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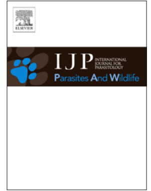

Trypanosomes genetic diversity, polyparasitism and the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*)

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ABSTRACT

While much is known of the impact of trypanosomes on human and livestock health, trypanosomes in wildlife, although ubiquitous, have largely been considered to be non-pathogenic. We describe the genetic diversity, tissue tropism and potential pathogenicity of trypanosomes naturally infecting Western Australian marsupials. Blood samples collected from 554 live-animals and 250 tissue samples extracted from 50 carcasses of sick-euthanized or road-killed animals, belonging to 10 species of marsupials, were screened for the presence of trypanosomes using a PCR of the 18S rDNA gene. PCR results revealed a rate of infection of 67% in blood and 60% in tissues. Inferred phylogenetic trees using 18S rDNA and glycosomal glyceraldehyde phosphate dehydrogenase (*gGAPDH*) sequences showed the presence of eight genotypes that clustered into three clades: a clade including *Trypanosoma copemani*, a new clade closely related to *Trypanosoma gilletti*, and a clade including *Trypanosoma* H25 from an Australian kangaroo. Trypanosome infections were compared in a declining and in a stable population of the endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*). This marsupial showed high rates of infection with Clade A genotypes (96%) in the declining population, whereas in the stable population, Clade B genotypes were predominant (89%). Mixed infections were common in woylies from the declining but not from the stable population. Histopathological findings associated with either mixed or single infections involving Clade A genotypes, showed a strong inflammatory process and tissue degeneration predominantly in heart, oesophagus and tongue. Trypanosomes were successfully grown in culture and for the first time we demonstrate that a genotype within Clade A has the capacity to not only colonize different tissues in the host but also to invade cells *in vitro*. These results provide evidence for the potential role of trypanosomes in the decline of a formerly abundant marsupial that is now critically endangered.
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Introduction

Trypanosomes are flagellated blood parasites that are capable of infecting virtually all classes of vertebrates. They range from non-pathogenic species to those that are highly pathogenic and are the causative agents of many diseases of medical and veterinary importance, including Chagas disease in South America and sleeping sickness and Nagana in Africa (Hoare, 1972). Some trypanosomes, normally considered as non-pathogenic, are capable of inducing detrimental effects in the host, particularly when the parasite encounters new or naïve host species following their introduction into a new habitat (Maraghi and Molyneux, 1989) or when an infected host is exposed to additional or increased levels of stress (Brown et al., 2000; Wyatt et al., 2008). This may have been the case with the introduction of *Trypanosoma lewisi* to immunologically naïve rodent hosts on Christmas Island, which caused a collapse in the population of the endemic rat *Rattus macleari* to the point of complete extinction (Pickering and Norris, 1996; Wyatt et al., 2008). Studies have also shown that *Trypanosoma theileri*, a non-pathogenic trypanosome of bovids that infects most cattle worldwide, may be considered potentially pathogenic in the presence of stress, gestation, poor nutritional status, and concurrent infections (Hussain et al., 1985; Doherty et al., 1993; Seifi, 1995).

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Within Australia, numerous trypanosome species and genotypes have been described naturally infecting a wide range of native marsupials. These include *Trypanosoma thylacis* in northern brown bandicoots (*Isoodon macrourus*) (Mackerras, 1959), *Trypanosoma irwini* and *Trypanosoma gilletti* in koalas (*Phascolarctos cinereus*) (McInnes et al., 2009, 2011a), *Trypanosoma binneyi* in platypus (*Ornithorhynchus anatinus*) (Noyes et al., 1999) and *Trypanosoma copemani* in quokkas (*Setonix brachyurus*), Gilbert's potoroo (*Potorous gilbertii*) (Austen et al., 2009) and koalas (McInnes et al., 2011a,b). Different genotypes are also seen in the woylie (*Bettongia penicillata*), common wombat (*Vombatus ursinus*), eastern grey kangaroo (*Macropus giganteus*), swamp wallaby (*Wallabia bicolor*) brush tailed possum (*Trichosurus vulpecula*), chuditch (*Dasyurus geoffroii*), golden bandicoot (*Isoodon auratus*), southern brown bandicoot (*Isoodon obesulus*) and burrowing bettong (*Bettongia lesueur*) (Bettiol et al., 1998; Noyes et al., 1999; Hamilton et al., 2005; Thompson et al., 2008; Smith et al., 2009; Paparini et al., 2011). Phylogenetic analysis has shown that most of these trypanosomes fall into several distinct clades. Some of them are closely related to trypanosomes from outside Australia, but the majority cluster with species/genotypes within Australia (Stevens et al., 2001; Hamilton et al., 2005; Thompson et al., 2008; Smith et al., 2009; Austen et al., 2009; McInnes et al., 2011a; Paparini et al., 2011). Despite the identification of this large list of trypanosomes and their marsupial hosts, there are many unanswered questions related to host–parasite interactions and their pathogenic potential during single and mixed natural infections.

A commonly infected Australian native marsupial is the woylie (Thompson et al., 2008; Smith et al., 2009), which occupied most of the southern half of the Australian mainland before European settlement in 1788. However by the 1970s, woylies were listed as critically endangered due to a drastic reduction in abundance from habitat destruction and introduced predators (Start et al., 1995; Orell, 2004; de Tores et al., 2008). The geographical distribution of the woylie became confined to three locations in Western Australia (WA), the Upper Warren Region, Tutanning Nature Reserve and Dryandra Woodland (Fig. 1) (DEC, 2008; de Tores et al., 2008). Significant conservation efforts, which included predator control and captive breeding and release, led to an increase in abundance from the mid 1970s into the early 2000s. As a consequence of these efforts, woylies were removed from the endangered species list in 1996 (Start et al., 1998). However, since 1999, remaining populations have undergone a dramatic 90% 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 et al., in press a,b). As a consequence of these population declines, woylies were included once again on the endangered species list (Wayne et al., 2008; Groom, 2010). Karakamia wildlife sanctuary, a predator-proof fenced reserve located 50 km north-east of Perth, is the only substantial translocated population of woylies that has remained stable in Western Australia. Disease, possibly caused by *Trypanosoma* parasites shown to be at high prevalence levels, has been under consideration as an important factor associated with the decline (Thompson et al., 2008).

Considering the pathogenic potential of trypanosomes when encountering new host species or when an infected host is exposed to increased levels of stress, it is becoming increasingly important to establish their presence, rate of infection, genetic diversity and phylogenetic status within Australian wildlife. To help clarify these issues, this study aims to: (i) determine the genetic variability and phylogenetic relationships of trypanosomes circulating in Western Australia marsupials; (ii) investigate the life cycle of trypanosomes in the marsupial host; (iii) characterize trypanosome behavior in axenic culture; and (iv) evaluate the pathogenicity of trypanosomes and their influence in the decline of the woylie. Together these data will help clarify the impact of trypanosomes on the health of native Australian marsupials.

Materials and methods

Sample collection

Tissue and blood samples were collected from 10 species of marsupials at different locations throughout Western Australia (WA) as part of the WA Department of Environment and Conservation (DEC) fauna research and monitoring program (Fig. 1, Table 1). A total of 554 heparinized peripheral blood samples were collected from trapped and released marsupials during ongoing trapping sessions by DEC. 237 of these samples were collected from woylies at the stable population in Karakamia Sanctuary and 257 from woylies at the declining population in the Upper Warren Region. Tissue samples were collected from sick-euthanized animals that were presented to Perth Zoo Veterinary Department for treatment and from dead (accidently killed on roads) animals sent to Murdoch University for necropsy. Sick animals were euthanized due to very poor body condition, marked lethargy and poor prognosis for return to the wild. A total of 250 tissue samples were collected from 50 carcasses and at least two of the following tissues were collected from each animal: spleen, liver, lung, heart, kidney, brain, oesophagus, tongue, skeletal muscle and bone marrow. Wildlife sampling was carried out under Murdoch University animal ethics approval permit numbers NS1182-06, W2172-08 and W2350-10, and DEC animal ethics approval permit number DECAEC/52/2009. All tissue samples were extensively washed with phosphate buffered saline (PBS) and stored in 100% ethanol and 10% formalin for DNA isolation and histopathological analysis respectively.

Isolation, growth behavior and morphology of trypanosomes

Cultures were established by inoculation of approximately 50 μl of peripheral blood in biphasic media containing Brain-Heart Infusion (BHI) medium with agar and 10% defibrinated horse blood as a solid phase, and either RPMI or HMI9 medium supplemented with 10% heat-inactivated Fetal Calf Serum (FCS) and 50 μg/ml Penicillin–Streptomycin as a liquid phase. All isolates were expanded in liquid media until they reached a density of approximately 1×10^6 parasites/ml and were subsequently maintained with successive passages every week at 27 °C in the dark. Thin blood smears taken from naturally infected marsupials and smears of logarithmic and stationary phase cultures were fixed in methanol and stained with the commercial Romanowsky-type stain 'Diff-Quik' for examination by light microscopy. For scanning electron microscopy (SEM), culture forms were fixed in a 1:1 mixture of 5% glutaraldehyde in 0.01 M PBS: cell culture media (pH 7.2), before being mounted on poly-L-lysine coated coverslips, progressively dehydrated through a series of ethanols and critical point dried as previously described (Edwards et al., 2011). Coverslips were mounted on stubs with adhesive carbon, coated with 2 nm Pt and imaged at 3 kV using the in-lens secondary electron detector on a Zeiss 55VP field emission SEM.

Cell infection

Metacyclic trypomastigotes (1.4×10^4/well) from one week old cultures growing in liquid media were used to infect monolayers of L6 (skeletal muscle) and Vero (kidney epithelial) cells grown to 50% confluency in RPMI media supplemented with 10% FCS, on 16 well glass coverslips. In parallel, cells were infected with *Trypanosoma cruzi* Tulahuen strain for comparison. All cells were maintained at 37 °C and 5% CO_2. Two days post-infection, cells

Fig. 1. Geographical origin of the different species of marsupials trapped in this study.

Table 1
Overall prevalence of *Trypanosoma* infection in blood and tissues of different species of Western Australian marsupials.

Marsupial species	Blood samples (%, 95% CI)	Carcasses (%, 95%CI)	Tissue samples (%, 95% CI)
Woylie (*Bettongia penicillata*)	335/494 (68, 63–71)	18/27 (67, 46–81)	67/154 (43, 35–51)
Quenda *(Isoodon obesulus)*	0/2	2/2	8/11
Quokka (*Setonix brachyurus*)	–	1/3	2/7
Tammar wallaby (*Macropus eugenii*)	–	1/3	3/7
Banded hare wallaby (*Lagostrophus fasciatus*)	1/1	–	–
Boodie (*Bettongia lesueur*)	1/7	0/1	0/3
Chuditch (*Dasyurus geoffroii*)	1/2	–	–
Common brush tailed possum (*Trichosurus vulpecula*)	3/7	–	–
Western grey kangaroo (*Macropus fuliginosus*)	29/41	4/9	9/38
Quoll (*Dasyurus maculatus*)	–	4/4	17/30
Total	370/554 (67, 63–71)	30/50 (60, 48–75)	106/250 (42, 36–48)

The rate of infection is given by: number of *Trypanosoma*-positive samples or carcasses/total number of samples or carcasses. 95% CI (95% confidence interval).

were washed three times with PBS to remove extracellular parasites, the coverslips were then removed, air-dried and stained with Romanowsky 'Diff-Quik' staining system for optical microscopy. For SEM, glass cover slips with attached trypanosome-infected cells were removed from the culture plates and processed and imaged as described above. For transmission electron microscopy (TEM), trypanosome-infected cells were washed three times with PBS, trypsinized with 0.25% trypsin/EDTA (GIBCO) for a few minutes at 37 °C to detach the cells from the culture plates and resuspended in fresh media. This cell suspension was then fixed in the glutaraldehyde:culture media mixture. 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 epoxy resin Procure-Araldite. Sections 120 nm thick were cut on a diamond knife and mounted on copper grids. Digital images were collected from unstained sections at 120 kV on a JEOL 2100 TEM fitted with a Gatan ORIUS1000 camera.

PCR amplification and sequencing of 18S rDNA and gGAPDH loci

Genomic DNA from blood, tissue and cultured trypanosomes was obtained using the QIAamp blood and tissue DNA MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A nested PCR that targets a variable region of the 18S rDNA locus using generic primers that have the potential to recognize different species of trypanosomatids was performed as described previously (Maslov et al., 1996; McInnes et al., 2011a). Positive samples with 18S rDNA PCR were screened with a second PCR of the *gGAPDH* locus using modified heminested reactions (McInnes et al., 2011a). To investigate the genetic diversity of trypanosomes infecting Western Australian marsupials, 200 positive PCR products from blood and tissue and 28 PCR products from trypanosomes grown in culture were chosen, purified using Agencourt AMPure PCR Purification system (manufacturer's instructions) and sequenced (18S rDNA: ~1.5 kb and *gGAPDH*: ~810 bp) using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Bio-systems, California, USA) on an Applied Bio-system 3730 DNA Analyzer. Chromatograms, derived from complete sequences, were used for contiguous assembly using the DNA sequence analysis program Sequencher version 5.0.1.

DNA sequence alignments and phylogenetic inferences

18S rDNA and g*GAPDH* sequences obtained from blood, tissue and cultured trypanosomes, were aligned using MUSCLE (Edgar, 2004) and then manually refined using BioEdit 7.0.9.0 (Hall, 1999). Three different alignments were created for phylogenetic inference. First, 1410 bp sequences of 18S rDNA from trypanosomes obtained in the current study were aligned with 31 *Trypanosoma* spp. sequences representing all known trypanosome clades, and five other trypanosomatid sequences for use as outgroups obtained from GenBank. Secondly, truncated (786 bp) sequences of 18S rDNA were aligned with those previously published from different Western Australian marsupials (Thompson et al., 2008; Smith et al., 2009; Austen et al., 2009). Finally, 810 bp g*GAPDH* sequences obtained in the current study were aligned with 26 *Trypanosoma* spp. sequences representing all major trypanosome clades, and five other trypanosomatid sequences for use as out-

groups. All *Trypanosoma* spp. and outgroup sequences obtained from GenBank are shown in Table 2. Phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian methods, implemented using MEGA 5 (Tamura et al., 2011) and Mr Bayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Genetic distances were also calculated using the Kimura 2 parameter model within MEGA 5. Nodal support was estimated with 10,000 bootstrap replicates. JModelTest 2.1.1 was used to find the most appropriate nucleotide substitution model for ML and Bayesian analyses (Posada, 2008). The models of nucleotide substitution chosen were: TIM3ef+I+G (equal-frequencies transition model plus gamma) for the first alignment and GTR+I+G (general time reversible gamma proportion of invariant sites) for the other two alignments. The Markov chain Monte Carlo 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.

Trypanosome clade-specific PCR

Trypanosome clade-specific primers were constructed based on the aggregation of the different genotypes in the phylogenetic tree obtained from the 1410 bp 18S rDNA sequences (see Results). Specific primers that amplify *T. copemani* and could recognize genotype 1 and genotype 2 (both in Clade A) were used as described previously (McInnes et al., 2011a). Two sets of specific primers that amplify the other major genotype groups, genotypes 3–7 (Clade B) and G8 (Clade C) were constructed as shown in Table 3. PCR reactions for all blood and tissue samples were performed in a total volume of 25 µl containing 0.2 U Taq DNA Polymerase, 200 M of dNTPs, 0.8 µM of each primer and 1 µl of DNA template. Amplification was performed in a PT100 thermocycler (MJ Research) and consisted of a pre-PCR step at 94 °C for 5 min, 50 °C for 2 min and primer extension at 72 °C for 4 min followed by 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature described in Table 3 for each clade, 50 s at 72 °C and a final extension step at 72 °C for 7 min. PCR products were run on a 1.5% agarose gel stained with SYBR safe (Invitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). Prevalence of infection with trypanosomes was expressed as the percentage of samples found positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000).

Histopathological analysis

PCR positive tissues were paraffin embedded and 3 µm-thick sections were cut and stained with hematoxylin and eosin (H&E). Each section was examined microscopically for inflammatory lesions and the presence of trypomastigotes in blood vessels and/or capillaries and amastigotes in tissue cells. Sections for histopathological analysis were only collected from tissue of woylies, quokkas, quendas and Western grey kangaroos.

Results

General rate of infection in blood and tissue revealed by PCR

Blood samples from 554 trapped and released marsupials and 250 tissue samples extracted from 50 carcasses of dead or euthanized marsupials were screened for the presence of trypanosomes using generic primers for 18S rDNA. At least one individual of each marsupial species examined was positive for *Trypanosoma* infection either in blood or tissue. The rate of infection over all host species was 67% in blood of trapped and released animals and 60% in carcasses of dead animals, where at least one tissue sample was positive (Table 1).

Table 2
GenBank accession number of the sequences used in the phylogenetic analysis.

Isolates	18S rDNA Accession number	*gGAPDH* Accession number
T. rotatorium	AJ009161	AJ620256
AAT (Currawong)	AJ620557	
AAI	AJ620559	–
KG1 (Tick)	–	FJ649492
T. mega	AJ009157	AJ620253
T. binneyi	AJ132351	AJ620266
T. granulosum	AJ620551	–
T. avium Rook	U39578	–
T. theileri	AJ009164	AJ620282
T. cyclops	AJ131958	
ABF (Wallaby)	AJ620564	AJ620278
H25 (Kangaroo)	AJ009168	AJ620276
H26	AJ009169	–
T. dionisii	AJ009151	FN599054
T. cruzi marinkellei	AJ009150	AJ620270
T. cruzi COLOMBIANA	AF239980	–
T. cruzi SLU31 clone2	AY785586	–
T. cruzi VINCH89	AJ009149	AJ620269
T. rangeli	AJ009160	AF053742
T. vespertilionis	AJ009166	AJ620283
T. conorhini	AJ012411	AJ620267
T. pestanai	AJ009159	AJ620275
AAP	AJ620558	AJ620277
T. lewisi	AJ009156	AJ620272
T. microti	AJ009158	AJ620273
T. gilletti Lanie	GU966589	GU966587
T. copemani Charlton (Koala)	GU966588	GU966585
T. copemani Mika (Koala)	–	GU966585
T. copemani Harrison (Koala)	–	GU966586
T. irwini	FJ649479	FJ649485
T. bennetti	AJ223562	FJ649486
T. copemani Q3 (Quokka)	EU571232	–
T. copemani Q10 (Quokka)	EU571234	–
T. copemani GP94 (Gilbert's potoroo)	EU571231	–
T. copemani GP63 (Gilbert's potoroo)	EU571233	–
TRY1 (Woylie)	EU518939	–
TRY2 (Woylie)	EU518940	–
WYA1 (Woylie)	FJ823116	–
WYA2 (Woylie)	FJ823121	–
CHA1 (Chuditch)	FJ823120	–
T. sp. AP2011 isolate27 clone4	JN315394	–
T. sp. AP2011 isolate4 clone6	JN315392	-
T. sp AP2011 isolate28 clone11	JN315387	-
T. sp. AP2011 isolate 15	–	JN315395
T. sp. AP2011 isolate 17	–	JN315396
Leptomonas sp.	AF153043	AF339451
Phytomonas serpens	U39577	EU084892
H. muscarum	L18872	DQ092548
H. samuelpessoai	U01016	AF047494
H. megaseliae	U01014	DQ092547

DNA sequences and phylogenetic analysis

Eight different genotypes (G1 to G8) were found at the 18S rDNA locus among the 200 blood and tissue samples chosen for sequencing (Table 4). The sequences obtained from trypanosomes grown in culture revealed all of them were G1 and G2. Very similar phylogenetic relationships were found using both Bayesian and ML analyses between these eight genotypes and the 31 trypanosome sequences obtained from GenBank; we show only the results from the Bayesian analysis here. The eight genotypes found in our study were grouped into three distinct clades (Fig. 2A). The first two genotypes (G1 and G2) clustered within Clade A; G1 showed a 99% and 98% similarity to *T. copemani* previously described from

Table 3
Clade-specific PCR conditions and primer sequences.

PCR	Primer name	Primer sequence	$MgCl_2$ concentration	Annealing temperature	Band size
Clade C External	H25EF	GCCGACAGTGCATTTTGT	1.0 mM	60	~750 bp
	H25ER	GAGCGAGATGAACTCGACC			
Clade C Internal	H25IF	TTTGAGGCGCAATGGTTTAG	1.0 mM	60	~400 bp
	H25IR	CGAGTTGAGGGAAGGTGGC			
Clade B External	TVEF	GGGGTCCTTTTATTTTATTTG	1.5 mM	58	~750 bp
	TVER	TAATTTATTGGCCAGACAAA			
Clade B Internal	TVIF	GACCAAAAACGTGCACGTG	1.0 mM	58	~350 bp
	TVIR	AAATCGTCTCCGCTTTAAC			

Table 4
GenBank accession number of the new reported sequences and origin of the isolates.

GenBank Accession number		*Trypanosoma* Genotype	*Trypanosoma* Clade	Hosts
18S rDNA	*gGAPDH*			
KC753530	KC812982	Genotype 1 (G1)	Clade A	Woylie, common brush-tailed possum
KC753531	KC812983	Genotype 2 (G2)	Clade A	Woylie, quokka, quoll, quenda
KC753533	KC812984	Genotype 3 (G3)	Clade B	Woylie, Western grey kangaroo
KC753532	KC812985	Genotype 4 (G4)	Clade B	Woylie, quenda
KC753534	KC812986	Genotype 5 (G5)	Clade B	Woylie, tammar wallaby
KC753535	-	Genotype 6 (G6)	Clade B	Woylie, chuditch
KC753536	KC812987	Genotype 7 (G7)	Clade B	Woylie
KC753537	KC812988	Genotype 8 (G8)	Clade C	Woylie, banded hare wallaby, boodie

a wombat and koalas (Noyes et al., 1999; McInnes et al., 2011a), and G2 was closely related to G1 but differed in 13 nucleotides among the 1410 bp of sequence. Five genotypes (G3 to G7) clustered in Clade B together with *T.* sp AP2011 isolate 27, *T.* sp. AP2011 isolate 28 and *T.* sp. AP2011 isolate 4 (Paparini et al., 2011). In all analyses, the closest relative of this clade among previously described trypanosome species was *T. gilletti* from koalas, although the nucleotide distance between *T. gilletti* and the genotypes within this clade was considerably greater than distances between many closely related *Trypanosoma* species (Table 5). The last genotype found (G8) presented 99% similarity with a trypanosome isolated from an Australian kangaroo – *Trypanosoma* H25 (Noyes et al., 1999). Genotypes within this clade (Clade C) were more closely related to trypanosomes from outside Australia such as *T. cruzi* and *Trypanosoma rangeli* from South America and were previously positioned in a monophyletic assemblage designated as "*T. cruzi* clade" (Hamilton et al., 2012). Phylogenetic analysis of the eight new genotypes found in this study and previously published, truncated 18S rDNA sequences from trypanosomes isolated from Western Australian marsupials showed close relationships between them. Trypanosome isolates from a chuditch (CHA1) and woylies (TRY1, TRY2, WYA1, WYA2), clustered within Clade B, while *T. copemani* isolates from quokkas (Q3 and Q10) and Gilbert's potoroos (GP63 and GP94) were more closely related to G1 than to G2 (Fig. 2B).

Additional support for the phylogenetic positioning of the new sequences was provided by the phylogenies derived from the g*GAPDH* sequences (Fig. 3), which showed the same topology as the 18S rDNA derived tree, although only seven genotypes were included due to the lack of PCR amplification of G6.

Trypanosome clade-specific PCR

To confirm reliability of trypanosome clade-specific primers and PCRs, DNA sequencing was carried out from 16 blood samples infected with genotypes representative of the three different clades. These sequences showed a 100% similarity with the full 18S rDNA sequences, and confirmed the high specificity of the clade-specific primers and PCRs.

Out of the 370 blood samples positive for trypanosome infection, 53% (95% CI = 49–57) were positive for Clade A, 32% (95% CI = 27–37) for Clade B, 2% (95% CI = 1–3) for Clade C and 13% (95% CI = 10–17) were mixed infections with two or more genotypes representative of different clades. From the 106 tissues infected, 47% (95% CI = 38–57) were positive for Clade A, 29% (95% CI = 21–39) for Clade B, 2% (95% CI = 0.3–7) for Clade C and 22% (95% CI = 14–31) for mixed infections. Although PCR using clade-specific primers showed the presence of mixed infections in some of the samples, sequencing of the same samples using the 18S rDNA trypanosome generic primers revealed infections with only one genotype.

Parasite genotypes were not host-specific, with most being found in at least two host species (Table 4). Whereas single infections were found in blood and different tissues of all marsupials examined, mixed infections were only present in blood and tissues of woylies. This host species presented a total of 13% (95% CI = 10–17) and 1% (95% CI = 0.2–3) of dual infections with genotypes belonging to Clade A–Clade B and Clade B–Clade C respectively. Two percent of the samples (95% CI = 0.8–4) presented triple infections with genotypes from the three clades.

All trypanosome genotypes were present in peripheral blood. However, only G2 (Clade A), G3–G5 (Clade B) and G8 (Clade C) were found infecting host tissues, including tissues from woylies, quokkas, quendas, tammar wallabies, quolls and western grey kangaroos. Trypanosomes within Clade A exhibited a preferential tissue distribution for skeletal muscle, tongue, oesophagus, and heart, while trypanosomes within Clade B showed predilection for skeletal muscle, heart and lung. Table 6 shows the rate of infection among all different tissues collected, including tissues infected with more than one genotype.

Comparison of trypanosome infections between stable and declining populations of woylies

Woylies showed a rate of infection of 68% in blood of trapped and released animals and 67% in carcasses of dead and euthanized animals (Table 1). There was a significantly higher prevalence of trypanosome infection in sick and euthanized woylies compared with those found dead on roads; while only four out of 12 road kills were infected with trypanosomes, almost all (nine out of 10) euthanized animals were positive for trypanosome infection (Fisher exact test, $P = 0.01$). There was a signifi-

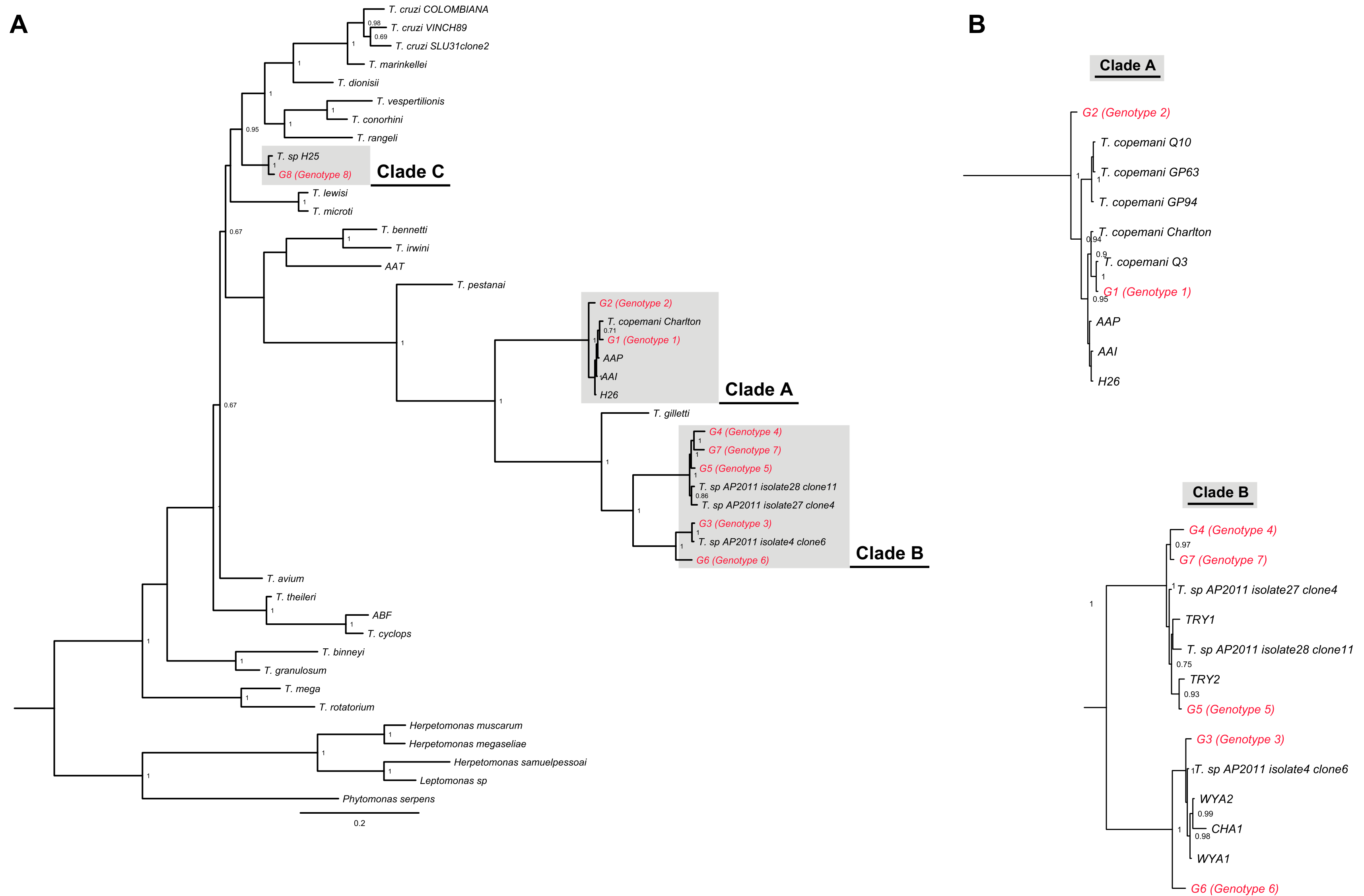

Fig. 2. Phylogenetic analysis of the relationships between Australian trypanosomes based on 18S rDNA sequences. Phylogenetic trees were constructed by the Bayesian method. (A) Phylogenetic position of longer 18S rDNA sequences (~1410 bp). (B) Phylogenetic position of shorter 18S rDNA sequences (786 bp) CHA1, TRY1, TRY2, WYA1, WYA2, BDA1, Q3, Q10, GP63 and GP94. Threes were rooted with five sequences as outgroups. Bootstrap values from Bayesian posterior probabilities are shown at nodes. In red: trypanosome genotypes found in this study. Bar, 0.2 substitutions per site.

Table 5
Genetic distances based on the 18S rDNA sequences from representative genotype sequences of each *Trypanosoma* clade found in this study; sequences from other *Trypanosoma* species are also shown. Analyses were conducted using the Kimura 2-parameter model within MEGA5. Genotype 2 (Clade A), genotype 3 (Clade B), genotype 8 (Clade C).

	Genotype 2	Genotype 3	Genotype 8	*T. gilletti*	*T. cruzi*	*T. rangeli*	*T. lewisi*
Genotype 2	–	0.154	0.132	0.120	0.152	0.157	0.139
Genotype 3	–	–	0.162	0.104	0.189	0.203	0.178
Genotype 8	–	–	–	0.137	0.052	0.048	0.052
T. gilletti	–	–	–	–	0.153	0.171	0.154
T. cruzi	–	–	–	–	–	0.087	0.069
T. rangeli	–	–	–	–	–	–	0.076
T. lewisi	–	–	–	–	–	–	–

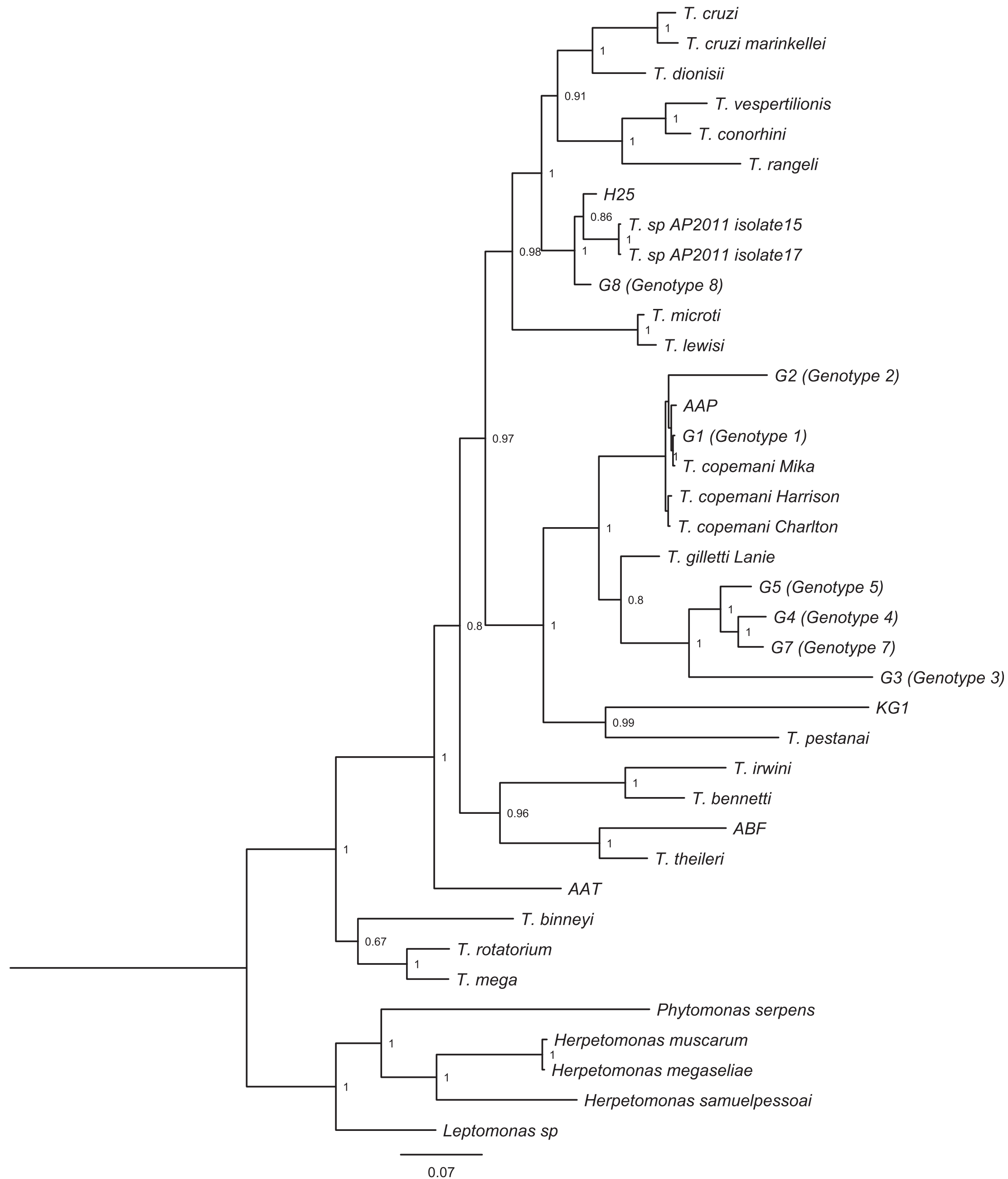

Fig. 3. Phylogenetic relationships of the new trypanosome isolates from Western Australian marsupials based on *gGAPDH* sequences (~810 bp) using Mr Bayes. The tree was rooted with five sequences as outgroups. Bayesian posterior probabilities are shown at nodes. Bar, 0.07 substitutions per site.

cant difference in the prevalence of infection with genotypes from Clade A and Clade B in the stable and declining populations (Fisher exact test, $P = 0.0001$). Among positive samples, the genotypes most frequently found in the stable population were from Clade B, present in 89% (95% CI = 82–94) of the samples. In contrast, genotypes from Clade A were most frequently found in the declining population, present in 96% (95% CI = 93–98) of the samples. Mixed infections were also found more frequently in the declining than in the stable population (Fisher exact test, $P = 0.001$) (Fig. 4).

Table 6
Differential tissue distribution of trypanosomes within the different clades.

Trypanosoma clades	Spleen	Kidney	Liver	Lung	Heart	Tongue	Skeletal muscle	Oesophagus	Brain	Bone marrow
Clade A	10% (2–26)	17% (5–37)	25% (13–43)	21% (9–37)	37% (19–58)	40% (16–68)	41% (25–59)	38% (14–68)	0%	9% (2–41)
Clade B	7% (1–22)	12% (26–32)	8% (16–21)	16% (60–31)	18% (6–38)	13% (16–40)	47% (29–65)	15% (19–45)	0%	9% (2–41)
Clade C	3% (0.1–17)	4% (0.1–21)	3% (0.1–14)	5% (0.6–18)	0%	0%	0%	0%	0%	0%
TOTAL	20% (8–38)	33% (16–55)	37% (22–54)	42% (26–59)	56% (35–74)	53% (26–79)	88% (72–97)	54% (25–81)	0%	18% (2–52)

The rate of infection (%) in each organ is given by the number of *Trypanosoma*-positive organs/total number of organs. Between brackets: 95% confidence interval (95% CI).

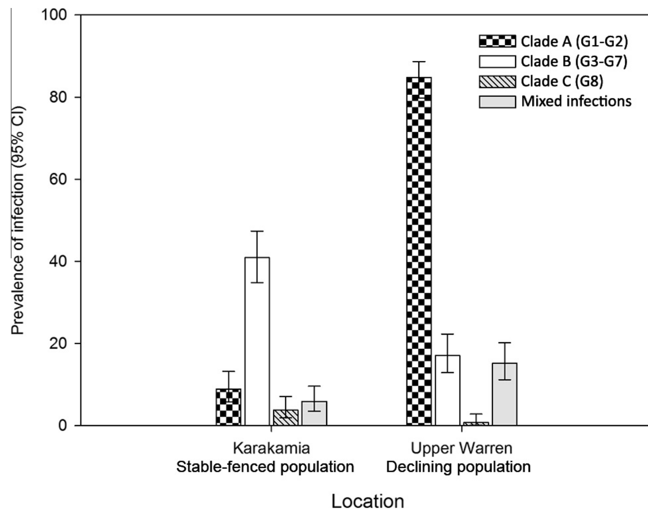

Fig. 4. Prevalence of infection with trypanosomes within the different clades in woylies from the stable and declining populations. 95% confidence intervals (95% CI).

Histopathological analysis

Gross pathology from 13 woylie carcasses that were infected with trypanosomes showed poor body and coat conditions and heavy infestation with ectoparasites (lice and ticks); most of them exhibited areas of alopecia accompanied by chronic multifocal exudative dermatitis.

Tissue sections taken from animals infected with genotypes either from Clade B or Clade C, including the woylie, Western grey kangaroo, and quenda, showed an absence of tissue lesions associated with trypanosomiasis. However, a mild inflammatory process was seen in tissues from woylies with a dual infection of genotypes from these two trypanosome clades. A moderate to marked inflammatory process was seen in tissues infected either with genotypes from Clade A or in co-infections involving trypanosomes from this clade. This process consisted, in general, predominantly of plasma cells, lymphocytes, macrophages, neutrophils and mast cells. The pathology seen in heart sections of three woylies showed a multifocal, severe and chronic pyogranulomatous myocarditis and endocarditis accompanied by muscle degeneration and necrosis (Fig. 5A–C). A multifocal, chronic, pyogranulomatous oesophagitis and glossitis, both accompanied by prominent skeletal muscle degeneration was also seen (Fig. 5D and E). Occasionally, inflammatory cells clustered around blood vessels (Fig. 5F). Trypanosomes were not observed intravascularly. However three woylies and one quenda presented structures suggestive of amastigotes in heart tissue (Fig. 6A and B). Histopathology of 10 sections taken from different tissues of two non-infected road kill woylies showed an absence of tissue lesions.

Growth behavior in culture and morphology of trypanosomes

Because woylies exhibited high levels of parasitemia and were detected harboring all different trypanosome genotypes found in this study, additional peripheral blood samples were taken and cultured in biphasic media from animals at Karakamia (stable population) and Upper Warren (declining population).

From 30 haemocultures established from blood of the stable population, no evidence of protozoa morphologically similar to trypanosomes was seen after 30 days of culture in biphasic media, either with RPMI or HMI9 media as a liquid phase. Furthermore, no trypanosomes were detected in direct peripheral blood smears from these animals, but PCR of extracted DNA from blood revealed 21 of them were infected with genotypes belonging to Clade B. In contrast, from the declining population, trypanosomes were detected in 24 of 30 haemocultures after day 8 to day 20 post-inoculation. PCR and sequencing of the 18S rDNA locus revealed all isolates were G1 and G2 (Clade A). Peripheral blood smears from the declining population showed blood forms consisted of large trypomastigotes, a small kinetoplast positioned far from the nucleus, and a well developed undulating membrane and flagellum (Fig. 7A). Dividing trypomastigote forms were never observed in blood smears.

Both genotypes in Clade A were successfully subcultured in biphasic media for up to 6 months at weekly intervals, but they differed in growth requirements when only liquid media was used. G1 showed a growth rate significantly higher in RPMI media while the growth of G2 was much more efficient in HMI9 media. Nevertheless, morphological features were almost identical between them. Eight-day-old cultures showed slender trypomastigotes that exhibited a rounded kinetoplast positioned close to the posterior end (Fig. 7B). Epimastigotes presented highly variable shapes with the kinetoplast positioned adjacent to the nucleus (Fig. 7C and D); some of the forms were spheromastigotes that were often seen to be dividing (Fig. 7E and F). Eight-day-old cultures also presented epimastigotes that were longer and thinner, most were undergoing binary division, giving rise to rosettes and transition forms of variable shape, length of body and flagellum (Fig. 8A). All forms growing in culture presented numerous acidocalcisomes randomly distributed along the whole body (Fig. 8B).

Cell infection

Trypanosome isolates G1 and G2 (Clade A) were used to infect Vero and L6 cells *in vitro*. Only G2 was able to infect cells. Amastigotes of G2 and *T. cruzi* (our positive control of infection) were observed inside cells at day 2 post-infection (Fig. 9A and B). Fig. 10A and B shows trypomastigotes invading a cell and dead cells surrounded by amastigotes and trypomastigotes that were frequently found after the second day of infection. Transmission electron microscopy of trypomastigotes of G2 confirmed the presence of acidocalcisomes and showed the flagellum at the surface of the cell containing the axoneme (AX) made of nine doublets of microtubules

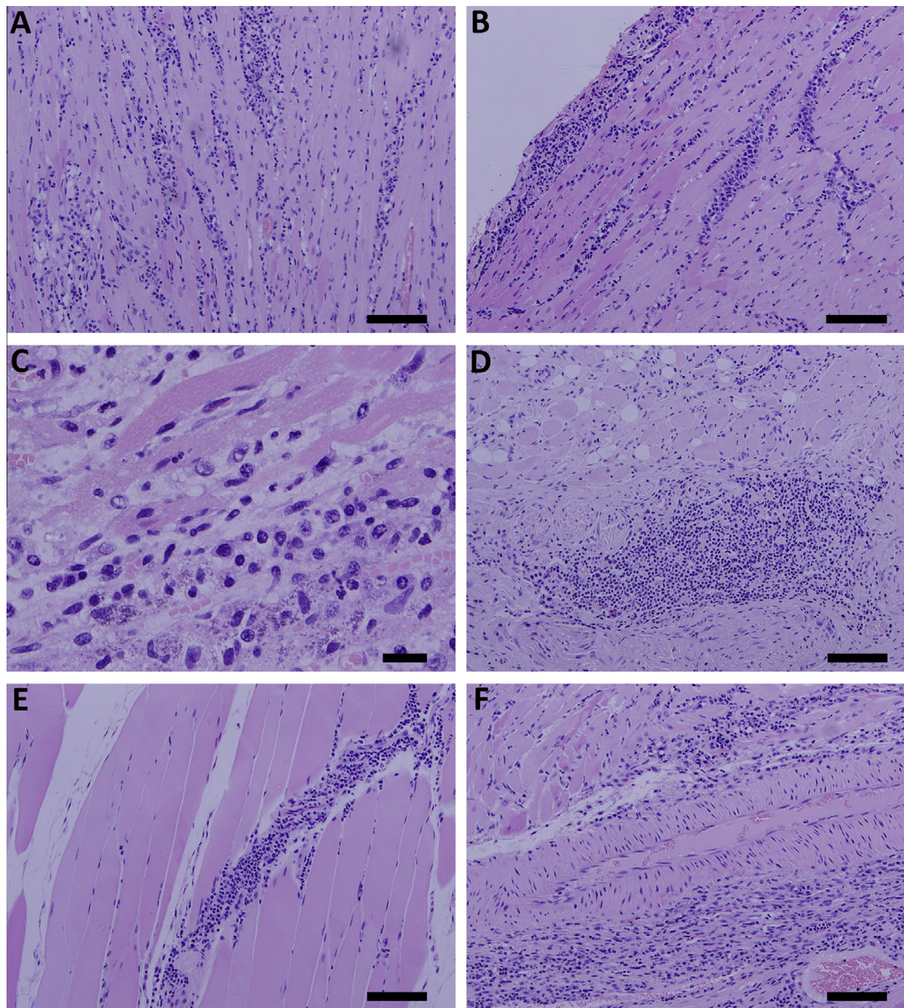

Fig. 5. Histopathology of two woylies naturally infected with G2 (Clade A) (H&E stained). (A) Multifocal, moderate to severe, chronic, pyogranulomatous myocarditis and (B) endocarditis. (C) Mineralisation of heart tissue. (D) Tongue showing multifocal, moderate, chronic, pyogranulomatous glossitis. (E) Skeletal muscle degeneration. (F) Inflammatory cells around a blood vessel. Scale bars = 20 µm.

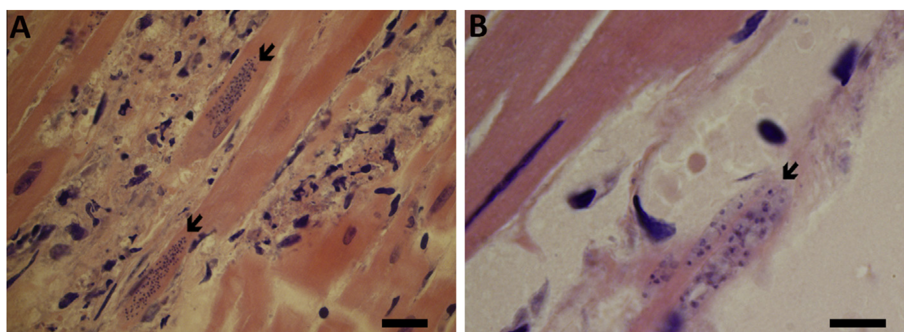

Fig. 6. Structures suggestive of amastigotes (arrows) of G2 (Clade A) in heart tissue positive by PCR (H&E stained). Scale bars = (A) 20 µm, (B) 10 µm.

surrounding a central pair as in most flagellated eukaryotes (Fig. 11A). Fig. 11B shows an amastigote inside a Vero cell.

Discussion

This study has confirmed previous findings that trypanosomes in Australian marsupials comprise a heterogeneous community, with low levels of host specificity and no evidence of restricted geographical distribution. *T. copemani* has been found in the blood of quokkas and Gilbert's potoroos from Western Australia (Austen et al., 2009), in koalas from Queensland (McInnes et al., 2011a,b), and in a wombat from Victoria (Noyes et al., 1999). *T. gilletti* and numerous closely related trypanosome genotypes have been reported in the blood of koalas from Queensland (McInnes et al., 2011a,b) and woylies from Western Australia (Thompson et al., 2008; Smith et al., 2009; Paparini et al., 2011). *Trypanosoma* H25, which was found to cluster on a phylogeny with *T. cruzi*, has been reported in the blood of a kangaroo from Victoria (Noyes et al., 1999) and in possums from Western Australia (Paparini et al., 2011). Here, we have found genotypes closely related to *T. copemani* in woylies, quendas, quolls and common brush tailed possums; a number of genotypes related to *T. gilletti* in the Western

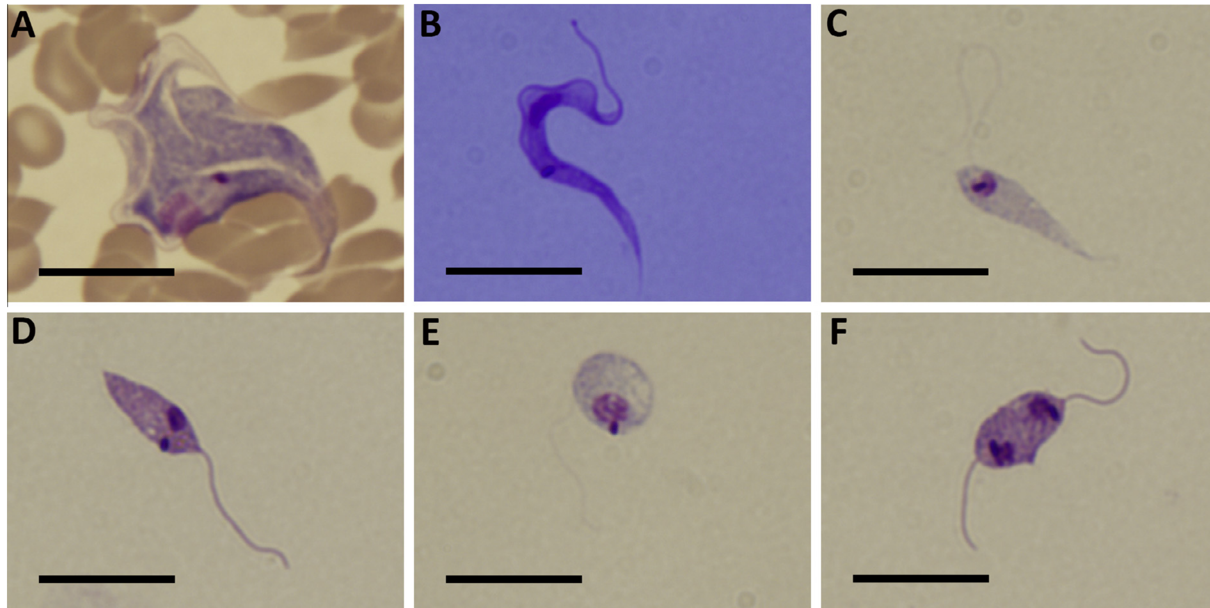

Fig. 7. Light microscopy of Diff-Quick stained blood and culture forms of G1 and G2 (Clade A) (A) Trypomastigote in blood of a woylie naturally infected; (B) slender epimastigote in culture; (C and D) shaped epimastigote in culture; (E) spheromastigote in culture; (F) spheromastigotes dividing in culture. Scale bars = 10 µm.

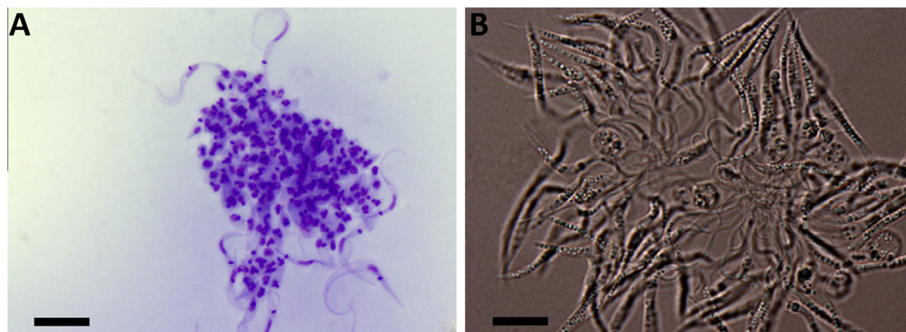

Fig. 8. Epimastigotes of G1 and G2 (Clade A) arranged in rosettes in culture. (A) Diff-Quick stained rosettes. (B) Rosettes in fresh wet preparations showing numerous intracellular acidocalcisomes. Scale bars = 10 µm.

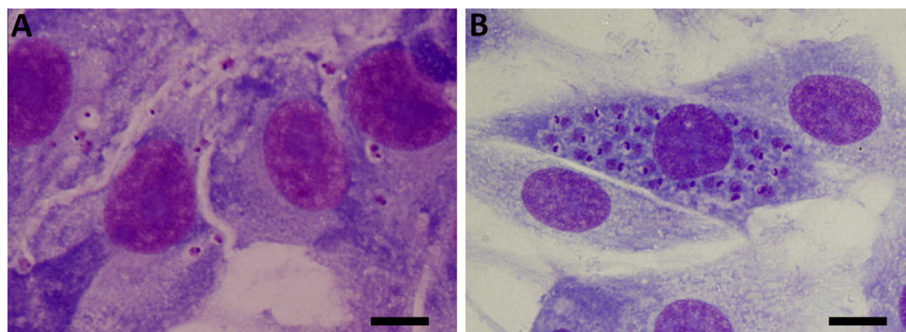

Fig. 9. Infection of Vero (A) and L6 cells (B) with G2 (Clade A) and *T. cruzi* as a positive control of infection (Diff-Quick stained). (A) Intracellular amastigotes of G2. (B) Intracellular amastigotes of *T. cruzi*. Scale bars = 10 µm.

grey kangaroos, quenda, tammar wallaby and chuditch; and a genotype closely related to *Trypanosoma* H25 in woylies, banded hare wallabies and boodies from Western Australia. Our data also demonstrate that marsupials belonging to several families in the same locality could be infected with the same trypanosome genotype. This lack of host specificity in Australian trypanosomes may have important consequences for the evolutionary and ecological interactions between trypanosomes and their marsupial hosts.

To infer phylogenetic relationships between the eight trypanosome genotypes found in this study and previously described species, we sequenced both 18S rDNA and *gGAPDH* genes. The use of partial 18S rDNA sequences alone is considered inadequate for inferring deep levels of phylogenetic relationships and additional gene sequences are needed to help resolve polytomies in the Trypanosomatidae (Hamilton et al., 2004, Hamilton and Stevens, 2011; Lymbery et al., 2011). Phylogenetic trees inferred in this study using both loci yielded almost identical topologies, and the general branching pattern was in agreement with those shown in previous analyses based on single and combined 18S rDNA and *gGAPDH* sequences (McInnes et al., 2009; Paparini et al., 2011;

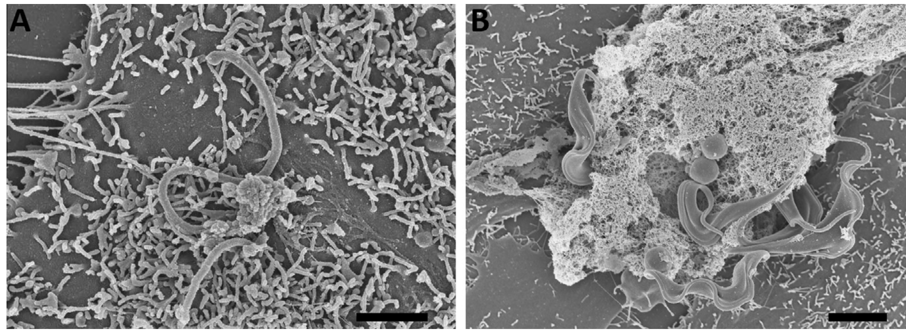

Fig. 10. Scanning electron micrograph of G2 (Clade A) grown in culture with Vero cells. (A) Trypomastigote invading a cell, with the flagella still external to the cell. (B) Dead cell(s) surrounded by amastigotes and trypomastigotes. Scale bars = 2 μm (A), 4 μm (B).

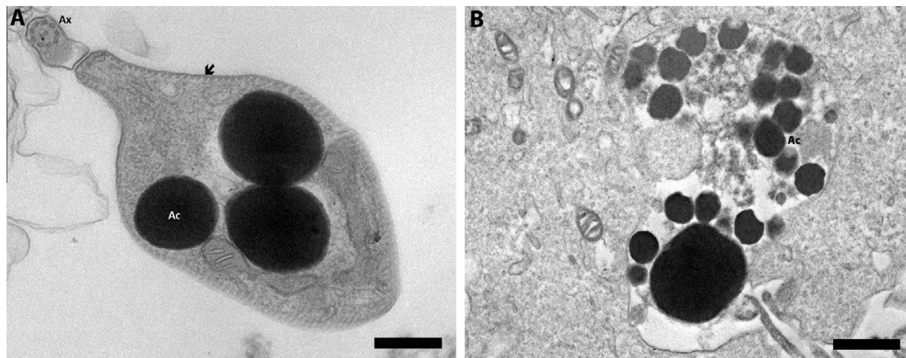

Fig. 11. Transmission electron micrograph of an epimastigote and an amastigote of G2 (Clade A). (A) Epimastigote in culture; Ax: Axoneme showing nine doublets of microtubules surrounding a central pair; Ac: Acidocalcisomes; Arrow: Subpellicular microtubules. (B) Amastigote inside a VERO cell. Scale bars = 0.5 μm (A), 1 μm (B).

Hamilton et al., 2012). Our analysis showed two of the genotypes firmly clustered in a monophyletic assemblage with *T. copemani*. A third genotype was almost identical to *Trypanosoma* H25, previously described in an Australian kangaroo, within a clade containing *T. cruzi*, *Trypanosoma marinkellei* and *T. rangeli* – all trypanosomes from South America (Stevens et al., 1999). The relationship supports the hypothesis that this clade may have evolved in ancestral marsupials before the separation of South America, Antarctica and Australia (Hamilton et al., 2009). The five remaining genotypes clustered together in a clade that was most closely related to *T. gilletti*; analysis of a shorter region of the 18S rDNA sequences, including previously described trypanosome sequences from a chuditch and woylies in Western Australia (Smith et al., 2009), placed these genotypes within this clade.

Using a similar nested PCR from the 18S rDNA locus as used in previous studies we found substantially higher levels of trypanosome infection: 67% prevalence in blood from 10 different species of marsupials. In contrast, Paparini et al. (2011) reported a prevalence of only 5% in blood from 11 species of Western Australian marsupials. Within woylies, we found a prevalence of 46% and 88% at Karakamia and the Upper Warren Region respectively, compared to previous detection rates of 14% and 35% for the same areas (Thompson et al., 2008). These differences may be partly attributable to the sensitivity of the PCR used and to the temporal fluctuation of trypanosomes in blood during the natural course of infection. Most of the trapped and released animals screened in the present study may have been in the active acute phase of infection where animals present high parasitemias easily detectable by PCR or microscopy. Previous studies have shown that in the early acute stage of *T. cruzi* infection, diagnosis is straightforward due to high levels of parasitemia but in the chronic stages, low parasitemia often precludes detection in fresh blood (Russomando et al., 1992; Vago et al., 2000; Lane et al., 2003; Campos et al., 2010). Another possible explanation for differences in prevalence may be the demonstrated capacity of Australian trypanosomes to migrate to different organs in the host; in some stages of infection parasites may be more frequently found in tissues than in peripheral blood, reducing the chances of detection by PCR. This could explain why in three woylie carcasses from which we could collect both blood and tissues, trypanosomes were detected by PCR in tissues but not in blood (data not shown). We also found a relatively high prevalence of mixed infections in woylies, but not in other host species (although sample sizes for other hosts were much smaller than for woylies). However, due to the low number of samples examined from the other marsupial species, except blood samples from western grey kangaroos and quolls, we cannot completely exclude the possibility that mixed infections can also occur in other species.

The fact that genotype, clade or species-specific molecular tools were not used previously (Smith et al., 2009; Paparini et al., 2011) may have masked the presence of less abundant trypanosomes in mixed infections. Results of PCR and sequencing using the 18S rDNA generic primers compared with clade-specific primers showed that more abundant Clade A genotypes in mixed infections masked the presence of less abundant Clade B and C genotypes. These results confirm the importance of the use of specific primers for rapid identification of trypanosomes in naturally infected marsupials, especially since sequencing directly from amplicons alone may not be able to detect mixed infections.

Despite PCR results revealed mixed infection in some of the blood samples, only trypanosomes from Clade A were able to be grown in culture. It has been shown that during the isolation and amplification of *T. cruzi* in culture, a selection of genotypes may occur (Bosseno et al., 2000) and that may be the case here. However, cultures from blood samples of animals infected only with trypanosomes from Clade B were unsuccessful indicating that possibly the medias used may not fulfill the nutritional growth needs of Clade B genotypes.

Although all trypanosome genotypes were found in the blood, only some genotypes were seen to colonize tissues. This suggests

a genetic basis for the heterogeneity in the biological behavior of trypanosomes in their marsupial host. We also found, for the first time in trypanosomes infecting Australian marsupials, a differential tissue tropism of trypanosomes within Clades A and B. Several studies have demonstrated that the genetic variability of *T. cruzi* may determine the different histotropism observed during infections and consequently the diverse clinical forms of the disease (Andrade et al., 1999, 2002; Vago et al., 2000; Macedo et al., 2004; da Silva Manoel-Caetano et al., 2008; Ramírez et al., 2010). However, the influence of the host genetic background in this process has been demonstrated as well (Freitas et al., 2009; Andrade et al., 2002). Botero et al. (2007) found a differential tissue tropism of two genetically different clones of *T. cruzi* in experimentally infected mice and Mantilla et al. (2010) reported a mixed infection in a chagasic patient from Colombia, consisting of two genetically diverse *T. cruzi* populations, one of them infecting the oesophagus and the other infecting the heart.

It has been demonstrated that trypanosome multiplication in the vertebrate host occurs extracellularly in the bloodstream or intracellularly in tissues. *T. lewisi* division occurs extracellularly in visceral capillaries, while *T. cruzi* uses host cells to replicate intracellularly and escape from the immune system to continue its life cycle, resulting in the infection of many tissues and organs (De Souza et al., 2010). Our finding of DNA of some genotypes representative of all three trypanosome clades in different marsupial organs, plus the discovery of intracellular amastigotes in some tissues where G2 (Clade A) DNA was present, confirmed that at least this genotype is able to invade cells. It has been shown that intact amastigotes are not easy to find during a chronic *Trypanosoma* infection, possibly due to the damage to host tissues and destruction of intracellular parasites caused by a strong inflammatory process (Higuchi et al., 1993; Zhang and Tarleton, 1999). However, we demonstrated that this genotype was able to invade and replicate in cultured Vero cells, although at a slower rate when compared to *T. cruzi* (data not shown).

Woylies had a greater prevalence of trypanosome infections than any other marsupial species examined and were also the only species found with multiple infections of two or more parasite genotypes. Our study correlates, for the first time, the presence of Australian trypanosomes and pathological changes in woylie tissues, during single infections with parasites belonging to Clade A and also during mixed infections involving two or three trypanosome genotypes. The pattern of inflammatory reactions during these infections was very similar and showed frequent muscle degeneration and occasional necrosis. The pathological changes seen in woylie tissues are similar to those seen in *Didelphis marsupialis* infected with *T. cruzi* from South and North America: myocarditis characterized by inflammatory infiltrates in skeletal muscles and oesophagus (Teixeira et al., 2006). The fact that pathological changes were seen during single and mixed infections involving G2 (Clade A) may merely reflect the virulence of this genotype. However, pathological changes were also observed in mixed infections involving trypanosomes that clustered within Clade B and C (in the absence of Clade A genotypes), suggesting alternative scenarios where the woylie immune system may be less efficient at resolving multiple infections or when mixed infections have potentiated pathogenicity. This latter result is in agreement with a previous study that suggested *T. gilletti* might have the ability to potentiate pathogenicity during concomitant infections in koalas (McInnes et al., 2011b).

Natural mixed infections between different species or genotypes of trypanosomes occur frequently in nature, with both parasite and host factors determining the overall parasitemia, virulence and pathogenicity (Martins et al., 2006; Pinto et al., 2006; Pollitt et al., 2011). Although several studies have reported the intrinsic characteristics of single trypanosome infections, it has not been clearly demonstrated whether the interaction of different trypanosomes in a single host can affect or modify the infection dynamics by either reducing or enhancing parasitemia, virulence and pathogenicity (Reifenberg et al., 1997). Rodrigues et al. (2010) showed that the combination of two strains of *T. cruzi* was able to trigger both protective inflammatory immunity and regulatory immune mechanisms that attenuate damage caused by inflammation in experimentally infected mice. However, in natural infections, virulence is difficult to predict when interactions are not restricted to a single host and parasite, but involve multiple infections as in the case of woylies.

There was a marked difference in the composition of trypanosome infections between woylies in the stable and the declining population. The greater prevalence of genotypes belonging to Clade A and mixed infections in the declining population of woylies, together with the demonstrated potential of these trypanosomes to cause pathological changes in woylies, suggests that infections with trypanosomes within this clade could be important contributors to the dramatic decline of the woylie. Given that we only examined woylies from a single stable population and a single declining population, there is a clear need for further studies to test this hypothesis, although it is noteworthy that *T. copemani* (Clade A) has been reported in the blood of other critically endangered and vulnerable Australian marsupials including Gilbert's potoroos, and quokkas from Western Australia (Austen et al., 2009) and koalas from Queensland (McInnes et al., 2011b).

In summary, this study highlights a wide genetic diversity of trypanosomes within Australian wildlife and reveals for first time the capacity of Australian trypanosomes to infect cells and their pathogenic potential either in single or mixed infections, supporting previous hypotheses (Thompson et al., 2008) associating trypanosome infections with the decline of woylies.

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