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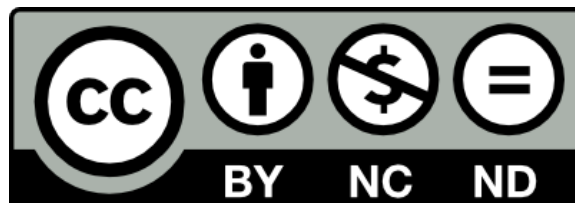
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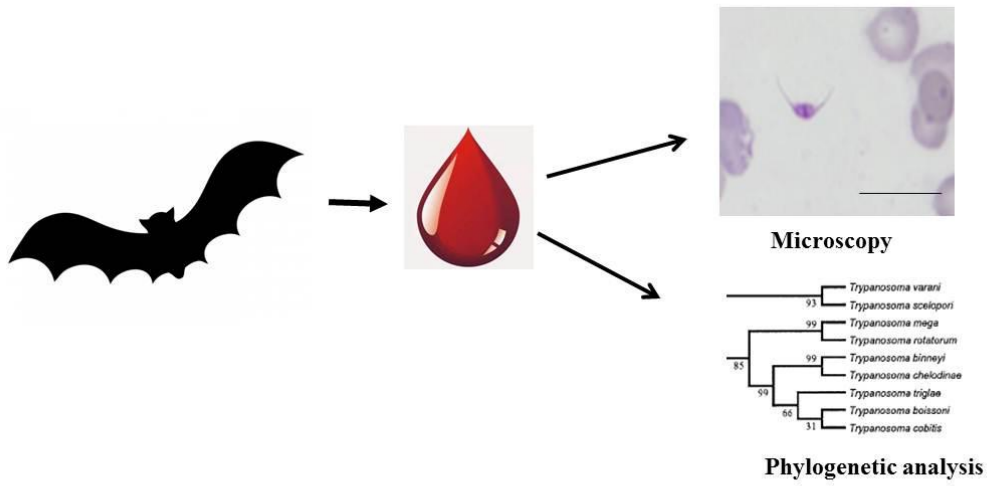
High prevalence of *Trypanosoma vegrandis* in bats from Western Australia

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Graphical Abstract

High prevalence of *Trypanosoma vegrandis* in bats from Western Australia

Highlights

- First report of *Trypanosoma vegrandis* in bats
- Morphological and molecular analysis
- High prevalence of *T. vegrandis* in bats
- Bats may play an important role in epidemiology

Abstract

The present study describes the first report of *Trypanosoma vegrandis* in bats using morphology and sequence analysis of the 18S rRNA gene. The PCR prevalence of *T. vegrandis* in bats was 81.8% (18/22). The high prevalence of *T. vegrandis* in the present study suggests that bats may play an important role in the epidemiology of *T. vegrandis* in Australia. *Trypanosoma vegrandis* appears to be geographically dispersed, has a wide distribution in Australia and low levels of host specificity.

Keywords: Trypanosome; *T. vegrandis*; bats; 18S rRNA

1.0 Introduction

Bats (Order Chiroptera) are divided into two suborders, Yangochiroptera and Yinpterochiroptera (Springer, 2013). They are considered reservoirs of multiple infectious diseases and zoonotic pathogens (Calisher et al 2006) and due to their broad distribution, social behavior and classification as the only mammals capable of sustained flight, have been implicated in the transmission of a range of microorganisms including rabies, severe acute respiratory syndrome (SARS), henipaviruses, ebola and pathogenic and non-pathogenic trypanosomes (Guyatt et al., 2003; Li et al., 2005; Halpin et al., 2000; Hamilton et al., 2012a; Hamilton et al., 2012b). Trypanosomes are blood-borne parasites, and while the majority of species appear to asymptomatically infect the host, *T. brucei rhodesiense* and *T. b. gambiense* are known to cause severe disease in humans and *T. cruzi* causes severe disease in both humans and animals, resulting in very large economic losses worldwide (Thompson et al., 2014). Bats are among the most common hosts of a large variety of trypanosomes in Africa, Asia, South America and Europe (Hoare, 1972; Baker, 1973; Marinkelle, 1976, Marinkelle, 1979; Gardner and Molyneux, 1988a; Gardner and Molyneux, 1988b; Molyneux, 1991; Hamanaka et al., 1993; Steindel et al., 1998; Grisard et al., 2003; Barnabe et al., 2003; Lisboa et al., 2008; Cottontail et al., 2009; Maia da Silva et al., 2009; Cavazzana et al., 2010; García et al., 2012; Hamilton et al., 2012a; 2012b; Lima et al., 2012; Pinto et al., 2012; Lima et al., 2013; Marcili et al., 2013; Silva-Iturriza et al., 2013; Cottontail et al., 2014; Ramírez et al., 2014). However, knowledge of the genetic diversity, hosts, vectors, life-cycles, pathology, distribution and phylogenetic relationships of trypanosome species infecting bats is restricted to a few species.

Three trypanosome species have been identified in Australian bats; *T. pteropi* from the black flying-fox (*Pteropus alecto*) (Breinl, 1913; Mackerras, 1959), *T. hipposideri* from the dusky horse-shoe bat (also known as the dusky leaf-nosed bat) (*Hipposideros ater*) (Mackerras, 1959) and more recently a novel trypanosome most similar genetically to *Trypanosoma rangeli* and *Trypanosoma minasense* was identified in an adult female little red flying-fox (*Pteropus scapulatus*) (Mackie et

al., 2015). In the present study, we screened Australian bats for trypanosomes using morphological and molecular methods.

2.0 Materials and methods

2.1 Samples

Both pteropid bats (Yangochiroptera) and microbats (Yinpterochiroptera) were screened (Table 1). Within the pteropids, black flying-fox (*Pteropus alecto*) (n=5) and little red flying-fox (*Pteropus scapulatus*) (n=7), blood and heart tissues were obtained from the Kimberley region of Western Australia. These bats were euthanased by local veterinary officers following reports of neurological disease, or interaction with members of the public resulting in bites or scratches. The carcasses were then necropsied in biological safety cabinets; the brain tissue sent to the CSIRO Australian Animal Health Laboratories (for Australian bat lyssavirus testing), and the remaining tissues stored at -80°C. Blood samples from microbats were sampled at the request of local wildlife carers as part of routine veterinary investigation. Two species were screened; Gould's wattled bats (*Chalinolobus gouldii*) (n=9) and a lesser long-eared bat (*Nyctophilus geoffroyi*) (n=1). All microbats were from the outer Perth metropolitan area and had been taken to carers as a result of habitat loss or suspected misadventure. Bats were anaesthetized with isoflurane and approximately 12 µL of blood was drawn from a blood vessel in the uropatagium (wing membrane vein) using a 26 gauge needle, before immediately being diluted 1:10 in phosphate buffered saline.

2.2 Microscopy

Bat blood samples were examined for the presence of trypanosomes using Wright-Giemsa stained (Hematek® Stain Pak) thin-blood smears. Slides were stained using a Hema-Tek Slide Stainer (Ames Company Division, Miles Laboratories Pty Ltd, Springvale Victoria, Australia). The slides were then air-dried and a coverslip mounted using DePeX mounting medium Gurr (Merck Pty. Limited, Kilsyth, Victoria, Australia). Digital light micrograph images of any trypanosomes

observed in blood films were taken at $\times 1000$ magnification. Morphological measurements (total length, breadth, nucleus to anterior, kinetoplast to nucleus, posterior to kinetoplast and free flagellum of the trypanosomes were made using Image-Pro Express software (Media Cybernetics, Inc., Bethesda, Maryland, U.S.A.) and the means and standard errors (\pm) were calculated. Morphological features were measured using Image J and compared with available morphological measurements for trypanosomes infecting Australian mammals.

2.3 DNA Extraction

Whole genomic DNA was extracted from bat blood using a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, Wisconsin, U.S.A.) and from bat tissue using a Qiagen DNeasy blood and tissue kit (Qiagen, Victoria, Australia). DNA was eluted in 50 μ l of water and stored at -20°C until use. A DNA extraction blank (with no blood or tissue added) was included with each batch of DNA extractions.

2.4 18S rRNA amplification and sequencing

Both generic *Trypanosoma* sp. primers and *T. vegrandis*-specific primers were used. For amplification of *T. vegrandis*, external forward primer S-823 and external reverse primer S-662 were used as previously described by McInnes et al. (2009) and Maslov et al. (1996) respectively. The PCR product from these external primers was run on a 1.5% agarose gel stained with SYBR safe (Invitrogen, USA). The gel band was excised and purified using a Promega Wizard SV Gel and PCR Clean-up System (Promega, Wisconsin, USA) and 1 μ l of gel-purified PCR was used in the secondary reaction. The secondary reaction consisted of the internal nested primers TRYall 1 Forward 5' ACCGTTTCGGCTTTTGTGG 3' and the *T. vegrandis* specific TVEG Reverse 5' AAATCGTCTTCGCTTTAACTTT 3' which amplify an ~468 bp fragment of the *T. vegrandis* 18S rRNA. PCR amplification with the *T. vegrandis*-specific primers was performed with the final mix containing 1 μ L of first round gel purified PCR product, 0.8 μ M of each primer, 0.02 U/ μ l Kapa

Taq (Kapa Biosystems, Massachusetts, USA), a final concentration of 2.5 mM of each dNTP and 2.5 μ L of 10 \times buffer (Kapa Biosystems, Massachusetts, USA) with 1.5 mM MgCl₂ (Kapa Biosystems, Massachusetts, USA) and Baxter Ultra-Pure H₂O to a volume of 25 μ L. Cycling conditions consisted of an initial PCR round of 94°C for 5 min, 50°C for 2 min and 72°C for 4 min followed by 45 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s. The PCR was completed with a final extension of 7 min at 72°C.

For amplification of *Trypanosoma* spp. external forward primer S-LF and external reverse primer S-762 was used in the primary PCR while two pairs of internal primers S-825 and S-LIR (producing a ~959 bp) and S-823 and S-662 (producing a ~ 900 bp) were used in secondary reactions as previously described by Maslov et al. (1996) and McInnes et al. (2009).

All controls (negative and positive PCR controls and DNA extraction blanks) produced appropriate PCR results. A representative sample of 13/18 *T. vegrandis*-specific PCR positive amplicons was gel purified using a Promega Wizard SV Gel and PCR Clean-up System (Promega, Wisconsin, USA) and submitted to the Australian Genome Research Facility (Ltd) for unidirectional sequencing using the *T. vegrandis*-specific internal reverse primer TVEG-Reverse.

2.5 Phylogenetic analysis

Nucleotide sequences generated for the 18S rRNA locus were imported into Geneious R7 and aligned with a number of closely related 18S trypanosome sequences from GenBank, with *T. irwini* (FJ649479) used as an outgroup (Table 2). Alignments based on 421bp, were obtained by MUSCLE (Edgar, 2004) using the default settings, and then curated by Gblocks (Castresana, 2000), remotely, choosing the low stringency options available on the Phylogeny.fr platform (Dereeper et al., 2008). The curated alignments were then exported in MEGA 6 (Tamura et al., 2013) for the selection of the most appropriate nucleotide substitution model (Tamura-Nei), using the dedicated function. The evolutionary history was inferred by distance (NJ) (in MEGA 6) and by maximum likelihood method implemented in the PhyML program (v3.0), on the Phylogeny.fr platform

(Dereeper et al., 2008). Specifically, the (Tamura-Nei) substitution model was selected assuming an estimated proportion of invariant sites and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data. Reliability for internal branch was assessed using the bootstrapping method (500 bootstrap replicates). Genetic distances were generated in MEGA 6 based on the Tamura-Nei algorithm.

2.6 Statistical analysis

Prevalences were expressed as the percentage of samples positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000).

3.0 Results

3.1 Microscopy

The morphology of *T. vegrandis* in blood films from the bats, represented short stumpy lanceolate to oval shaped trypanosomes, with absent undulating membranes (Fig. 1). The trypanosomes generally lacked a clearly defined kinetoplast and nucleated region and were observed as polymorphic (Fig. 1A-D). The posterior end was drawn out and tapered into a point while the anterior end was slightly rounded with either an absent or short free flagellum. A kinetoplast and nuclear region was identifiable in one trypanosome isolated from bat 15-4 (Fig. 1A). The kinetoplast in this isolate was round and situated close to the anterior nucleus. The morphological parameters for this isolate were 13.8 μm in total length, 6.7 μm posterior to kinetoplast, 1.0 μm in breadth, 0.7 μm for kinetoplast to nucleus, 0.8 μm for nucleus to anterior and the free flagellum measured 2.8 μm . In addition, morphological measurements for total length and free flagellum were made from 13 and 10 trypanosomes respectively from bat isolate 15-4. The total length ranged between 6.7 - 13.8 μm with a mean of $9.0 \pm 0.54 \mu\text{m}$ while the free flagellum ranged between 1.6 - 3.7 μm with a mean of $2.6 \pm 0.2 \mu\text{m}$.

3.2 Prevalence of trypanosomes in bat populations

The overall PCR prevalence of trypanosomes in bats was 81.8% (18/22) (65.7 - 97.9% 95 CI). For pteropids, the prevalence was 85.7 % (59.8 - 111.6% 95 CI) for the little red flying-fox (*Pteropus scapulatus*) and 60% (17.1 - 102.9% 95 CI) for the black flying-fox (*Pteropus alecto*). For microbats, the prevalence was 88.9% (68.4 - 109.4% 95 CI) for Gould's wattled bats (*Chalinolobus gouldii*) and the single blood sample from a lesser long-eared bat (*Nyctophilus geoffroyi*) was also positive.

3.3 Phylogenetic analysis of trypanosome isolates from bats at the 18S locus

Amplicons were only produced using the *T. vegrandis*-specific primers. No other trypanosome species were detected. Sequences were obtained for thirteen of the eighteen *T. vegrandis*-specific PCR positives (Table 1). Bat-derived trypanosome sequences from all four species were 100% identical to each other and one representative isolate (15-5) from a Gould's wattled bat was included in the phylogenetic analysis. Based on Gblocks, of the 421 bp of sequence available, 397 bp were phylogenetically useful. Concordant tree topologies from ML and NJ analysis were obtained (data not shown). Phylogenetic analysis of the relationships between the bat-derived *T. vegrandis* isolate with *T. vegrandis* 18S genotypes on GenBank and closely related trypanosome species (Fig. 2 NJ tree shown) confirmed that all the sequences isolated from bats belonged to the *T. vegrandis* clade. The bat-derived *T. vegrandis*, grouped most closely with *T. vegrandis* genotype 5 from a woylie/brush-tailed bettongs (*Bettongia penicillata*), (KC753534) and exhibited four single nucleotide polymorphisms (SNP's) from this genotype across 421 bp of sequence.

4.0 Discussion

In the present study, morphological and molecular analysis was used to identify *T. vegrandis* infecting bats from the outer Perth metropolitan area in the south-west of Western Australia and the

Kimberley region in the north of Western Australia. *Trypanosoma vegrandis* has previously been identified in woylies/brush-tailed bettongs, western grey kangaroos (*Macropus fuliginosus*), southern brown bandicoots (*Isodon obesulus*), the tammar wallaby (*Macropus eugenii*) and western quolls (*Dasyurus geoffroii*) in Western Australia (Averis et al., 2009; Papparini et al., 2011; Botero et al., 2013; Thompson et al., 2013). More recently it has been identified in koalas (*Phascolarctos cinereus*) from south-east Queensland and northern New South Wales (Barbosa et al., unpublished). Data from the present and previous studies therefore suggest that *T. vegrandis* is geographically dispersed, has a wide distribution in Australia and low levels of host specificity.

Three trypanosome species have been described in Australian bats; *T. pteropi*, *T. hipposideri* and an un-named species (Breinl, 1913; Mackerras, 1959; Mackie et al., 2015). *Trypanosoma hipposideri* was described as very small and slender with a large kinetoplast located near the posterior end and a delicate short free flagellum at the anterior end and measured between 10.5 – 13.0 μm in total length and 1.5- 2 μm in breadth. *Trypanosoma pteropi*, first described by Breinl (1913) in flying-foxes (*Pteropus gouldii*), represented a slender trypanosome with a long pointed posterior end, which had an under-developed undulating membrane and a long free flagellum. The original measurements by Breinl. (1913) for total length was between 20-22 μm , 2.5-3.0 μm in breadth, 2.5-3.0 μm from the posterior to the kinetoplast and 11-12 μm in free flagellum. Mackerras (1959) later described trypanosomes isolated from a flying-fox (*Pteropus* sp.) as morphologically similar to *T. pteropi*, however they were slightly smaller with a sharply pointed anterior end and measured 18-20 μm in total length, 2-4 μm in breadth, 1.5-4 μm from the posterior end to the kinetoplast, 4-5 μm from the kinetoplast to the nucleus with the free flagellum measuring 8-10 μm (Mackerras, 1959). In the most recent study, the novel trypanosome identified in the adult female little red flying fox (Mackie et al., 2015), measured 20.4-30.8 μm in length and 1.3-2.3 μm in breadth, with tapered ends, and is therefore much larger than *T. vegrandis*.

In the present study, *T. vegrandis* from bats ranged between 6.7 - 13.8 μm in total length (mean 9.0 μm), with one isolate measuring 1.0 μm in breadth at the level of the nucleus. The

overlap in size between *T. hipposideri* and *T. vegrandis* suggests that these may be the same species. However, in contrast to *T. hipposideri*, the trypanosomes identified in bats in the present study generally lacked a clearly defined kinetoplast and nucleated region. Unfortunately genetic sequences from *T. pteropi* and *T. hipposideri* are not available and therefore cannot be compared genetically with *T. vegrandis*. *Trypanosoma vegrandis* was first described in woylies as a small curved trypanosome with a pointed posterior and an anterior nucleus in which the kinetoplast is closely situated. In woylies, *T. vegrandis* measured 6.9-10.5 μm in total length (mean 8.3 μm), (1.0-1.6 μm in breadth (mean 1.3 μm), with a free flagellum length of 1.2 - 2.8 μm (mean 1.8 μm), a posterior to kinetoplast length of 2.7-3.8 μm (mean 3.3 μm), a kinetoplast to nucleus length of 0.8-1.9 μm (mean 1.2 μm) and a nucleus to anterior length of 1.5-3.2 μm (mean 2.1 μm) (Thompson et al., 2013). In the present study, the mean total length (9.0 μm) and mean free flagellum length (2.6 μm) of the bat isolates are morphologically similar to *T. vegrandis* from woylies. Only one bat isolate was observed with a defined kinetoplast and nuclear region and similar to the woylie isolates, contain an anterior nucleus with a kinetoplast situated adjacently. For this bat isolate, the posterior to kinetoplast length (6.7 μm) and total length (13.8 μm) were larger compared to the woylie isolates, yet smaller in kinetoplast to nucleus (0.7 μm) and nucleus to anterior (0.8 μm).

The morphological differences observed between the marsupial and mammalian *T. vegrandis* isolates are possibly due to this species being polymorphic in nature with reports of multiple genotypes (Botero et al., 2013; Thompson et al., 2013). Trypanosomes have few morphological features detectable using light microscopy, which can adequately delimit species (Gibson, 2009) and the very small size of *T. vegrandis* (8.3 μm in length and 1.3 μm in width), renders this even more difficult. In the present study, morphological analysis was also limited due to the low levels of true blood stream trypomastigotes. This may have been due to the life-cycle phase at the time of sampling. Typically trypanosomes within the vertebrate host exhibit *T. cruzi*-like multiplication, with division taking place in the amastigote stage, followed by epimastigote forms that later develop into trypomastigote blood stream stages (Hoare, 1972). The small

trypanosome stages observed in the bat blood (Fig. 1B-D) may represent epimastigote or transitional forms, while the more defined trypanosome (Fig. 1A) may represent the true blood stream life-cycle. Additional research however is required to understand the life-cycle stages of *T. vegrandis*.

The identity of *T. vegrandis* was confirmed using sequence and phylogenetic analysis of the 18S rRNA gene, which clearly showed that the bat-derived isolates grouped within the *T. vegrandis* clade. No other trypanosome species were identified. The bat-derived *T. vegrandis* isolates grouped most closely with *T. vegrandis* genotype 5 from a woylie.

In the present study, the PCR prevalence of *T. vegrandis* in Australian bats was 81.8% (65.7-97.9% 95 CI), which was much higher than the 4% (0.1-20.4% 95 CI) reported in pteropids by Mackeras (1959). Previous studies have reported a prevalence of 20% (0.0-40.2% 95 CI) in woylies (Paparini et al., 2011) and up to 32% (27.1-36.6% 95 CI) in various other marsupial species (Botero et al., 2013). The very high prevalence of *T. vegrandis* in Australian bats suggests that bats may be the primary host for *T. vegrandis*, however further research is required to confirm this. In other countries, a range of both zoonotic and bat-specific trypanosome species have been identified in bats. For example, studies in Colombian and Brazilian bats have reported trypanosome prevalences of 2.4-80% using microscopy (D'Alessandro et al., 1971; Marinkelle, 1972; Marinkelle, 1976). Molecular studies have identified a trypanosome prevalence of 61.2% (53.6-68.5% 95 CI) in bats in Colombia (Ramírez et al., 2014) and 11.6% (7.7-16.6% 95 CI) in Panamanian bats (Pinto et al., 2012). Recently a novel trypanosome was identified in the blood of an adult female little red flying-fox (*Pteropus scapulatus*) from south-eastern Queensland (Mackie et al., 2015). The novel trypanosome was most similar to *Trypanosoma rangeli* and *Trypanosoma minasense* (1% genetic distance at the 18S rRNA locus and 14-15% at the gGAPDH locus respectively) (Mackie et al., 2015).

Further investigations comprising larger sample sizes and additional species of bats are required to determine and compare the prevalence of *T. vegrandis* and other trypanosome species in

bats more accurately. However the high prevalence of *T. vegrandis* in the present study suggests that bats may play an important role in the epidemiology of *T. vegrandis* in Australia. The impact of *T. vegrandis* on bat and marsupial health is currently unknown, but mixed *T. vegrandis* and *Trypanosoma copemani* co-infections were found more frequently in declining compared to stable woylie populations ($p=0.001$) (Botero et al., 2013). All of the flying-foxes in the present study were clinically infected with Australian bat lyssavirus, and the microbats were from a wildlife care facility and so may be viewed as a population under a degree of stress. Further studies are essential to better understand the clinical significance of *T. vegrandis*, particularly in populations subject to stress from environmental factors or infectious agents, which may lead to expression of trypanosome-associated disease or increased shedding of the organism.

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Figure Captions

Fig. 1. Light photomicrograph of trypanosome stages of *T. vegrandis* in a Modified Wright's stained blood films from a bat (isolate 15-4). Scale bars represent 10 μ m.

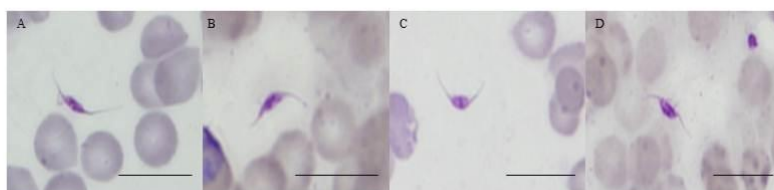
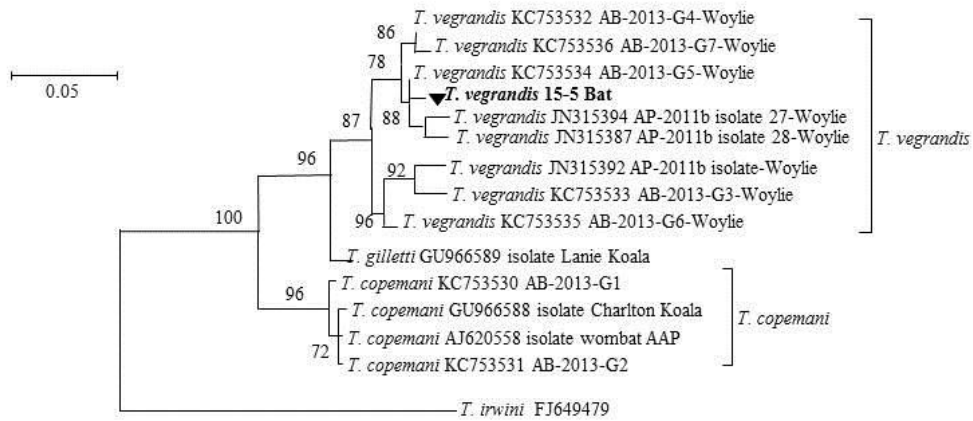


Fig. 2. Neighbour Joining (NJ) analysis of the relationships between *Trypanosoma* spp. and bat-derived *T. vegrandis* at the 18S rRNA locus (421 bp). Percentage support (>50%) from 500 replicates is indicated at the left of the supported node.



Tables

Table 1. Pteropid bats (Yangochiroptera) and microbats (Yinpterochiroptera) screened by PCR for the presence of *T. vegrandis* either in blood or heart tissue.

Bat ID	Species	Sample type	Positive via <i>T. vegrandis</i> specific PCR	Sequenced product
15-3	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-4	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-5	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-6	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-7	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-8	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-9	<i>Chalinolobus gouldii</i>	blood	positive	yes
15-10	<i>Chalinolobus gouldii</i>	blood	positive	no
15-11	<i>Nyctophilus geoffroyi</i>	blood	positive	yes
14-2549	<i>Pteropus alecto</i>	blood	negative	-
14-2382	<i>Pteropus scapulatus</i>	blood	positive	yes
14-2259 #1	<i>Pteropus scapulatus</i>	heart tissue	positive	yes
14-2259 #2	<i>Pteropus scapulatus</i>	heart tissue	positive	no
14-2259 #4	<i>Pteropus scapulatus</i>	heart tissue	positive	yes
14-2347	<i>Pteropus scapulatus</i>	heart tissue	negative	-
14-2382	<i>Pteropus scapulatus</i>	heart tissue	positive	yes
14-2430	<i>Pteropus scapulatus</i>	heart tissue	positive	no
14-2549	<i>Pteropus alecto</i>	heart tissue	negative	-
14-2566	<i>Pteropus alecto</i>	heart tissue	positive	no
14-3739 #1	<i>Pteropus alecto</i>	heart tissue	positive	yes
14-3739 #2	<i>Pteropus alecto</i>	heart tissue	positive	no
15-0092	<i>Chalinolobus gouldii</i>	heart tissue	negative	-

Table 2. GenBank accession number of the 18S rDNA sequences used in the phylogenetic analysis.

Isolates	Accession number
<i>T. irwini</i>	FJ649479
<i>Trypanosoma copemani</i> AB-2013-G2,	KC753531
<i>Trypanosoma copemani</i> isolate AAP (wombat)	AJ620558
<i>T. copemani</i> isolate Charlton (koala)	GU966588
<i>Trypanosoma copemani</i> isolate AB-2013-G1	KC753530
<i>T. gilletti</i> isolate Lanie (koala)	GU966589
<i>T. vegrandis</i> AP-2011b isolate 4 clone 6 (woylie)	JN315392
<i>T. vegrandis</i> AB-2013-G3 (woylie)	KC753533
<i>T. vegrandis</i> AB-2013-G6 (woylie)	KC753535
<i>T. vegrandis</i> AP-2011b isolate 27 clone 4 (woylie)	JN315394
<i>T. vegrandis</i> AP-2011b isolate 28 clone 11 (woylie)	JN315387
<i>T. vegrandis</i> AB-2013-G5 (woylie)	KC753534
<i>T. vegrandis</i> AB-2013-G4 (woylie)	KC753532
<i>T. vegrandis</i> AB-2013-G7 (woylie)	KC753536