authors (Hamilton and Stevens, 2011), who noted that the short (<500 bp) V7–V8 region of the18S rDNA is "unsuitable for resolving relationships between distantly related trypanosomes, as it evolves quickly, and sequences from distantly related taxa cannot be aligned with confidence".

In the southwest region of Western Australia, the woylie has recently undergone a dramatic reduction in abundance, despite no apparent increase in the number or type of predators in the region, and no apparent decrease in natural resources (Wayne, 2008; Groom, 2010). Phylogenetic analysis of woylie-derived isolates identified two genetically distinct trypanosomes (ML bootstrap value 85%) within woylie D4, indicating that individuals from this species can be infected by multiple genotypes (Figure 2). All the woylie-derived trypanosome genotypes grouped with

genotypes found in Australian marsupials, suggesting that they are likely to belong to an endemic or Australasian species.

The phylogenetic relationships of possum-derived trypanosome isolates may be further clarified once more morphological data is available, and more Australian native mammal trypanosome species are genetically characterised at both 18S rDNA and gGAPDH loci. Specifically, more genetic and morphological data on the trypanosome genotypes BRA2 and BDA1, from a bush rat and a boodie (Averis et al., 2009), which appear to associate with the possum-derived isolates (bootstrap values >95%), is needed to confirm their placement and relationship to other trypanosome species.

The vector for possum-derived trypanosome isolates is unknown and identification of vectors for a novel trypanosome is difficult due to the range of vectors known to transmit trypanosomes. Sometimes it is possible to infer vectors of a novel trypanosome from the types of vectors transmitting closely related trypanosomes. However, the vector(s) of the closest relatives of the possum-derived trypanosome isolates (H25 and BRA2) is also currently unknown.

In conclusion, the genetic analysis of two unlinked loci confirms the identification of novel trypanosome genotypes in common brushtail possums and woylies. No particular clinical signs have been observed in any of the infected animals, and the clinical significance of individual trypanosomes (or of mixed infections) is currently unknown. An increased understanding of the diversity of *Trypanosoma* spp. associated with native wildlife will contribute to the conservation efforts and translocation programmes of endangered species in Australia in the future.

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391 **Figure captions**

392 **Figure 1**

393 Trypomastigote in a peripheral blood film stained with a modified Wright's stain, from a wild
394 common brushtail possum (*Trichosurus vulpecula*), captured in Dwellingup, WA.

395

396 **Figure 2**

397 Phylogenetic analysis of the relationships of *Trypanosoma* spp. with woylie and novel possum-
398 derived isolates, based on 18S rDNA partial sequences. Evolutionary history was inferred using the
399 Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). Branch
400 support/bootstrap values for Maximum Likelihood, Maximum Parsimony and Distance analyses
401 respectively, are indicated at the left of each node. a) Phylogenetic position of possum-derived
402 trypanosome sequences with shorter sequences from other marsupial-derived trypanosome species.
403 b) Phylogenetic position of woylie-derived trypanosome sequences with shorter sequences from
404 other marsupial-derived trypanosome species.

405

406 **Figure 3**

407 Phylogenetic analysis of the relationships of *Trypanosoma* spp. with woylie and novel possum-
408 derived isolates, based on gGAPDH partial sequences. Evolutionary history was inferred using
409 Maximum Likelihood analysis based on the Kimura 2-parameter model (Kimura, 1980). Branch
410 support/bootstrap values for Maximum Likelihood, Maximum Parsimony and Distance analyses
411 respectively, are indicated at the left of each node.

Table 1

Prevalence of trypanosome 18SrDNA in a range of marsupial hosts from different geographic locations in Australia. Molecular analyses of glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) were performed only on samples providing 18S rDNA positive results. Abbreviations: n.a. = not applicable; n.p. = not provided; n.p.o. = no parasites observed.

SAMPLE GROUPS		MICROSCOPY		MOLECULAR ANALYSIS			
Source	**Species (Nr. of animals)**	**Blood smears (Nr.)**	**Observations**	**Blood samples (Nr.)**	**18s PCR (Nr. positives)**	**GAPDH PCR (Nr. positives)**	**Prevalence % (95% C.I.)**
DWELLINGUP, WA	Chuditch (18)	14	n.p.o.	18	0	0	0.
	Common brushtail possum (24)	21	Five trypomastigotes	24	3	3	12.5 (0-25.7)
	Southern brown bandicoot (4)	3	n.p.o.	4	0	0	0
	Woylie (15)	12	n.p.o.	15	3	0	20 (0-40.2)
Total	**61**	**50**		**61**	**6**	**3**	**9.8 (2.4-17.3)**
KANYANA, WA	Boodie (3)	3	n.p.o.	3	0	0	n.a.
	Western red juvenile (5)	n.p.	n.a.	5	0	0	0
	Western grey juvenile (4)	n.p.	n.a.	4	0	0	0
	Wallaroo (2)	n.p.	n.a.	2	0	0	0
	Woylie (8)	n.p.	n.a.	8	0	0	0
	Bilby (7)	n.p.	n.a.	7	0	0	0
	Western barred bandicoot (3)	n.p.	n.a.	3	0	0	0
	Chuditch (1)	n.p.	n.a.	1	0	n.a.	n.a.

Total	**33**	**3**		**33**	**0**	**n.a.**	**n.a.**
CAIRNS, QL	Quoll (6)	n.p.	n.a.	6	0	0	0
Total	**6**	**n.a.**		**6**	**0**	**0**	**n.a.**
BUSSELTON, WA	Ringtail possum (13)	n.p.	n.a.	13	0	0	0
Total	**13**	n.a.	n.a.	13	0	0	0
Grand total	**113**	**53**		**113**	**6**	**3**	**5.3 (1.2-9.4)**

Table 2

GenBank Accession numbers and sources (where known) of the sequences used for the phylogenetic analyses.

18S rDNA *Trypanosoma* spp.				
Acc. Nr.	Species	Isolate code	Isolation source	Location
FJ649480	*T. avium*	A1073		
FJ649482	*T. avium*	sp30		
AJ009140	*T. avium*	LSHTM 144B	Chaffinch *Fringilla coelebs*	Czech Republic
AJ223562	*T. bennetti*	KT-2 (ATCC 50102)	American kestrel *Falco sparverius*	America
AJ620565	*T. binneyi*	AAW	Platypus *Ornithorhynchus anatinus*	Australia
U39580	*T. boissoni*	ITMAP 2211	Senegal marine ray *Zanobatus atlanticus*	Senegal
NC005063	*T. brucei*	TREU927		
AJ009143	*T. cobitis*	LUMP 1243	Freshwater fish *Noemacheilus barbatulus*	England
AJ012411	*T. conorhini*	USP	Rat *Rattus rattus*	Brazil
GU966588	*T. copemani*	Charlton	Koala *Phascolarctos cinereus*	Australia
AY461665	*T. corvi*	ITMAP 180795	Raven *Corvus fugilegus*	England
AJ009149	*T. cruzi*	VINCH 89	Triatomine bug *Triatoma infestans*	Chile
FJ649484	*T. cruzi marinkellei*	B3	Bat *Phyllostomum discolor*	Brazil
AJ131958	*T. cyclops*		Macaque *Macaca* sp.	Malaysia
AJ009151	*T. dionisii*	P3	Bat *Pipistrellus pipistrellus*	England
D89527	*T. evansi*	Tansui-Taiwan		Taiwan
GU966589	*T. gilletti*	Lanie	Koala *Phascolarctos cinereus*	Australia
AJ620552	*T. granulosum*	Portugal	Eel *Anguilla anguilla*	Portugal
AJ620546	*T. grayi*	BAN1	Tsetse fly *Glossina palpalis gambiensis*	Africa
AJ223565	*T. grayi*		Tsetse fly *Glossina palpalis gambiensis*	Africa
FJ649479	*T. irwini*		Koala *Phascolarctos cinereus*	Australia
AJ223566	*T. lewisi*	ATCC 30085	Rat Rattus rattus	England
AJ009157	*T. mega*	ATCC 30038	African toad *Bufo regularis*	Africa
AJ009158	*T. microti*	TRL 132	Vole *Microtis agrestis*	England
AJ009159	*T. pestanai*	LEM 110	Badger *Meles meles*	France
AJ009160	*T. rangeli*	RGB Basel	Dog *Canis* sp.	Venezuela
AJ009161	*T. rotatorium*	B2-II	Bullfrog *Rana catesbeiana*	Canada
U67182	*T. scelopori*		Lizard *Sceloporus occidentalis*	America
EU596252	*T.* sp.	610	Caiman *Caiman yacare*	Brazil

EU596253	*T.* sp.	624	Caiman *Caiman yacare*	Brazil
EU596254	*T.* sp.	1092	Caiman *Caiman yacare*	Brazil
AJ620558	*T.* sp.	AAP	Wombat *Vombatus ursinus*	Australia
AJ620557	*T.* sp.	AAT	Currawong *Strepera sp.*	Australia
AJ620564	*T.* sp.	ABF	Wallaby *Wallabia bicolor*	Australia
AJ620548	*T.* sp.	Gecko	Gecko *Tarentola annularis*	Senegal
AJ009168	*T.* sp.	H25	Kangaroo *Macropus giganteus*	Australia
AB281091	*T.* sp.	KG1	Tick *Haemaphysalis hystricis*	Japan
AB447493	*T.* sp.	Python	Python *Python regius*	Ghana
AJ009164	*T. theileri*	K127	Ox *Bos taurus*	Germany
U39584	*T. triglae*	ITMAP 2212	Trigla *Trigla lineata*	
AJ005279	*T. varani*	V54	Monitor lizard *Varanus exanthematicus*	Senegal
AJ009166	*T. vespertilionis*	P14	Bat *Pipistrellus pipistrellus*	England

Outgroup *taxa*

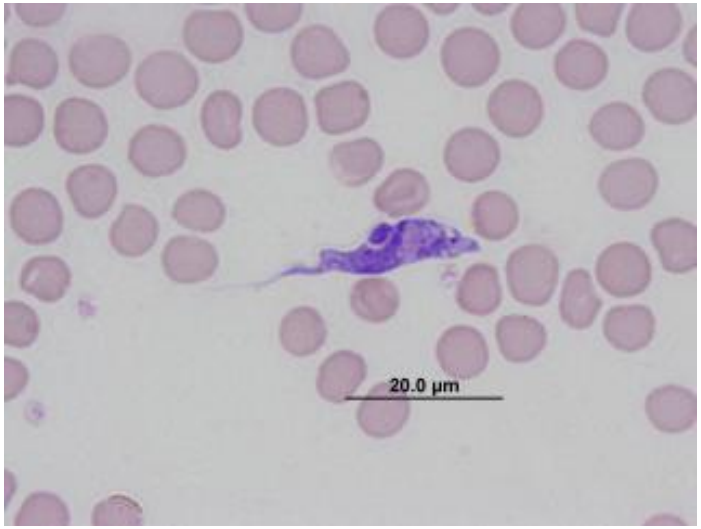
Isolate code	Species	Isolate code	Species
L29266	*Blastocrithidia culicis*	AF153038	*Leptomonas collosoma*
Y00055	*Crithidia fasciculata*	AF153040	*Leptomonas seymouri*
L29264	*Crithidia oncopelti*	U39577	*Phytomonas serpens*
L18872	*Herpetomonas muscarum muscarum*	AF153044	*Wallaceina inconstans*

gGAPDH *Trypanosoma* spp.				
Acc. Nr.	Species	Isolate code	Isolation source	Location
FJ649488	*T. avium*	A1073		
FJ649490	*T. avium*	sp30		
AJ620263	*T. avium*	LSHTM 144B	Chaffinch *Fringilla coelebs*	Czech Republic
FJ649486	*T. bennetti*			
AJ620266	*T. binneyi*	AAW	Platypus *Ornithorhynchus anatinus*	Australia
AJ620245	*T. boissoni*	ITMAP 2211	Senegal marine ray *Zanobatus atlanticus*	Senegal
XM_840454	*T. brucei*	TREU927		
AJ620284	*T. bruce irhodesiense*	058	Human *Homo sapiens*	Zambia
AJ620290	*T. congolense*	Savannah GAM2	Ox *Bos taurus*	The Gambia
AJ620267	*T. conorhini*	USP	Rat *Rattus rattus*	Brazil
FJ649496	*T. corvi*			
AJ620269	*T. cruzi*	VINCH 89	Triatomine bug *Triatoma infestans*	Chile
FJ649495	*T. cruzi marinkellei*	B3		
FJ649493	*T. cyclops*			
FJ649494	*T. dionisii*			
AF053743	*T. evansi*			
FJ649485	*T. irwini*		Koala *Phascolarctos cinereus*	Australia
AJ620272	*T. lewisi*	L32	Rat Rattus rattus	England
AJ620253	*T. mega*	ATCC 30038	African toad *Bufo regularis*	Africa
AJ620273	*T. microti*	TRL 132	Vole *Microtis agrestis*	England
AJ620275	*T. pestanai*	LEM 110	Badger *Meles meles*	France
AF053742	*T. rangeli*			
AJ620256	*T. rotatorium*	B2-II	Bullfrog *Rana catesbeiana*	Canada
AJ620293	*T. simiae*	KEN2	Tsetse fly *Glossina morsitans*	The Gambia
AB362559	*T. sp.*	Python	Python *Python regius*	Ghana
AM503075	*T. sp.*	Msubugwe-2006	Tsetse fly *Glossina spp.*	Tanzania

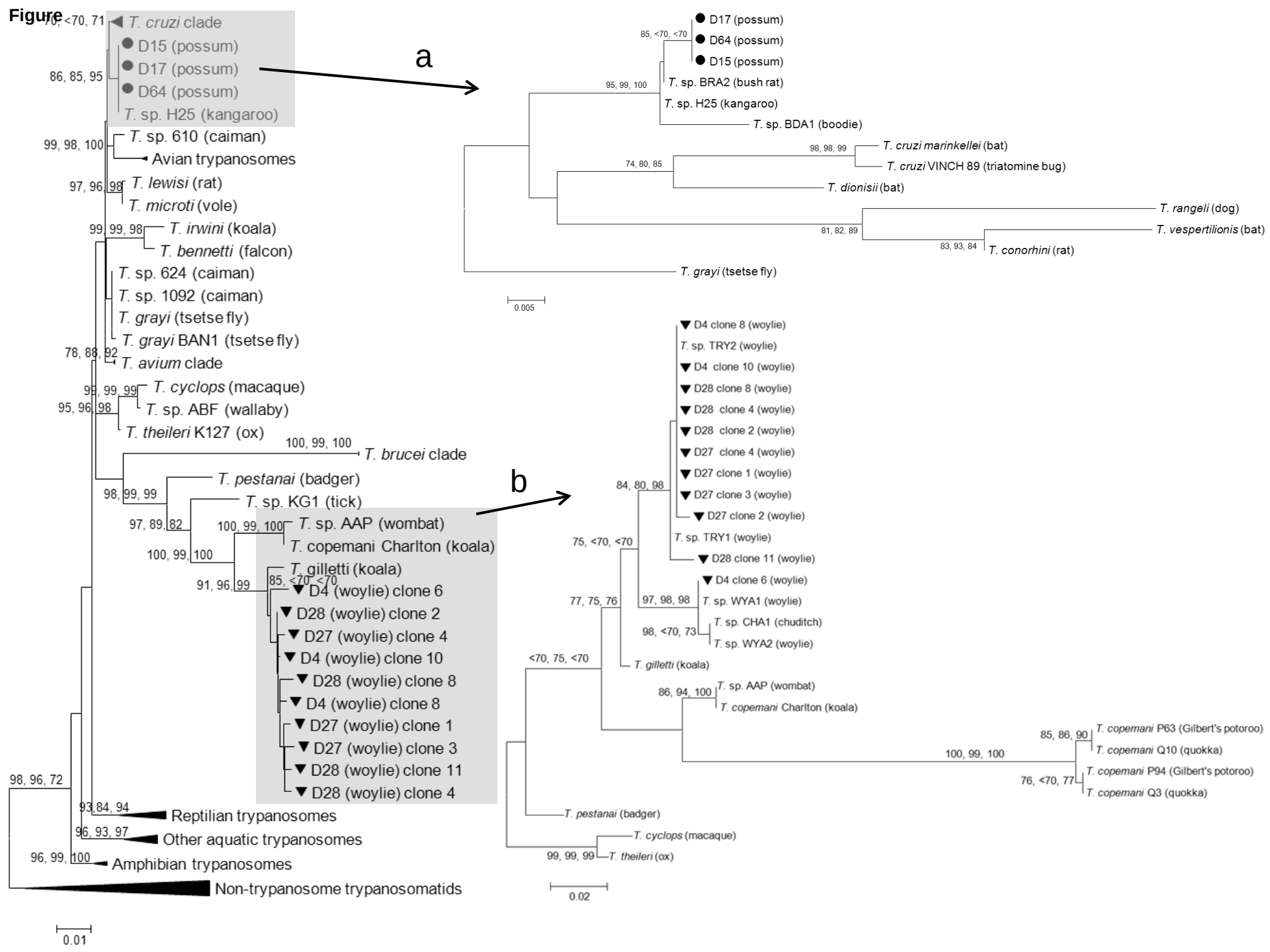
FJ649492	*T. sp.*	KG1		
AJ620264	*T. sp.*	AAT	Currawong *Strepera sp.*	Australia
AJ620259	*T. sp.*	Gecko	Gecko *Tarentola annularis*	Senegal
AJ620276	*T. sp.*	H25	Kangaroo *Macropus giganteus*	Australia
AJ620278	*T. sp.*	ABF	Wallaby *Wallabia bicolor*	Australia
AJ620277	*T. sp.*	AAP	Wombat *Vombatus ursinus*	Australia
AJ620282	*T. theileri*	K127	Ox *Bos taurus*	Germany
AJ620261	*T. varani*	V54	*Varanus exanthematicus*	Senegal
AJ620283	*T. vespertilionis*	P14	Bat *Pipistrellus pipistrellus*	England
AF053744	*T. vivax*		Cattle	

Outgroup *taxa*			
Isolate code	Species	Isolate code	Species
EU079137	*Blastocrithidia culicis*	EU084898	*Leptomonas collosoma*
AF047493	*Crithidia fasciculata*	AF047495	*Leptomonas seymouri*
EU079134	*Crithidia oncopelti*	EU084892	*Phytomonas serpens*
DQ092548	*Herpetomonasmuscarum*	EU076608	*Wallaceina inconstans*

Figure



Figure


a
b
T. cruzi clade
D15 (possum)
D17 (possum)
D64 (possum)
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T. sp. 610 (caiman)
Avian trypanosomes
T. lewisi (rat)
T. microti (vole)
T. irwini (koala)
T. bennetti (falcon)
T. sp. 624 (caiman)
T. sp. 1092 (caiman)
T. grayi (tsetse fly)
T. grayi BAN1 (tsetse fly)
T. avium clade
T. cyclops (macaque)
T. sp. ABF (wallaby)
T. theileri K127 (ox)
T. brucei clade
T. pestanai (badger)
T. sp. KG1 (tick)
T. sp. AAP (wombat)
T. copemani Charlton (koala)
T. gilletti (koala)
D4 (woylie) clone 6
D28 (woylie) clone 2
D27 (woylie) clone 4
D4 (woylie) clone 10
D28 (woylie) clone 8
D4 (woylie) clone 8
D27 (woylie) clone 1
D27 (woylie) clone 3
D28 (woylie) clone 11
D28 (woylie) clone 4
Reptilian trypanosomes
Other aquatic trypanosomes
Amphibian trypanosomes
Non-trypanosome trypanosomatids
0.01
D17 (possum)
D64 (possum)
D15 (possum)
T. sp. BRA2 (bush rat)
T. sp. H25 (kangaroo)
T. sp. BDA1 (boodie)
T. cruzi marinkellei (bat)
T. cruzi VINCH 89 (triatomine bug)
T. dionisii (bat)
T. rangeli (dog)
T. vespertilionis (bat)
T. conorhini (rat)
T. grayi (tsetse fly)
0.005
D4 clone 8 (woylie)
T. sp. TRY2 (woylie)
D4 clone 10 (woylie)
D28 clone 8 (woylie)
D28 clone 4 (woylie)
D28 clone 2 (woylie)
D27 clone 4 (woylie)
D27 clone 1 (woylie)
D27 clone 3 (woylie)
D27 clone 2 (woylie)
T. sp. TRY1 (woylie)
D28 clone 11 (woylie)
D4 clone 6 (woylie)
T. sp. WYA1 (woylie)
T. sp. CHA1 (chuditch)
T. sp. WYA2 (woylie)
T. gilletti (koala)
T. sp. AAP (wombat)
T. copemani Charlton (koala)
T. copemani P63 (Gilbert's potoroo)
T. copemani Q10 (quokka)
T. copemani P94 (Gilbert's potoroo)
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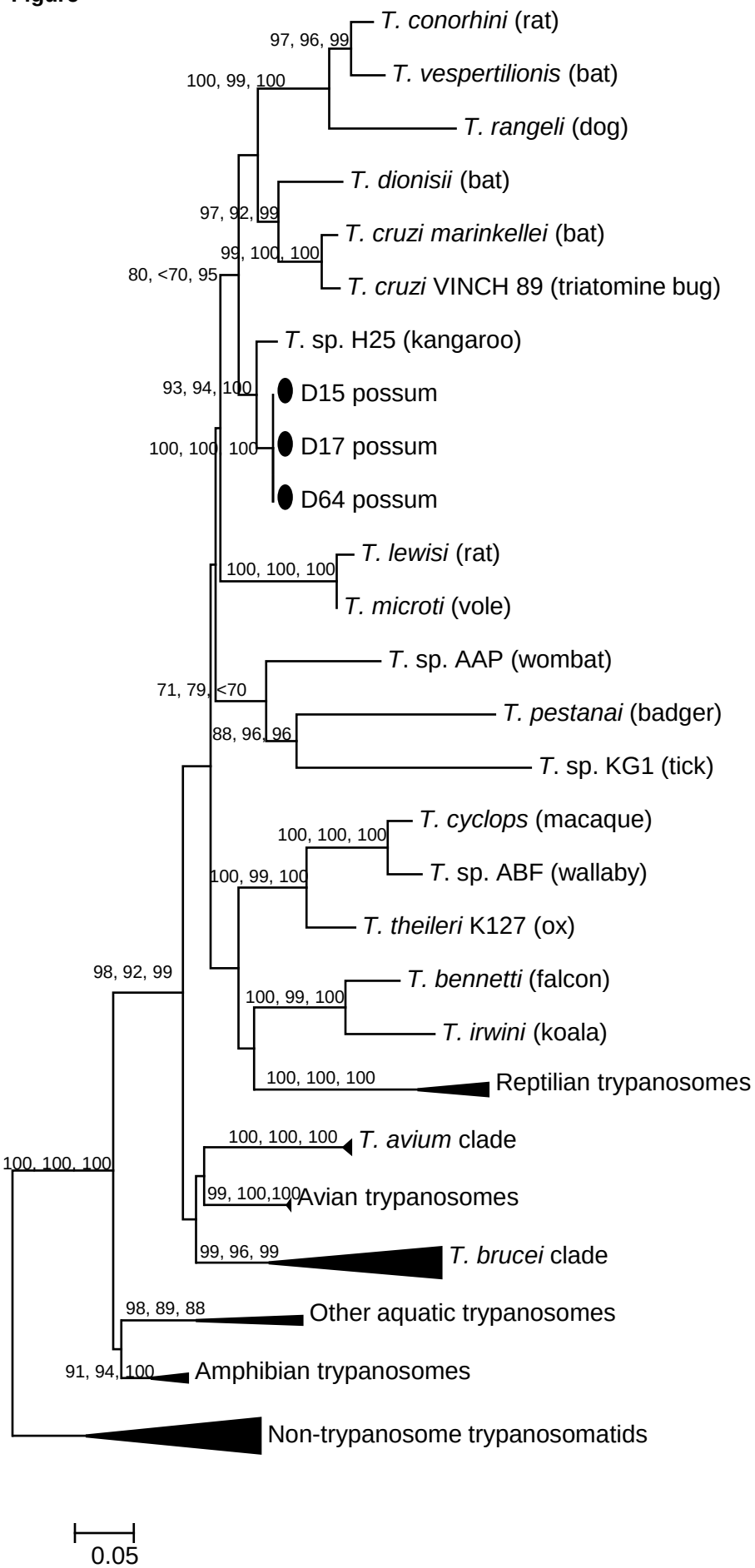
T. pestanai (badger)
T. cyclops (macaque)
T. theileri (ox)
0.02

Figure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97, 96, 99
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100, 99, 100
T. rangeli (dog)
T. dionisii (bat)
97, 92, 99
T. cruzi marinkellei (bat)
99, 100, 100
80, <70, 95
T. cruzi VINCH 89 (triatomine bug)
T. sp. H25 (kangaroo)
93, 94, 100
D15 possum
100, 100, 100
D17 possum
D64 possum
T. lewisi (rat)
100, 100, 100
T. microti (vole)
T. sp. AAP (wombat)
71, 79, <70
T. pestanai (badger)
88, 96, 96
T. sp. KG1 (tick)
T. cyclops (macaque)
100, 100, 100
T. sp. ABF (wallaby)
100, 99, 100
T. theileri K127 (ox)
98, 92, 99
T. bennetti (falcon)
100, 99, 100
T. irwini (koala)
100, 100, 100
Reptilian trypanosomes
100, 100, 100
T. avium clade
100, 100, 100
99, 100,100
Avian trypanosomes
99, 96, 99
T. brucei clade
98, 89, 88
Other aquatic trypanosomes
91, 94, 100
Amphibian trypanosomes
Non-trypanosome trypanosomatids
0.05