Novel trypanosome *Trypanosoma gilletti* sp. (Euglenozoa: Trypanosomatidae) and the extension of the host range of *Trypanosoma copemani* to include the koala (*Phascolarctos cinereus*)

L. M. McINNES[1]*, J. HANGER[2], G. SIMMONS[3], S. A. REID[1] *and* U. M. RYAN[1]

[1] *Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Perth, WA 6150, Australia*
[2] *The Australian Wildlife Hospital, Beerwah, Qld 4519 and* [3] *School of Veterinary Science, University of Queensland, St Lucia, Qld 4072, Australia*

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SUMMARY

Trypanosoma irwini was previously described from koalas and we now report the finding of a second novel species, *T. gilletti*, as well as the extension of the host range of *Trypanosoma copemani* to include koalas. Phylogenetic analysis at the 18S rDNA and gGAPDH loci demonstrated that *T. gilletti* was genetically distinct with a genetic distance (±S.E.) at the 18S rDNA locus of 2·7±0·5% from *T. copemani* (wombat). At the gGAPDH locus, the genetic distance (±S.E.) of *T. gilletti* was 8·7±1·1% from *T. copemani* (wombat). *Trypanosoma gilletti* was detected using a nested trypanosome 18S rDNA PCR in 3/139 (~2%) blood samples and in 2/29 (~7%) spleen tissue samples from koalas whilst *T. irwini* was detected in 72/139 (~52%) blood samples and *T. copemani* in 4/139 (~3%) blood samples from koalas. In addition, naturally occurring mixed infections were noted in 2/139 (~1·5%) of the koalas tested.

Key words: *Trypanosoma* spp., *Trypanosoma gilletti*, *Trypanosoma copemani*, *Trypanosoma irwini*, koala, *Phascolarctos cinereus*, phylogeny, 18S rDNA, gGAPDH.

INTRODUCTION

Trypanosomes are flagellated blood parasites transmitted primarily by haematophagous arthropods and able to infect all classes of vertebrates. There are limited data on trypanosomes in Australian mammals, with only 6 species named and a number of novel trypanosome genotypes, most with limited genetic data with the exception of isolates from a wallaby and a kangaroo. The 6 trypanosome species identified in Australian mammals are *T. thylacis* from the short-nosed bandicoot (*Isoodon macrourus*) (Mackerras, 1959), *T. pteropi* from the black flying fox (*Pteropus gouldii*) (Mackerras, 1959), *T. hipposideri* from the dusky horse-shoe bat (*Hipposideros bicolor albanensis*) (Mackerras, 1959), *T. binneyi* from the platypus (*Ornithorhynchus anatinus*) (McMillan and Bancroft, 1974), *T. copemani* from the Gilbert's potoroo (*Potorous gilbertii*), the quokka (*Setonix brachyurus*) and common wombat (*Vombatus ursinus*) (Noyes *et al*. 1999; Austen *et al*. 2009) and *T. irwini* from the koala (*Phascolarctos cinereus*) (McInnes *et al*. 2009). In addition to these named species there are also good genetic data for 2 novel trypanosome genotypes reported from a wallaby (ABF) (Hamilton *et al*. 2004) and a kangaroo (H25) (Stevens *et al*. 1998; Hamilton *et al*. 2004) as well as some limited genetic data from a range of other native Australian mammals.

The koala has a fragmented distribution in the eastern states of Australia ranging from north-east Queensland (Qld) to the Eyre Peninsula in South Australia (S.A.). European settlement had a dramatic effect on the range and population numbers of koalas. Factors which have affected koala abundance include land clearance for agriculture and urban development, hunting for the fur industry (historical event), spread of infectious diseases (notably chlamydiosis), increased incidents of motor vehicle strike and dog attacks in combination with natural phenomena such as wildfires and drought. As a result, the regional conservation status of the koala varies from secure in some areas to vulnerable or extinct in others. The koala is currently classified as vulnerable to extinction in New South Wales (N.S.W.) under the N.S.W. Threatened Species Conservation Act 1995 No. 101, as Schedule 2 'Vulnerable species' and in parts of Qld under the Qld Nature Conservation

* Corresponding author: Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Perth, WA 6150, Australia. Tel: +61 893602495. Fax: +61 893104144. E-mail: LindaMMcInnes@gmail.com

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(Wildlife) Regulation 2006 as Schedule 3 'Vulnerable wildlife'.

The present study describes the genetic characterization of a novel trypanosome in koalas as well as the host range extension of *T. copemani* to include the koala. We consider the new trypanosome described in koalas to be a novel trypanosome species and propose the name *Trypanosoma gilletti* sp. n.

MATERIALS AND METHODS

Sources of isolates and sample collection

A total of 139 blood samples (99 from Qld and 40 from N.S.W. koalas) were collected as part of routine procedures from koalas that were presented to the Australian Wildlife Hospital at Beerwah, Qld during 2006–2009. More than 600 koalas per annum are presented to the hospital from south-east Qld and northern N.S.W. In addition to the blood samples, 29 spleen tissue samples were collected from koalas presented to the Moggil Koala Hospital, operated by the Queensland National Parks and Wildlife during 2006–2007. Koalas were either dead at the time of presentation or euthanased on humane grounds. These koalas came from south-east Qld with most originating from the greater Brisbane area. Details of the geographical origin (state and shire; local government defined area) of each of the koalas as well as sex, age and reason for admission were recorded.

For clinical examination and blood collection, koalas were anaesthetized with an intramuscular administration of alfaxalone (Alfaxan® CD RTU, Jurox Australia) at an approximate dose rate of 3 mg/kg. Anaesthesia was maintained with a combination of oxygen and isoflurane delivered by either mask or endotracheal intubation. Blood samples of approximately 0·5–1 ml were collected by venepuncture of the cephalic vein. The blood was mixed with EDTA in a Vacutainer® tube (Becton-Dickinson, NJ, USA) and stored at −20 °C. All veterinary procedures were performed by registered wildlife veterinarians in accordance with standard veterinary practice.

Morphological measurements

Thin-blood smears were stained with Wright-Giemsa stain (Hematek® Stain Pak) using a Hema-Tek Slide Stainer (Ames Company Division, Miles Laboratories Pty Ltd, Springvale Victoria, Australia). The slides were then air-dried and a cover-slip mounted using DePeX mounting medium Gurr (Merck Pty. Limited, Kilsyth, Victoria, Australia).

Digital light micrograph images of any trypomastigotes observed in blood films were taken at ×1000 magnification. Morphological measurements (total length, breadth, kinetoplast to anterior (KA), kinetoplast to nucleus (KN), posterior to kinetoplast (PK) and free flagellum (FF)) of the trypomastigotes were made using Image-Pro Express software (Media Cybernetics, Inc., Bethesda, MD, USA) and the means and standard errors calculated. Morphological features were measured and compared to the published *T. copemani* from the quokka and Gilbert's potoroo (Austen *et al*. 2009) and *T. irwini* from the koala (McInnes *et al*. 2009). The statistical significance of any differences was tested using a one-way analysis of variance (ANOVA) and the Tukey's Honestly Significant Difference test at a 95% confidence limit using the software Statistics Package for Social Sciences (SPSS Inc, Chicago, IL, USA). The appropriateness of using the ANOVA was assessed by applying Levene's test for the homogeneity of variances. Measurements which demonstrated differences in variances were analysed with Tamhane's P2 test and Dunnett's T3 test in SPSS.

In vitro *culturing*

Attempts were made to isolate trypanosomes from whole blood samples of 17 koalas into *in vitro* cultures. Twenty microlitres of blood were placed into 2 ml Cryo.s™ cryopreservation vials (Greiner Bio-One GmbH, Solingen, Germany) containing 1 ml of Modified Sloppy Evans Medium (MSEM) (Noyes *et al*. 1999). Inoculated vials were maintained in the dark at room temperature for 10–14 days. Microscopic examination of wet-smear preparations from the medium was performed for the detection of motile trypanosomes at 200× and 400× magnification once a week, for 3 weeks, after the first 10–14 day incubation.

DNA extraction

Whole genomic DNA was extracted from koala blood using a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, WI, USA). DNA was eluted in 50 μl of water and stored at −20 °C until use. A DNA extraction blank (with no blood added) was included with each batch of DNA extractions. DNA from spleen tissue was extracted using a QIAGEN DNeasy Blood & Tissue Kit (QIAGEN Pty Ltd, Doncaster Victoria, Australia) and stored at −20 °C until use.

18S rDNA amplification and sequencing

DNA was screened for the presence of trypanosomatid 18S rDNA as previously described by McInnes *et al*. (2009) with the correction of primer S823 (cgaacaactgccctatcagc) sourced from Maslov *et al*. (1996) rather than S825 being used in conjunction with primer S662 as incorrectly stated. The approximate 1500 bp fragment of 18S rDNA generated includes variable regions V3, V4, V6 and the V7–V8 variable region used by Da Silva *et al*. (2004) to distinguish trypanosomes. All controls

(negative and positive PCR controls and DNA extraction blanks) produced appropriate PCR results. All positive PCR products were purified using a MO BIO UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories Inc. West Carlsbad, CA, USA) and sequenced using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystem 3730 DNA Analyzer. All sequences that produced mixed chromatograms indicating mixed DNA sequence templates were cloned using a pGEM®-T Easy Vector System II (Promega Corporation, Madison, WI, USA) and at least 10 clones picked and DNA sequenced.

gGAPDH amplification and sequencing

DNA samples positive by 18S rDNA PCR were screened with glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) primers as previously described in McInnes *et al*. (2009). Trypanosome phylogenetic analysis now routinely incorporates analysis of the gGAPDH gene, a tool pioneered by Hamilton *et al*. (2004), as the utility of 18S rDNA for deep level phylogeny has been questioned (Hamilton *et al*. 2004). All negative and positive PCR controls produced appropriate PCR results. DNA sequencing was conducted as described above.

Phylogenetic analysis

Nucleotide sequences generated for the 18S rDNA (1582 bp) and gGAPDH (810 bp) loci of 1 *T. gilletti* and 4 *T. copemani* (koala) isolates were aligned with sequences from a number of related trypanosomatids from GenBank and their phylogeny inferred using Maximum Likelihood analysis by the program PhyML (Dereeper *et al*. 2008) using the GTR (+I+G) model of evolution (Lanave *et al*. 1984). The reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006), a statistical test of branch support and an alternative to non-parametric bootstrap branch support estimation. The appropriate model of nucleotide substitution (GTR+I+G) for ML analysis was chosen using the Akaike information criterion (Akaike, 1974) in the software program jModeltest 0.1.1 (Posada, 2008).

Parsimony analysis and measurement of genetic distance using the Jukes-Cantor model (±standard error) (Jukes and Cantor, 1969) were conducted using Mega version 4 (Mega4: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, AR, USA) (Tamura *et al*. 2007). All codon positions were included and all positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Bootstrap analysis was conducted using 1000 replicates to assess the reliability of inferred tree topologies. The Jukes-Cantor model was chosen following the guidelines of Nei and Kumar (2000).

The relationship between *T. gilletti* and the 4 *T. copemani* (koala) isolates, a chuditch (*Dasyurus geoffroii*) (CHA1) and a number of woylie (*Bettongia penicillata*) derived trypanosome isolates (*T.* sp. TRY1, TRY2, WYA1 and WYA2) (Smith *et al*. 2008; Averis *et al*. 2009) were also analysed. The analysis was only based on a short region (~480 bp) of 18S rDNA sequence because only small 18S rDNA fragments are presently available from GenBank for the chuditch and woylie derived trypanosome isolates. An analysis with a 1019 bp sequence of 18S rDNA was also performed to investigate the relationship of the *T. copemani* (koala) isolates and *T. copemani* genotypes A and B from the quokka and Gilbert's potoroo (Austen *et al*. 2009) because longer (1550 bp) sequences were not available for the *T. copemani* genotypes A and B.

The GenBank nucleotide sequences used in the 18S rDNA and gGAPDH phylogenetic analysis are presented in Table 1.

RESULTS

Trypanosoma 18S rDNA PCR screen

Screening for the presence of trypanosome DNA using an 18S rDNA PCR detected the presence of a trypanosomatid in 78/139 (56·1%) blood samples and 2/29 (6·9%) koala spleen samples tested (Table 2). Single trypanosome infections were determined in 76/139 blood samples; 71/139 (51·1%) were *T. irwini*, 2/139 (1·4%) were *T. gilletti* (Lanie and Timbo) and 3/139 (2·2%) were *T. copemani* (Harrison, Cameron and Mika). Dual infections were determined in 2/139 (1·4%) blood samples; one was a mixed infection of *T. irwini*/*T. copemani* (Charlton) and another was *T. irwini*/*T. gilletti* (Barbie). The 2 positive spleen DNA samples (K27 and K28) were determined to be single infections of *T. gilletti*. There were no koalas exhibiting co-infection of *T. copemani* and *T. gilletti*.

18S rDNA sequencing

ClustalW alignment (http://align.genome.jp/) of the partial 18S rDNA sequences revealed the 5 isolates of *T. gilletti* (K27, K28, Timbo, Lanie and Barbie) to be genetically distinct from other known trypanosome species and 100% identical to each other. A DNA sequence for the 18S rDNA locus of *T. gilletti* (Lanie) (1595 bp) was deposited in the GenBank database under Accession number GU966589.

A single 18S rDNA sequence for the 4 *T. copemani* (koala) isolates was deposited under Accession number GU966588 (isolate Charlton) as the sequences generated for the 4 isolates (Cameron, Charlton, Harrison and Mika) were 100% identical.

Table 1. The GenBank Accession numbers of the sequences used in the phylogenetic analysis

18S rDNA sequences	gGAPDH sequences
T. gilletti sp. n. (Lanie) GU966589	*T. gilletti* sp. n. (Lanie) GU966587
T. copemani (koala Cameron, Harrison, Mika, Charlton) GU966588	*T. copemani* (koala Mika) GU966585
T. copemani (wombat AAP) AJ620558	*T. copemani* (koala Cameron, Harrison) GU966586
T. irwini FJ649479	*T. copemani* (koala Charlton) GU966584
T. pestanai (badger LEM110) AJ009159	*T. copemani* (wombat AAP) AJ620277
T. sp. (tick KG1) AB281091	*T. irwini* FJ649485
T. brucei (TREU927) NC 005063	*T. pestanai* (badger LEM110) AJ620275
T. b. rhodesiense (UTRO2509) AJ009142	*T.* sp. (tick KG1) FJ649492
T. evansi (Tansui-Taiwan) D89527	*T. brucei* (TREU927) XM_ 840454
T. sp. (Msubugwe-2006 tsetse fly) AM503350	*T. b. rhodesiense* AJ620284
T. simiae (KEN2) AJ009162	*T. evansi* AF053743
T. congolense Savannah (WG81) AJ009146	*T.* sp. (Msubugwe-2006 tsetse fly) AM503075
T. vivax U22316	*T. simiae* (KEN2) AJ620293
T. avium (A1073) FJ649480	*T. congolense* Savannah (GAM2) AJ620290
T. avium (Chaffinch) AJ009140	*T. vivax* AF053744
T. avium (sp30) FJ649482	*T. avium* (A1073) FJ649488
T. corvi (ITMAP 180795) AY461665	*T. avium* (Chaffinch) AJ620263
T. sp. (currawong AAT) AJ620557	*T. avium* (sp30) FJ649490
T. sp. (python) AB447493	*T. corvi* (ITMAP 180795) FJ649496
T. varani (V54) AJ005279	*T.* sp. (currawong AAT) AJ620264
T. sp. (gecko) AJ620548	*T.* sp. (python) AB362559
T. bennetti (KT-2)AJ223562	*T. varani* (V54) AJ620261
T. sp. (wallaby ABF) AJ620564	*T.* sp. (gecko) AJ620259
T. cyclops AJ131958	*T. bennetti* (KT-2) FJ649486
T. theileri (K127) AJ009164	*T.* sp. (wallaby ABF) AJ620278
T. conorhini (USP) AJ012411	*T. cyclops* FJ649493
T. vespertilionis (P14) AJ009166	*T. theileri* (K127) AJ620282
T. rangeli (RGBBasel) AJ009160	*T. conorhini* (USP) AJ620267
T. sp. (H25 Kangaroo) AJ009168	*T. vespertilionis* (P14) AJ620283
T. cruzi (VINCH89) AJ009149	*T. rangeli* AF053742
T. cruzi marinkellei (B3) FJ649484	*T.* sp. (H25 Kangaroo) AJ620276
T. dionisii (P3) AJ009151	*T. cruzi* (VINCH89) AJ620269
T. microti (TRL132) AJ009158	*T. cruzi* marinkellei (B3) FJ649495
T. lewisi (ATCC 30085) AJ223566	*T. dionisii* (P3) FJ649494
T. grayi (BAN1) AJ620546	*T. microti* (TRL132) AJ620273
T. granulosum (Portugal) AJ620552	*T. lewisi* (L32) AJ620272
T. binneyi (AAW) AJ620565	*T. grayi* (BAN1) AJ620258
T. boissoni (ITMAP 2211) U39580	*T. granulosum* (Portugal) AJ620247
T. mega (ATCC 30038) AJ009157	*T. binneyi* (AAW) AJ620266
T. rotatorium (B2-II) AJ009161	*T. boissoni* (ITMAP 2211) AJ620245
Herpetomonas muscarum L18872	*T. mega* (ATCC 30038) AJ620253
Phytomonas serpens U39577	*T. rotatorium* (B2-II) AJ620256
Leptomonas collosoma AF153038	*Herpetomonas muscarum* DQ092548
Crithidia oncopelti L29264	*Phytomonas serpens* EU084892
Blastocrithidia culicis L29266	*Leptomonas collosoma* EU084898
Crithidia fasciculata Y00055	*Crithidia oncopelti* (ATCC 12982) EU079134
Wallaceina inconstans AF153044	*Blastocrithidia culicis* (ATCC 30268) EU079137
Leptomonas seymouri AF153040	*Crithidia fasciculata* AF047493
T. sp. (TRY1 woylie) EU518939	*Wallaceina inconstans* (ZK) EU076608
T. sp. (TRY2 woylie) EU518940	*Leptomonas seymouri* AF047495
T. sp. (WYA1 woylie) FJ823116	
T. sp. (WYA2 woylie) FJ823121	
T. sp. (CHA1 chuditch) FJ823120	
T. copemani (quokka Q3, genotype A) EU571232	
T. copemani (quokka Q10, genotype B) EU571234	
T. copemani (Gilbert's potoroo P94, genotype A) EU571231	
T. copemani (Gilbert's potoroo P63, genotype B) EU571233	

gGAPDH gene sequencing

Partial fragments of the gGAPDH gene were amplified from 2 of the *T. gilletti*-infected koalas, Lanie (895 bp) and Timbo (683 bp). The overlapping region (683 bp) of the gGAPDH sequences generated from the two koalas was 100% identical and therefore a single sequence from Lanie (895 bp) was submitted to GenBank under Accession number GU966587. Three *T. copemani* gGAPDH genotypes

Table 2. Details of the koalas from which spleen or blood samples tested positive by 18S rDNA PCR and DNA sequencing for *Trypanosoma copemani* and/or *Trypanosoma gilletti*

Sample ID	DNA extract source	Location	Sex/Age	Reason for hospital admission	18S DNA sequencing
K27	Spleen	Redlands Shire, Qld	M adult	Cystitis	*T. gilletti* sp. n.
K28	Spleen	Redlands Shire, Qld	F sub adult	Dog attack	*T. gilletti* sp. n.
Charlton	Blood	Caboolture Shire, Qld	M adult	Hit by car – released	*T. copemani*/*T. irwini*
Timbo	Blood	Redland Shire, Qld	M sub adult	Cystitis	*T. gilletti* sp. n.
Lanie	Blood	Gold Coast, Qld	F sub adult	Orphan	*T. gilletti* sp. n.
Mika	Blood	Lismore, N.S.W.	M sub adult	Orphan	*T. copemani*
Harrrison	Blood	Gympie Shire, Qld	M sub adult	Orphan	*T. copemani*
Cameron	Blood	Gympie Shire, Qld	M sub adult	Hit by car	*T. copemani*
Barbie	Blood	Logan City Council, Qld	F Adult	Hit by car – euthanased	*T. irwini*/ *T. gilletti* sp. n.

were successfully amplified from 4 koalas (Cameron, Charlton, Harrison and Mika). DNA sequences for the gGAPDH locus from koalas Mika and Charlton and the identical genotype determined from Harrison and Cameron were submitted to GenBank under Accession numbers GU966585, GU966584 and GU966586 respectively.

Phylogenetic analysis

A concatenated ML tree of 18S rDNA (1582 bp) and gGAPDH (810 bp) sequences revealed that *T. gilletti* was genetically distinct and grouped with the *T. copemani* clade which consisted of *T. copemani* isolates from quokkas and potoroos, *T. pestanai* isolated from badgers and an isolate (KG1) from a tick (Fig. 1). Maximum likelihood and parsimony analysis produced similar tree topology to the ML analysis and bootstrap values for these analyses were added to ML tree (Fig. 1). Analysis of 18S rDNA and gGAPDH loci individually revealed the same clade associations as the concatenated tree and these trees are therefore not included.

At the 18S rDNA locus, *T. gilletti* had a 2·7 ± 0·5% (2·9 ± 0·8% over V7–V8 region) and 4·0 ± 0·6% (3·3 ± 0·8% over V7–V8 region) genetic distance ± standard error (S.E.) from *T. copemani* (wombat AAP, H26) and *T. pestanai* respectively. At the gGAPDH gene the percentage genetic distance (± S.E.) between *T. gilletti* and *T. copemani* (wombat AAP) and between *T. gilletti* and *T. pestanai* was 8·6 ± 1·1% and 18·4 ± 1·6% respectively. The 18S rDNA genetic distance matrix for *T. gilletti*, *T. copemani* isolates and related trypanosomes are presented in Table 3A and B, depending on length of sequence available for analysis.

The phylogenetic relationship of *T. gilletti* and some Australian marsupial trypanosome isolates for which only short (~480 bp) 18S rDNA sequences were available is presented in Fig. 1a. The genetic distance (± S.E.) between *T. gilletti* and trypanosomes identified in the woylie (*T.* sp. TRY1, TRY2, WYA1 and WYA2) and chuditch (CHA1) over a shorter 18S rDNA sequence (~480 bp) was 3–4·6 ± 1·3% (Table 3B).

Novel species description

Trypanosoma gilletti sp. n.

Diagnosis: Phylogenetic analysis of partial fragments of the 18S rDNA and gGAPDH gene of the trypanosome.

Taxonomic summary

Vertebrate host: Koala (*Phascolarctos cinereus*)
Invertebrate host: Unknown
Type location: Redland Shire, Qld, Australia
Additional locations: Gold Coast, Qld, Australia
Site of infection: Blood and spleen tissue.
Pre-patent and patent periods: Unknown
Etymology: This species is named *Trypanosoma gilletti* after wildlife veterinarian Dr Amber Gillett who has contributed significantly to our knowledge of trypanosome infections in koalas.

Trypomastigote morphology

No trypomastigotes were observed in the blood smears of the koalas infected solely with *T. gilletti*. A koala (Barbie), determined using molecular analysis to have a mixed infection of *T. irwini* and the novel trypanosome *T. gilletti*, appeared to have 2 distinct trypomastigote morpho-types; one of which corresponded to *T. irwini* (Fig. 2b) and one which was unique (Fig. 2a). Trypomastigotes of *T. copemani* were noted in blood smears from 2 koalas (Harrison and Cameron). Measurements of morphological parameters (length, breadth, PK, NK, NA and FF) of 13 trypomastigotes from the koala Barbie and 25 trypomastigotes observed from a young male *T. copemani*-positive koala (Harrison) are listed in Table 4. Images of a selection of these measured

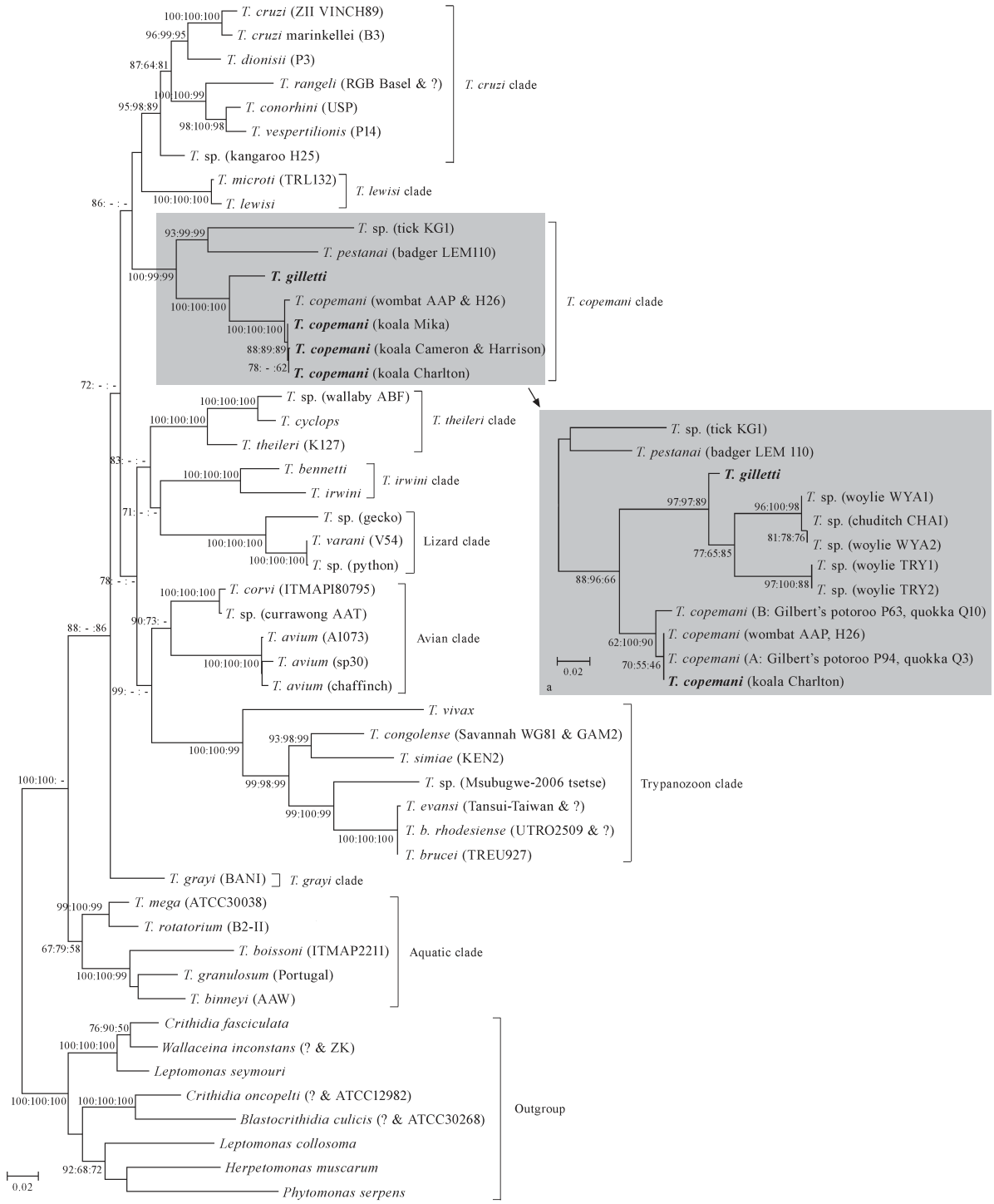

Fig. 1. Phylogenetic analysis of the relationships of *Trypanosoma* spp. and novel trypanosomatid *Trypanosoma gilletti* isolates from the koala based on composite gGAPDH and 18S rDNA partial sequences (~810 bp and ~1582 bp respectively) using Maximum Likelihood analysis performed by PhyML 3.0. Branch support/bootstrap values for PhyML, Maximum Parsimony and Distance analyses respectively are indicated at the left of each node. (a) Phylogenetic position of *T. copemani* isolates (Q10, Q3, P94 and P63), *T.* sp. TRY1, TRY2, WYA1, WYA2 and CHA1 (~480 bp 18S rDNA only). Scale bars represent substitutions/site.

trypomastigotes are presented in Fig. 3a and b. Morphological measurements of *T. copemani* trypomastigotes from koalas were significantly different ($P<0{\cdot}05$) to all the morphological features measured from *T. copemani* trypomastigotes from the Gilbert's potoroo and to PK and FF measurements from the quokka (Table 4). The morphological measurements of 12 trypomastigotes with similar morphology from the koala Barbie, when compared with *T. irwini* from the koala, were not significantly different ($P<0{\cdot}05$) in length, breadth, KN, NA or FF measurements. The morphological measurements of the unique

Table 3. Matrix of Jukes-Cantor percentage genetic distances (±standard error) of partial 18S rRNA gene sequence of various *Trypanosoma* spp. most closely related to the novel trypanosome *Trypanosoma gilletti*

A. Analysis based on 1019 bp of *T. gilletti* 18S rDNA with 682 informative positions.

	T. gilletti	*T. copemani* (koala)	*T. copemani* (A)	*T. copemani* (B)	*T. copemani* (wombat)
T. copemani (koala)	2·8±0·7				
T. copemani (genotype A, isolates Q3 & P63)	2·8±0·7	0·6±0·3			
T. copemani (genotype B, isolates Q10 & P94)	3·3±0·7	1±0·4	0·4±0·2		
T. copemani (wombat AAP, AAI & H26)	2·8±0·7	0·6±0·3	0·0±0·0	0·4±0·2	
T. irwini	6·1±0·9	5·8±0·9	5·8±0·9	5·9±0·9	5·8±0·9

B. Analysis based on ~482 bp of *T. gilletti* 18S rDNA with 271 informative positions.

	T. gilletti	*T. copemani* (koala)	*T. copemani* (A)	*T. copemani* (B)	*T. copemani* (wombat)	*T. irwini*	*T.* sp. (TRY1)	*T.* sp. (TRY2)	*T.* sp. (WYA1)	*T.* sp. (WYA2)
T. copemani (koala)	4·6±1·3									
T. copemani (genotype A)	4·6±1·3	0·0±0·0								
T. copemani (genotype B)	5·4±1·4	0·7±0·5	0·7±0·5							
T. copemani (wombat AAP, AAI & H26)	4·6±1·3	0·0±0·0	0·0±0·0	0·7±0·5						
T. irwini	8·6±1·8	9·0±1·8	9·0±1·8	9·0±1·8	9·0±1·8					
T. sp. (TRY1)	3·0±1·0	5·4±1·4	5·4±1·4	5·4±1·4	5·4±1·4	8·2±1·7				
T. sp. (TRY2)	3·4±1·1	5·7±1·4	5·7±1·4	5·7±1·4	5·7±1·4	8·6±1·8	0·7±0·5			
T. sp. (WYA1)	4·2±1·2	6·6±1·5	6·6±1·5	6·6±1·5	6·6±1·5	9·0±1·8	2·2±0·9	2·6±1·0		
T. sp. (WYA2)	4·6±1·3	7·0±1·6	7·0±1·6	7·0±1·6	7·0±1·6	9·4±1·9	2·6±1·0	3·0±1·0	0·4±0·4	
T. sp. (CHA1)	4·6±1·3	7·0±1·6	7·0±1·6	7·0±1·6	7·0±1·6	9·4±1·9	2·6±1·0	3·0±1·0	0·4±0·4	0·0±0·0

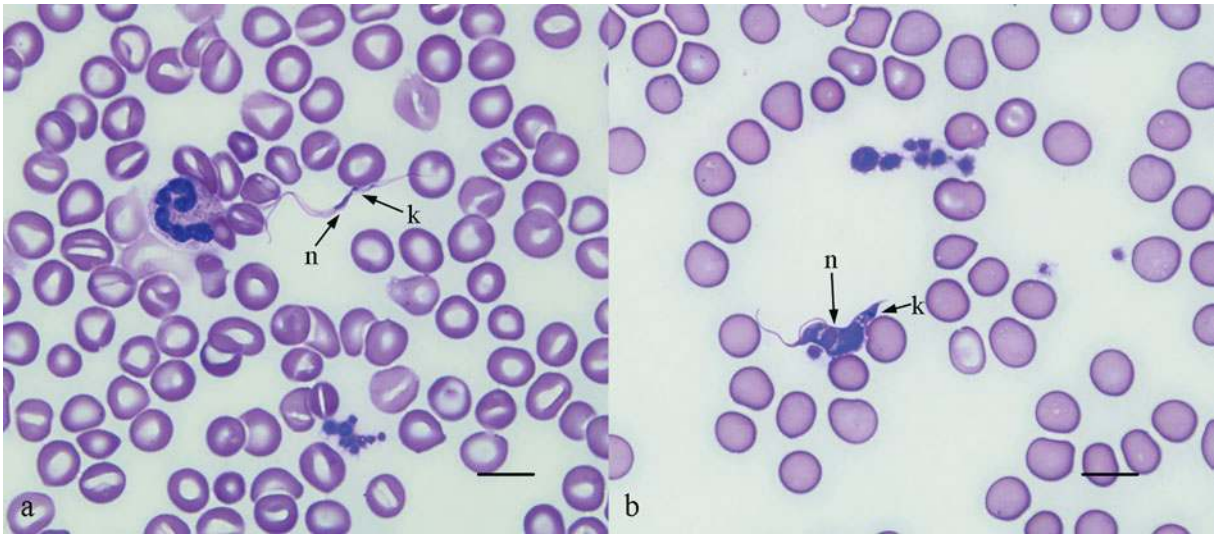

Fig. 2. (a and b) Trypomastigotes observed in thin blood smears from a koala (Barbie) stained with Wright-Giemsa. The trypomastigote nucleus stained pink (n) and the dark stained sphere at the distal end, the kinetoplast (k) are labelled in both figures. Scale bar represents 10 μm.

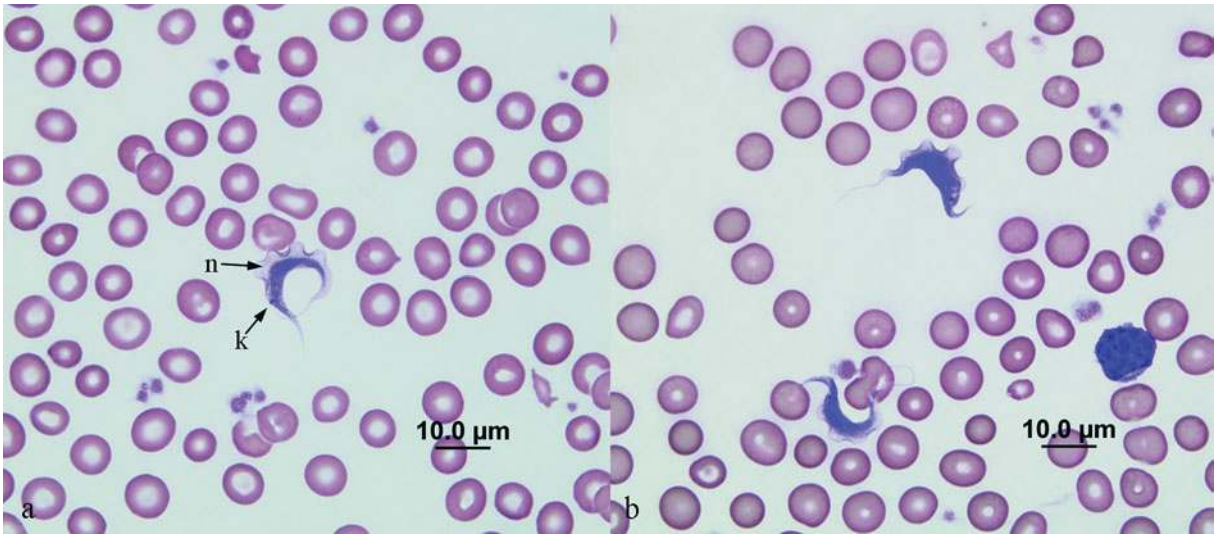

Fig. 3. (a and b) Trypomastigotes of *Trypanosoma copemani* in thin blood smears from a koala (Harrison) stained with Wright-Giemsa. The trypomastigote nucleus stained pink (n) and the dark stained sphere at the distal end, the kinetoplast (k) are labelled in (a).

trypomastigote from Barbie were outside of the 99% confidence intervals of morphological measurements from *T. copemani* from quokka, Gilbert's potoroo and koalas and *T. irwini* with the exception of measured FF (Table 4).

In vitro *culturing attempts*

Of the 17 koalas selected for *in vitro* culturing attempts, 7 were determined to be trypanosome negative, 8 were infected with *T. irwini*, 1 with *T. gilletti* (koala isolate Timbo) and none were infected with *T. copemani*. Examination of wet smears and stained smears did not detect any trypanosomes in any of the inoculated media.

DISCUSSION

The present study provides the description of a second novel *Trypanosoma* sp. in koalas and the extension of the host range of another Australian marsupial trypanosome *T. copemani* to include koalas. The study shows that *T. gilletti* is present in lower levels in the koala population compared with *T. irwini*, the first trypanosome to be described from koalas. The results of the present study extend the analysis presented in the previous description of *T. irwini* (McInnes *et al*. 2009), which reported a prevalence of 38·2% for *T. irwini* in 68 koalas examined. Analysis of a larger number of koalas has identified a higher prevalence of *T. irwini*, perhaps augmented by seasonal variation of vectors and/or koala habits.

Table 4. Mean measurements, range and standard error (S.E.) of morphological features of trypomastigotes observed in the blood of 2 koalas (*Phascolarctos cinereus*) (koala isolates Harrison and Barbie)

	Harrison (*T. copemani* trypomastigotes, $n=25$)		Barbie (*T. irwini* similar trypomastigotes, $n=12$)		Barbie (unique trypomastigote $n=1$)
Feature	Size range (μm)	Mean size (μm) ± S.E.	Size range (μm)	Mean size (μm) ± S.E.	size (μm)
Total length	33·4–48·1	39·5 ± 0·7	27·2–37·59	35·0 ± 0·9	41·7
Breadth	2·8–4·9	3·7 ± 0·1	3·4–4·5	3·9 ± 0·1	1·2
PK	7·5–14·3	10·1 ± 0·3	2–4·9	3·9 ± 0·3	15·6
KN	3·5–6·7	5·1 ± 0·1	9·8–13·3	11·3 ± 0·4	3·5
NA	11·3–21·9	16·1 ± 0·6	7·4–11·0	8·7 ± 0·4	23·1
FF	5·8–12·8	9·0 ± 0·3	7·2–12·7	10·5 ± 0·5	11·2

(Total length: length of body measured along the mid-line including free flagellum (total length). Breadth: maximum breadth measured at the level of the nucleus (including undulating membrane). PK: distance between the posterior end and the kinetoplast. KN: distance between the kinetoplast and posterior edge of the nucleus. NA: distance between the anterior edge of the nucleus and the anterior end of the body. FF: length of the free flagellum.)

The new koala trypanosome, *T. gilletti*, identified in the present study, is phylogenetically distinct from *T. irwini* and most closely related to the Australian marsupial trypanosome *T. copemani,* which has been isolated from common wombats, quokkas and Gilbert's potoroos (Noyes *et al*. 1999; Hamilton *et al*. 2005; Austen *et al*. 2009). However, analysis of a shorter region of the 18S rDNA sequence, which included isolates not included in the concatenated analysis (due to the small size of the 18S rDNA sequence available (~480 bp) and the lack of corresponding gGAPDH sequences for these isolates) suggests that *T. gilletti* is most closely related to trypanosome isolates from a chuditch (CHA1) and a number of woylies (*T.* sp. TRY1, TRY2, WYA1 and WYA2) in Western Australia (WA). The most informative phylogenetic analysis for *T. gilletti* and *T. copemani* (koala) therefore only includes in the *T. copemani* clade data from *T. copemani* (wombat AAP), *T.* sp. (KG1) and *T. pestanai,* as these are the only *T. copemani* clade isolates with available gGAPDH sequences. The overall topology of the concatenated 18S rDNA and gGAPDH ML tree produced in order to determine the evolutionary position of *T. gilletti* amongst the trypanosomes was similar to equivalent trees previously presented (Hamilton *et al*. 2007; McInnes *et al*. 2009; Viola *et al*. 2009). The placement of *T. gilletti* in the *T. copemani* clade was supported by high aLRT branch support and bootstrap values (99–100%) in the 18S rDNA and gGAPDH analysis individually and in the concatenated analysis.

Trypanosoma copemani which was identified for the first time in 3 koalas appears to be a somewhat ubiquitous Australian trypanosome infecting a range of Australian marsupials from different parts of Australia. To date it has been identified in 3 wombats (*Vombatus ursinus*) originating from Launching Place, Victoria (Vic.) (H26) (Noyes *et al*. 1999), from unknown locations in eastern Australia (AAP and AAI) (Hamilton *et al*. 2004, 2005), and in the Gilbert's potoroo (*Potorous gilbertii*) and the quokka (*Setonix brachyurus*) from Two Peoples Bay, WA (Austen *et al*. 2009; Noyes *et al*. 1999). Though strict host specificity is the rule for some trypanosomes, species such as *T. evansi*, *T. cruzi* (Burleigh and Andrews, 1995) and *T. rangeli* (Anez, 1982) are known to have wide host ranges. *Trypanosoma copemani* has now been identified in 4 species of Australian marsupials from 3 suborders of the order Diprotodontia from diverse locations in Australia.

The observation that koalas from the same geographical areas were simultaneously infected with up to 2 trypanosome species is interesting because it may impact the outcome of infection in these individuals. Mixed infections were identified in this study in 2 koalas and it is possible with the application of species-specific genetic tools that the prevalence of mixed infections in koalas is in fact higher. It is important to consider the effect mixed infections may have in modulating trypanosome infections in koalas when making inferences about the likely clinical significance of any individual trypanosome species. Animals experimentally infected with a mixture of *T. cruzi* strains with different levels of pathogenicity have been reported to show significantly different patterns or parasitaemia and/or pathogenicity (Franco *et al*. 2003; Martins *et al*. 2006).

No definitive morphological measurements of *T. gilletti* were able to be made as no trypomastigotes were observed in the blood smears of the 2 koalas with *T. gilletti* singular infections. Although a trypanosome was observed in a wet smear from one of these koalas (Timbo), no trypomastigotes were found in fixed and stained thin-blood smears and consequently no morphological measurements could be made. The only possible *T. gilletti* morphology comes from the observation of a uniquely-shaped

trypomastigote in the koala Barbie with a mixed infection of *T. gilletti* and *T. irwini*. A blood smear from this koala contained 2 morphologically different trypomastigote forms. The predominant trypomastigote form was found to be indistinguishable from *T. irwini* trypomastigotes measured from the koala (McInnes *et al*. 2009). The other trypomastigote form observed was very different to bloodstream forms of both *T. irwini* from koalas and *T. copemani* from koalas, quokkas and Gilbert's potoroos and is most probably *T. gilletti*. Further analysis is required to confirm the identity of this slender trypomastigote.

Although the importance of trypomastigote morphology should not be discounted, the utility of delineating trypanosome species based on morphometrics alone is highly questionable as pleomorphism of bloodstream trypomatigotes of numerous *T.* spp. has been documented (Hoare, 1972; Ziccardi and Lourenco-de-Oliveira, 1999; Zintl *et al*. 2000; Lainson *et al*. 2008). Trypomastigote pleomorphism was noted in the present study when comparisons were made of morphometric measurements of *T. copemani* from koalas with those of *T. copemani* from Gilbert's potoroo and quokka. Austen *et al*. (2009) also observed pleomorphism in the *T. copemani* trypomastigotes from both the Gilbert's potoroo and the quokka. Comparisons of the trypomastigotes from the *T. copemani* wombat isolates (AAP, AAI and H26) with those of *T. copemani* (koala, quokka and Gilbert's potoroo) are not appropriate as only *in vitro*-cultured wombat trypanosome forms were measured, and *in vitro* culture of bloodstream trypomastigotes stimulates change to vector infective forms (Hoare, 1972). However, the unique trypomastigote from Barbie had a similar morphology to *in vitro* cultured slender *T. copemani* trypomastigotes isolated from quokkas, Gilbert's potoroo and wombats (Noyes *et al*. 1999).

The description of a new trypanosome species based on molecular data alone is not ideal. However, trypanosomes have few morphological features detectable using light microscopy which can adequately delimit species (Gibson, 2009). Several new species of protozoan parasites have been described using only molecular data because of the limitations of the respective morphological characteristics. For example, for the genera *Theileria* and *Babesia*, a genetic distance of 0·7% and 3·4% respectively at the18S rRNA locus is sufficient to be classified as a distinct species (Schnittger *et al*. 2003). Similarly, for *Cryptosporidium*, if the genetic distance at 2 unlinked loci is equal to or greater than currently accepted species, then this is strongly supportive of species status (Xiao *et al*. 2004). The complex life cycles and inherent pleomorphism of trypanosomes necessitate more reliance on genetic characterization. For example, the recently described trypanosome species *T. copemani* has been isolated from diverse hosts and exhibits significant pleomorphism which would, without genetic data, raise the prospect of it being erroneously classified as more than one species. In order to safely delimit species based on genetic information reliance on 18S rDNA data alone are inappropriate due to intraspecies variation at this locus occasionally exceeding interspecies variation in some trypanosome clades. It is proposed that significant portions of the 18S rDNA (inclusive of the V7–V8 region) and gGAPDH gene (> 65%) are used 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 intraspecies variation noted in this study). These parameters will exclude many closely related species from being described on genetic distance alone. Species description of such trypanosomes would require added information to validate their species status.

The phylogenetic relationships of *T. gilletti* and *T. copemani* may be further clarified once more Australian native mammal trypanosome species are genetically characterized at both 18S rDNA and gGAPDH loci. Specifically, more genetic data on the trypanosome genotypes (TRY1, TRY2, WYA1, WYA2 and CHA1) from the woylie (*Bettongia penicillata*) and chuditch (*Dasyurus geoffroii*) (Smith *et al*. 2008; Averis *et al*. 2009), which appear to associate with the *T. copemani* clade, is needed to confirm their placement and relationship to *T. copemani* and *T. gilletti*. In addition, genetic information would be valuable for *T. thylacis* from the short-nosed bandicoot (*Isoodon macrourus*) (Mackerras, 1959), the *T.* spp. from the southern brown bandicoots (*Isoodon obesulus*) (Bettiol *et al*. 1998), the eastern barred bandicoots (*Perameles gunnii*) (Bettiol *et al*. 1996) and the bat trypanosomes *T. pteropi* and *T. hipposideri* (Mackerras, 1959) in order to develop an increased understanding of the evolutionary and phylogenetic relationships of Australian native mammal trypanosomes.

The vector for *T. gilletti* 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 vectors of the closest relatives of *T. gilletti* (*T. copemani* and *T. pestanai*) are currently unknown. There is a range of ectoparasites known to parasitise koalas, including ticks (*Ixodes* spp. and *Haemophysalis* spp.) (Roberts, 1970), fleas (e.g. *Ctenocephalus felis*), mites (*Koalachirus perkinsi, Sarcoptes scabiei, Demodex* spp. and *Notoedres cati*) (Jackson *et al*. 2003), mosquitoes and flies. The identification of *T. copemani* in quokkas, Gilbert's potoroos and koalas together with the host specificity of the wombat flea would not support the suggestion of Noyes *et al*. (1999) that the wombat flea is the

putative vector of *T. copemani*. Ticks are a more likely vector for koala trypanosomes, as ticks have been shown to be naturally infected with trypanosomes by a number of studies (el Kady, 1998; Latif *et al*. 2004; Thekisoe *et al*. 2007), and koalas are commonly infested with ticks (Roberts, 1970). A trypanosome isolate (KG1) isolated from a naturally infected *Haemaphysalis hystricis* tick in Japan (Thekisoe *et al*. 2007), which is associated with the *T. copemani* clade, suggests that ticks may be good candidate vectors. There is, however, no single tick species common to all the *T. copemani* hosts identified thus far. It is possible that the *T. copemani* vector is not a single tick species common to all the hosts but a range of tick species.

In conclusion, the genetic analysis of 2 unlinked loci confirms that the novel trypanosome identified in this study is a new species of trypanosome of the koala; *Trypanosoma gilletti*. In addition the host range of *T. copemani* is extended by the confirmation of the occurrence of this trypanosome in the koala.

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