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Short Communication

Molecular characterisation of native Australian trypanosomes in quokka (*Setonix brachyurus*) populations from Western Australia

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Abstract

The quokka, *Setonix brachyurus*, is a vulnerable, small marsupial endemic to Western Australia. Blood samples were collected from quokkas from three different geographical locations; Two Peoples Bay, Bald Island and Rottnest Island. The overall prevalence of trypanosomes by nested PCR at the 18S ribosomal RNA gene was 57.3% (63/110) with prevalences of 91.4%, 85.3% and 4.9% respectively for Two Peoples Bay, Bald Island and Rottnest Island. Phylogenetic analysis conducted on 47 18S PCR positives identified two *T. copemani* genotypes, with *T. copemani* genotype B, the most prevalent genotype infecting quokka populations from the three locations with an overall prevalence of 51.8% (24/47) compared to 34% for *T. copemani* genotype A (16/47). The overall prevalence of mixed *T. copemani* genotype A and B infections was 14.9% (7/47). Phylogenetic analysis of 26 quokka isolates at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) locus, largely supported the 18S analysis but identified a mixed infection in one quokka isolate (Q4112- 4117 from Two Peoples Bay). *Trypanosoma copemani* genotype B has previously only been isolated from quokkas and the Gilbert's potoroo whereas *T. copemani* genotype A has a wide host range and may be pathogenic. Further work is required to determine the clinical impact of *T. copemani* on marsupial populations.

Keywords: Trypanosome; *T. copemani*; *T. vegrandis*; quokka; 18S rRNA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

The first phylogenetic analysis of *Trypanosoma* sp. infecting native Australian mammals was conducted in 1999 [1,2]. More recent phylogenetic analysis has extended this analysis in a range of marsupial hosts [3-9]. In a study by Botero et al. [8], eight trypanosome genotypes designated G1 to G8 were reported from a variety of Australian marsupials. G1 and G2, group with *T. copemani* isolates previously described from wombats (*Vombatus ursinus*) and koalas (*Phascolarctos cinereus*) while G3-G7 clustered within the *T. vegrandis* clade [8,9] and Genotype 8 exhibited a 99% similarity to an Australian kangaroo (*Macropodidae*) trypanosome isolate (H25), which has previously been reported to be genetically similar to *T. cruzi* [2].

In the present study, molecular characterization of both the 18S rRNA and GAPDH loci were used to screen for and elucidate trypanosome species associated with quokka populations.

Blood samples examined in the present study were collected from 35, 34 and 41 quokkas from Two Peoples Bay (34º 58'S, 118º 11'E) (TPB) near Albany, Bald Island (34º 55' S, 118º 27'E) (BI) and Rottnest Island (32º 00'S, 115º 31'E) (RI) respectively. All work was carried out under Murdoch University animal ethics permit W2204/09 and Department of Parks and Wildlife permit number SC000767.

Whole genomic DNA was extracted from fresh blood samples using a MasterPure™ DNA Purification Kit (Epicentre® Biotechnologies, Madison, Wisconsin, U.S.A.). Nested PCR protocols were used to amplify the trypanosome 18S rDNA region using primers and PCR reactions as previously described [6,10]. An additional set of internal nested primers TRYall 1 Forward 5' ACCGTTTCGGCTTTTGTTGG 3' and TVEG Reverse 5' AAATCGTCTTCGCTTTAACTTT 3' (this study) which amplify ~468 bp of the 18S rRNA trypanosome gene were used on the RI quokka samples, as previous attempts to amplify these samples using the universal 18S rDNA primers were unsuccessful. Amplified products were purified using a QIAquick® PCR Purification kit (Qiagen) and sequenced directly using an ABI Prism Dye Terminator Cycle Sequencing Core kit (Applied Biosystems, USA). If mixed sequencing chromatograms resulted, then PCR products were

cloned into a pGEM®-T Easy vector (Promega, USA) and plasmid inserts (n=10 per isolate) re-sequenced. A subset of isolates (n=26), that were positive at the 18S locus, were amplified using a hemi-nested PCR of the GAPDH gene (~880 bp) as previously described [6].

After alignment, an 841 bp region of the 18S rRNA gene and a 664 bp region of the GAPDH gene (based on available shorter trypanosome sequences from this study and in GenBank), were analysed using MEGA6 (http://www.megasoftware.net/). After selection of the most appropriate evolutionary model (Kimura-2-parameter) using Model-Test in MEGA 6, Maximum Parsimony (MP), Maximum Likelihood (ML) and Distance analysis were conducted using MEGA 6. Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Bayesian Inference (BI) analysis was carried out by MrBayes (http://mrbayes.sourceforge.net), using the default options. For 18S, the Kimura 2-parameter + G (0.19) model was used and for GAPDH analysis, the GTR + G (0.30) was chosen. Trees were visualized by FigTree v1.4.0 (http://tree.bio.ed.ac.uk/).

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 [11].

The overall prevalence of trypanosomes in quokkas was 57.3% (48-65.5% CI) (63/110) and varied from 4.9% in RI to 91.4% in TPB (Table 1). Of the 32, 29 and 2 PCR positives at the 18S locus obtained from quokkas from TPB, BI and RI respectively, sequences were obtained for 21, 24 and 2 positives respectively. Sequence and phylogenetic analysis showed that all the trypanosome isolates from the quokkas were *T. copemani* (grouping into either *T. copemani* genotype A or B clades) (Table 1). The prevalence of *T. copemani* genotype B was higher for both TPB and BI compared to *T. copemani* genotype A.

The phylogenetic relationship of *T. copemani* to other trypanosome species at the 18S rRNA (Fig. 1) and GAPDH (Fig. 2) gene were analysed using Distance, Parsimony, ML and Bayesian analysis (Bayesian tree shown). For ease of phylogenetic analysis, two *T. copemani* genotype A

representative 18S rRNA sequences from quokka isolates Q2031 from TPB and Q3336-3325 from BI and two representative genotype B isolates from quokkas Q1051 from TPB and Q4471-3340 from BI were included in the tree, although phylogenetic analysis was conducted on all isolates. At the 18S locus, phylogenetic analysis produced trees with similar topographies with *T. copemani* genotypes A and B identified. Genotype B, formed a separate clade consisting of trypanosomes isolated from quokkas and a *T. copemani* sequence from a woylie (KC753530) previously designated as G1 by Botero et al. [8] which exhibited 1.9% genetic distance from *T. copemani* genotype B. Genotype A was 100% homologous with a *T. copemani* isolate from a woylie (*Bettongia penicillata* - KC753531), (previously referred to as G2 by Botero et al. [8] and also grouped with sequences from wombats (AA1- AJ620559, H26-AJ009169 and APP- AJ620558) [1]. *Trypanosoma copemani* isolates from koalas (GU966585- GU966586 and GU966588) [6] formed a separate clade. The quokka *T. copemani* genotypes A and B were 1.8% distant from each other. *Trypanosoma copemani* genotype C (represented by wombat isolates AAI, AAP, H26) as previously described [5] was not supported in this study at the 18S rRNA locus, when analysing larger datasets.

Sequences were obtained for twenty-six quokka isolates at the GAPDH locus; four representative genotype A sequences (from quokka isolates Q2031, Q3336-3325, Q1837-1464 and QBS5) and four genotype B quokka isolates (Q2367-2332, Q4478-3340, Q2088-2050 and Q1051) were included in the tree, although phylogenetic analysis was conducted on all twenty-six isolates.

Consistent with the 18S analysis, at the GAPDH locus, *T. copemani* genotype B isolates formed their own clade while *T. copemani* genotype A quokka isolates clustered with koala and wombat isolates and the RI quokka isolate (QBS5). In contrast to the 18S analysis, genotype A quokka isolates also grouped with a G1 sequence (woylie), which had grouped in a clade at the 18S locus with *T. copemani* genotype B. One *T. copemani* isolate from a woylie representing G2 (KC812983), which grouped with genotype A at the 18S locus, formed a separate clade at the GAPDH locus. The genetic distance between *T. copemani* genotypes A and B was 1.8% at the

GAPDH locus. The genetic distance between the sequence represented by G2 (KC812983) and *T. copemani* genotypes A and B were 2.9%, and 3.1% respectively.

One isolate, Q4112- 4117 from TPB, which grouped with *T. copemani* genotype B clade at the 18S rRNA locus, grouped by itself but close to a loose clade that included G8 from a woylie (KC812988) [8], H25 from a kangaroo (AJ620276) and sequences from brushtail possums (*Trichosurus vulpecula*) (JN315395 and JN315396) [1,7,12]. This isolate (Q4112-4117) exhibited 3.9% genetic distance from H25 and 1.8% genetic distance from brushtail possums (JN315395 and JN315396). The genetic distance between Q4112-4117 and H25 from *T. cruzi* at the GAPDH locus was 11.1% and 11% respectively.

Analysis of the *T. vegrandis* clade identified large genetic variation with genetic distances of 0.4-3.2% within *T. vegrandis* isolates at the 18S locus, with the latter distance of 3.2% between G3 (KC753533) and G4 (KC753532)/G7 (KC753536). At the GAPDH locus, genetic distances of up to 19.2 % were identified within the *T. vegrandis* clade, between *T. vegrandis* isolates G3 (KC812984) and G4 (KC812985).

Quokka trypanosome isolates grouped within the *T. copemani* marsupial clade together with isolates from wombats, koalas, woylies and a common brushtail possum, demonstrating that *T. copemani* is infective to a wide variety of Australia marsupials. Two main *T. copemani* genotypes were identified (genotype A and B). An 18S sequence from a woylie (KC753530), previously designated as G1 by Botero et al. [8] grouped in a clade with genotype B but exhibited 1.9% genetic distance.

Trypanosoma copemani genotype B was the most prevalent genotype infecting quokka populations. At the 18S rRNA locus, *T. copemani* genotype A isolates from quokkas were 100% homologous to the G2 isolate from a woylie [8], indicating that genotype A is prevalent in multiple marsupial species, with G2 previously detected in woylies, a quoll (*Dasyurus hallucatus*) and a southern brown bandicoot (*Isoodon obesulus*) [8].

In contrast to the 18S analysis however, at the GAPDH locus, the G2 genotype (KC812983) grouped separately from *T. copemani* genotypes A and B, which suggests that the G2 genotype may have been a mixed infection consisting of two genotypes, with different genotypes amplified at the 18S and GAPDH loci. A mixed infection in quokka isolate Q4112- 4117 was also identified, as at the 18S rRNA locus, this isolate grouped with *T. copemani* genotype B clade, but at the GAPDH locus, grouped with a clade by itself but closest to a clade that included the G8 genotype [8]. It has been previously reported that the G8 genotype is closely related to *T. cruzi* at the 18S locus [2,7,8,13], however in the present study, the genetic distance between this clade and *T. cruzi* at the 18S locus was 8.2-8.6%. At the GAPDH locus, the genetic distance was larger (11-11.1%), in agreement with a previous study by Botero et al. [8]. This phenomenon of mixed trypanosome infections in native Australian marsupials has previously been reported in woylies and koalas [6,8,14]. Identifying mixed infections has important clinical implications as mixed *T. copemani* and *T. vegrandis* infections are found more frequently in declining woylie populations compared to stable groups of woylies (p=0.001) [8]. Both *T. copemani* genotype G2 (which corresponds to *T. copemani* genotype A) and *T. vegrandis* have been identified within the internal organs of the woylie, where they are thought to adversely affect the fitness and coordination of the host, thus increasing their susceptibility to predation [9]. A recent study identified erythrocyte abnormalities, including microspherocytes and schistocytes in *T. copemani* infected quokka blood [15], which are changes typically associated with haemolytic anaemias [16]. This suggests that *T. copemani* may be a cause of anaemia in marsupials and it is possible that *T. copemani* may be the cause of seasonal anaemia and low red blood cell counts in quokkas from Rottnest Island [17]. However further clinical investigations are needed to determine the clinical impact of *T. copemani* on quokka populations.

Large genetic distances were evident within *T. vegrandis* isolates at both the 18S locus (0.4% to 3.2%) and the GAPDH locus (up to 19.2%). This indicates that *T. vegrandis* is not a uniform species but is in fact a species complex. Previous studies have reported that a genetic

distance of 3.75% at the GAPDH gene is sufficient to delimit a new trypanosome species [19]. By this criterion, there are multiple valid species within *T. vegrandis,* which need to be characterised more fully in the future.

In conclusion, *T. copemani* consists of multiple genotypes, of which genotype A has previously been associated with pathogenic effects in woylies. Mixed infections were common and further work is required to determine the clinical impact of *T. copemani* on marsupial populations.

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References

[1] Noyes HA, Stevens JR, Teixeira M, Phelan J, Holz P, A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp.in wombats and kangaroos in Australia. Int. J. Parasitol. 29, 331–339.

[2] Stevens, J.R., Noyes, H.A., Dover, G.A., Gibson, W.C., 1999. The ancient and divergent origins of the human pathogenic trypanosomes, *Trypanosoma brucei* and *T. cruzi*. Parasitology 118, 107-116.

[3] Stevens, J.R., Noyes, H.A., Schofield, C.J., Gibson, W., 2001. The molecular evolution of Trypanosomatidae. Adv. Parasitol. 48, 1-56.

[4] Hamilton, P. B., Stevens, J. R., Gidley, J., Holz, P. and Gibson, W. C., 2005. A new lineage of trypanosomes from Australian vertebrates and terrestrial bloodsucking leeches (Haemadipsidae). Int. J. Parasitol. 35, 431-443.

[5] Austen, J.M., Jefferies, R., Friend, J.A., Ryan, U., Adams, P., Reid, S.A., 2009. Morphological and molecular characterisation of *Trypanosoma copemani* n. sp. (Trypanosomatidae) isolated from Gilbert's potoroo (*Potorous gilbertii*) and quokka (*Setonix brachyurus*). Parasitol. 136, 783–792.

[6] McInnes, L.M., Hanger, J., Simmons, G., Reid, S.A., Ryan, U.M., 2010. Novel trypanosome *Trypanosoma gilletti* sp. (Euglenozoa: Trypanosomatidae) and the extension of the host range of *Trypanosoma copemani* to include the koala (*Phascolarctos cinereus*). Parasitol. 138, 59–70.

[7] Paparini, A., Irwin, P.J., Warren, K., McInnes, L.M., De Tores, P., Ryan, U.M., 2011. Identification of novel trypanosome genotypes in native Australian marsupials. Vet Parasitol. 183, 21–30.

[8] Botero, A., Thompson, C.K., Peacock, C., Clode, P.L., Nicholls, P.K., Wayne, A.F., Lymbery, A.J., Thompson, R.C.A., 2013. Trypanosomes genetic diversity, polyparasitism and the

population decline of the critically endangered Australian marsupial, the brushtailed bettong or woylie (*Bettongia penicillata)*. Int. J. Parasitol. 2, 77-89.

[9] Thompson, C.K., Botero, A., Wayne, A.F., Godfrey, S.S., Lymbery, A.J., Thompson, R.C.A., 2013. Morphological polymorphism of *Trypanosoma copemani* and description of the genetically diverse *T. vegrandis* sp. nov. from the critically endangered Australian potoroid, the brushtailed bettong (*Bettongia penicillata* (Gray, 1837)). Parasite Vectors 6, 121.

[10] Maslov, D. A., Lukes, J., Jirku, M. and Simpson, L., 1996. Phylogeny of trypanosomes as inferred from the small and large subunit rRNAs: implications for the evolution of parasitism in the trypanosomatid protozoa. Mol Biochem Parasitol 75, 197–205.

[11] Rozsa, L., Reiczigel, J., Majoros, G., 2000. Quantifying parasites in samples of hosts. J. Parasitol. 86, 228-232.

[12] Hamilton, P. B., Stevens, J. R., Gaunt, M.W., Gidley, J., and Gibson, W. C., 2004. Trypanosomes are monophyletic: evidence from genes for glyceraldehyde phosphate dehydrogenase and small subunit ribosomal RNA. Int. J. Parasitol. 34, 1393-1404.

[13] Hamilton, P. B., Teixeira, M. M. G., Stevens, J. R., 2012. The evolution of *Trypanosoma cruzi*: the 'bat seeding' hypothesis. Trends Parasitol. 28, 136-141.

[14] Barbosa, A., Austen, J., Gillett, A., Warren, K., Paparini, A., Irwin, P. and Ryan, U., 2015. First report of *Trypanosoma vegrandis* in koalas (*Phascolarctos cinereus*). Para. Int. In press.

[15] Austen, J. M., Reid, S. A., Robinson, D.R., Friend, J. A., Ditcham, W. G. F., Irwin, P.J., Ryan, U., 2015. Investigation of the morphological diversity of the potentially zoonotic *Trypanosoma copemani* in quokkas and gilbert's potoroos. Parasitology. 142(11), 1443-1452.

[16] Rodak, B.F., Fritsma, G.A., Keohane, E.M., 2012. Hematology: Clinical Principles and Applications (Fourth edition). Elsevier Saunders St. Louis, Missouri.

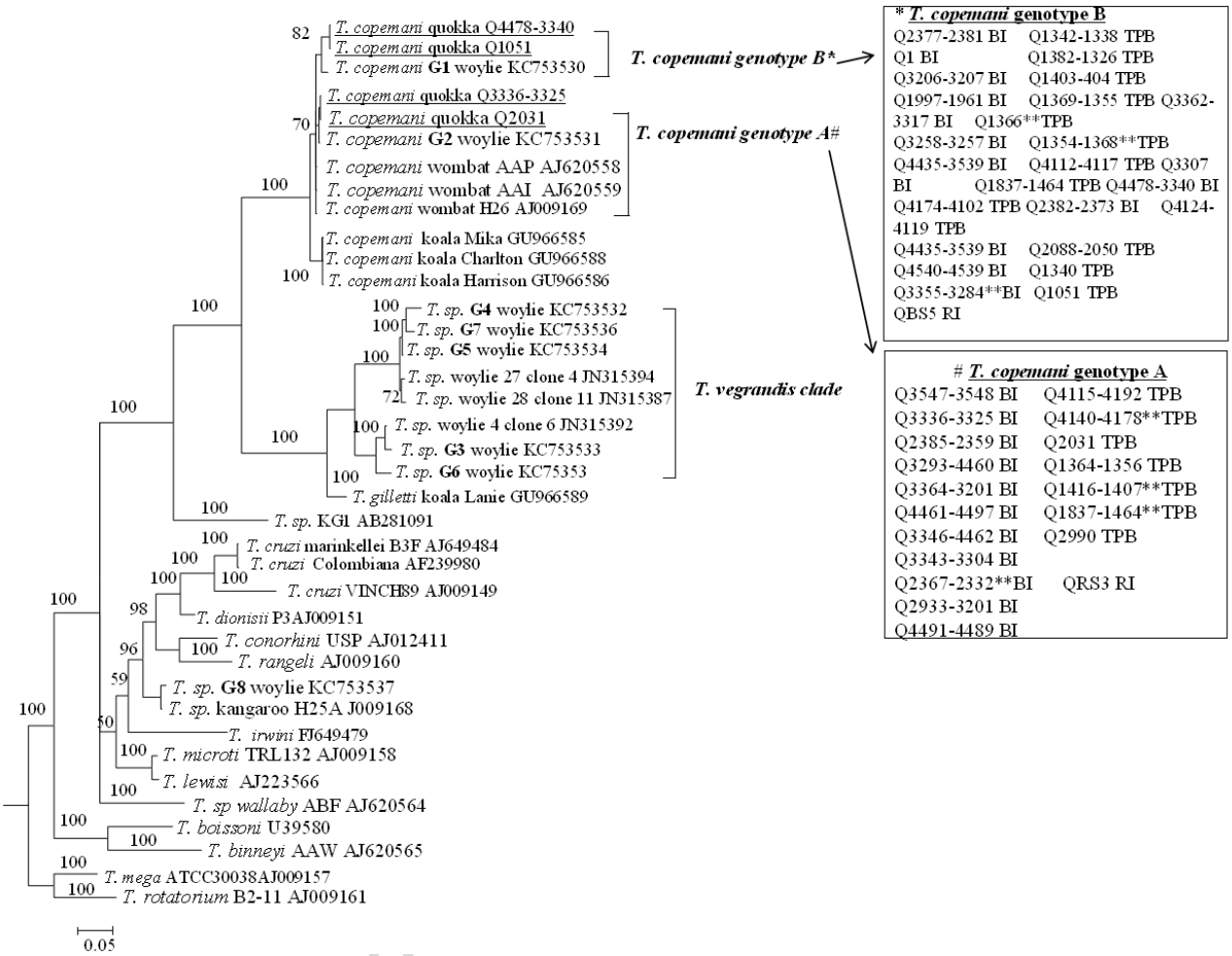
[17] Baker, S., Glover, R., Jacobsen, P., Kakulas, B. A., 1974. Seasonal anaemia in the Rottnest quokka, *Setonix brachyurus* (Quoy and Gaimard) (Marsupialia; Macropodidae). Comp. Biochem. Physiol. 49, 147-157.

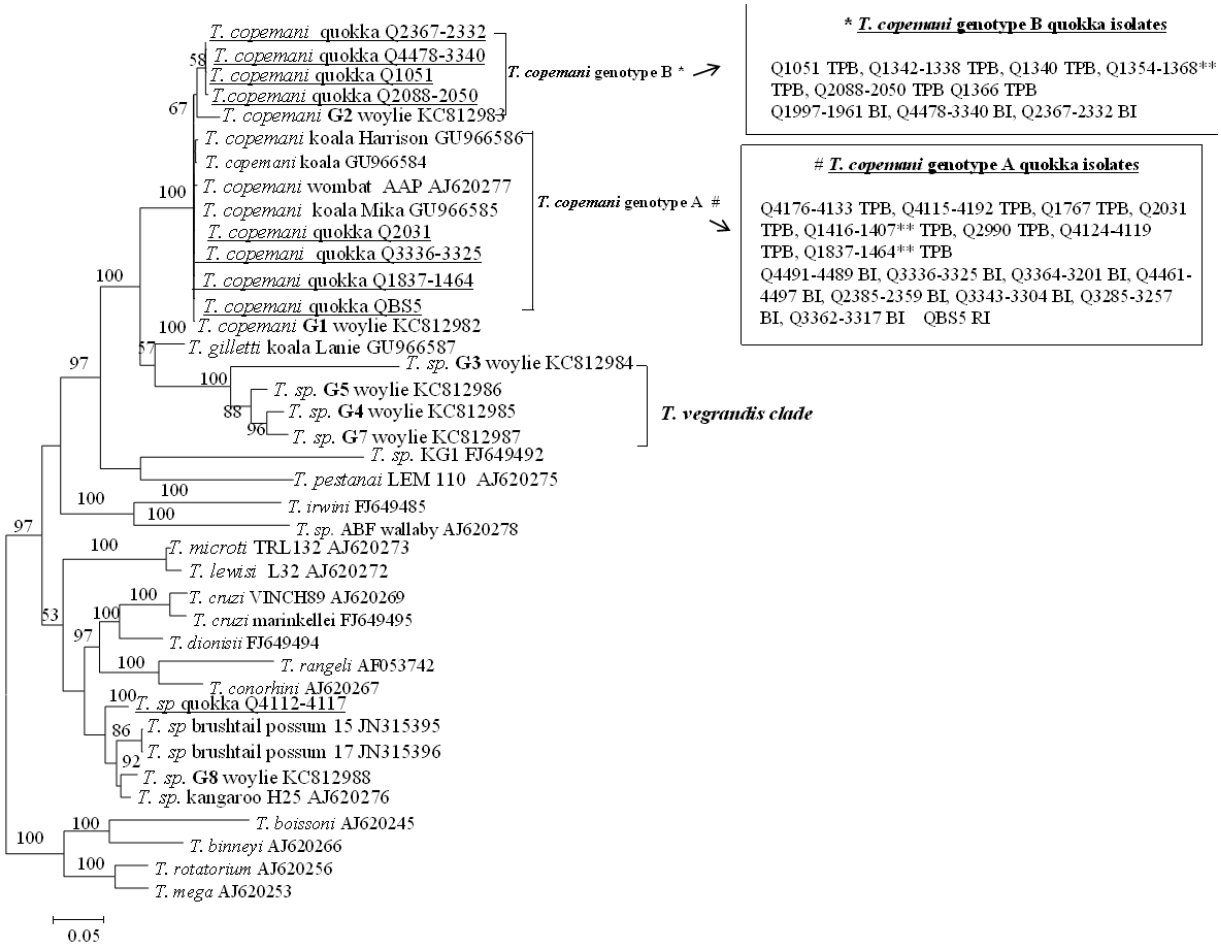
[18] Gibson, W., 2009. Species-specific probes for the identification of the African tsetse-transmitted trypanosomes. Parasitol. 136, 1501-1507.

[19] McInnes, L.M., Gillett, A., Hanger, J., Reid, S.A., Ryan, U.M., 2011. The potential impact of native Australian trypanosome infections on the health of koalas (*Phascolarctos cinereus*). Parasitol. 138, 1–11.

Fig. 1. Phylogenetic relationships of *T. copemani* genotype A and B quokka isolates from Two Peoples Bay (TPB), Bald Island (BI) and Rottnest Island (RI) using the Bayesian Inference method based on partial (841 bp) 18S rDNA sequences. Posterior probabilities are indicated on the main branches. GenBank accession numbers are given. Quokka isolates representing *T. copemani* genotype A(#) and *T. copemani* genotype B(*) are listed. Isolates designated ** were mixed *T. copemani* genotype A and B infections.

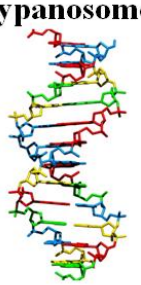
Fig. 2. Phylogenetic relationships of *T. copemani* genotype A and B quokka isolates from Two Peoples Bay (TPB), Bald Island (BI) and Rottnest Island (RI) using the Bayesian Inference method based on partial (641 bp) GAPDH sequences. Posterior probabilities are indicated on the main branches. GenBank accession numbers are given. Quokka isolates representing *T. copemani* genotype A(#) and *T. copemani* genotype B(*) are listed. Isolates designated ** were mixed *T. copemani* genotype A and B infections.





Quokka (*Setonix brachyurus*)

DNA extraction + PCR for trypanosomes



Phylogenetic analysis at 2 loci

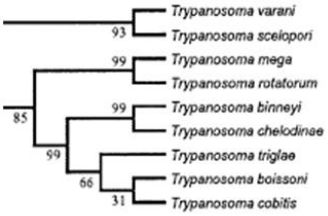


Table 1
Prevalence of *T. copemani* genotypes in quokka isolates from Two Peoples Bay (TPB), Bald Island (BI) and Rottnest Island (RI) as determined by PCR and sequencing of partial 18S rRNA fragments. 95% confidence intervals are given in parenthesis. Mixed infections were determined by cloning and sequencing of amplicons producing mixed sequencing chromatograms.

	Two Peoples Bay (TPB)	Bald Island (BI)	Rottnest Island (RI)
Overall Prevalence	91.4% - 32/35 (82.2-100.7% CI)	85.3% - 29/34 (73.4-97.2)	4.9% -2/41 (0-11.5)
T. copemani genotype A	23.8% - 5/21 (5.6-42)	41.7% -10/24 (21.9-61.4)	50% -1/2 (0-199.3)
T. copemani genotype B	52.4% -11/21 (31-73.7)	50% -12/24 (30-70)	50% -1/2 (0-199.3)
Mixed *T. copemani* genotype A and B*	23.8% - 5/21 (5.6-42)	8.3% -2/24 (0-19.4)	-

Highlights

- Molecular characterization of *Trypanosoma copemani* at 2 loci
- Identification of distinct genotypes within *T. copemani*
- Mixed infections with other trypanosome species identified
- *T. copemani* genotype A may be pathogenic
- *Trypanosoma vegrandis* is a species complex