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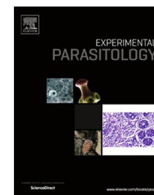

Novel genotypes of *Trypanosoma binneyi* from wild platypuses (*Ornithorhynchus anatinus*) and identification of a leech as a potential vector

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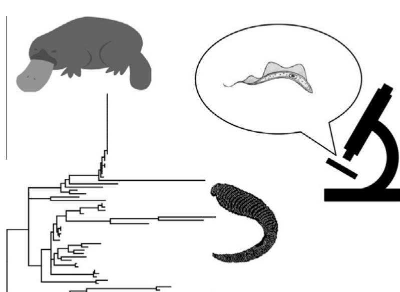
HIGHLIGHTS

- We detect high levels of *Trypanosoma binneyi* infections in wild Tasmanian platypuses.
- We detect multiple closely-related genotypes of *T. binneyi* in wild platypuses from Tasmania.
- We provide evidence to support the hypothesis that leeches are potential vectors of *T. binneyi.*
- In the sampled animals, no particular clinical signs were clearly associated with the infections.

GRAPHICAL ABSTRACT



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ABSTRACT

Little is known about the prevalence and pathogenesis of trypanosomes in Australian monotremes, and few genetic characterisation studies have been conducted with these haemoparasites. During the present investigation, molecular and microscopic methods were used to screen peripheral blood ($n = 28$) and ectoparasites ($n = 10$ adult ticks; $n = 5$ tick nymphs; $n = 1$ leech; and $n > 500$ tick eggs) collected from wild Tasmanian platypuses (*Ornithorhynchus anatinus*), for the presence of trypanosomatid-specific DNA and/or trypomastigotes. The genes for the small ribosomal subunit RNA (18S rDNA) and glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) were amplified and sequenced, prior to conducting phylogenetic analyses.

The detection rate of the parasite-specific 18S rDNA in platypus blood was 85.7% ($n = 24/28$), and the leech was also positive at both loci. Microscopically, high parasitaemia and the presence of abundant trypomastigotes, morphologically consistent with *Trypanosoma binneyi* Mackerras (1959), were observed in the blood films. Phylogenetic analyses at the 18S locus revealed the existence of four trypanosomatid-like genotypes, with variable similarity to two previously-described genotypes of *T. binneyi* (range of genetic p-distance: 0.0–0.5%). For the gGAPDH locus, for which only one *T. binneyi* sequence is available in GenBank, three genotypes closely related *T. binneyi* were identified (range of genetic p-distance: 0.1–0.4%). The leech-derived trypanosome isolate was virtually identical (at the two loci studied) to the other parasites sequenced from infected platypuses; however, the molecular or morphological identification of the leech species was not possible.

Although further studies are required, the molecular detection of trypanosomes in an aquatic leech removed from a platypus, suggests the possibility that these haematophagous hirudineans may be a vector for *T. binneyi* (and closely related genotypes).

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1. Introduction

Trypanosomes (phylum Euglenozoa) are parasitic haemoprotozoa that infect both humans and animals, causing morbidity, mortality and economic losses worldwide (Englund et al., 1982). Trypanosomes, usually transmitted by arthropod or leech vectors, are the aetiological agents of serious human diseases such as African and South American trypanosomiasis (sleeping sickness and Chagas disease, respectively) and diseases of veterinary importance such as African bovine trypanosomiasis, which are characterised by a range of clinical signs including fatigue, fever, anaemia, and death (Hamilton et al., 2004).

In addition to their medical, veterinary and economic relevance, their ubiquitous biogeography, as well as the broad host range of trypanosomes, has prompted molecular studies on their phylogeny and evolution (Haag et al., 1998; Hamilton et al., 2007, 2004). To date, numerous trypanosome species/genotypes have been reported from native Australian eutherians, marsupials and monotremes (rev. in Thompson et al., 2014) (Table 1). In addition to the potential relevance of these parasites on the conservation status of some of the critically endangered species (Paparini et al., 2011; Thompson et al., 2014), collection of DNA sequences from evolutionary distinct and/or geographically isolated hosts may shed light on the transmission, phylogeny, and evolution of these parasites. For these reasons, also exploiting the opportunity offered by a broader project underway, we decided to perform a survey in wild platypuses (*Ornithorhynchus anatinus*). In particular, we were interested in uncovering novel phenotypes or genotypes of trypanosomes, and their potential vectors.

In the platypus, early observations of un-named trypanosomes were made by Owen in 1933–1934 (Owen undated, ca. 1934–5). However, it was not until 1959 (and then 1974) that a description and a name were provided for *Trypanosoma binneyi* (Mackerras, 1959; McMillan and Bancroft, 1974). Preliminary molecular (and microscopic) studies conducted on this protozoan species revealed morphological peculiarities, a high frequency of infections, and a phylogenetic association with the cartilaginous fish-derived sequences of *Trypanosoma boissoni* (Jakes et al., 2001; Noyes et al., 1999). In the present study, we describe the molecular systematics of novel genotypes of *T. binneyi*, found in wild-trapped Tasmanian platypuses. This study also provides the first molecular identification of *T. binneyi*-like DNA in a leech, collected from a platypus. This observation suggests that, as for most members of the aquatic trypanosomes, leeches may act as a vector also of this species of parasite.

Table 1
Trypanosome species recorded in naturally-infected Australian animals (adapted from Thompson et al., 2014).

Subclass	Group	Common name	Species	*Trypanosome* species
Monotremes	Order: Monotremata	Platypus	*Ornithorhynchus anatinus*	*T. binneyi*
Marsupials	Orders: Dasyuromorphia and Peramelemorphia (Polyprotodont Marsupials)	Chuditch (Western quoll)	*Dasyurus geoffroii*	*T. vegrandis*
		Tiger quoll	*Dasyurus maculatus*	*T. copemani*
		Golden bandicoot	*Isoodon auratus*	*T.* sp.
		Northern brown bandicoot	*Isoodon macrourus*	*T. thylacis*
		Southern brown bandicoot	*Isoodon obesulus*	*T. vegrandis*, *T. copemani*
		Eastern barred bandicoot	*Perameles gunnii*	*T.* sp.
		Dibbler	*Parantechinus apicalis*	*T.* sp.
		Common planigale	*Planigale maculata*	*T.* sp.
		Common wombat	*Vombatus ursinus*	*T. copemani*
		Koala	*Phascolarctos cinereus*	*T. copemani*, *T. irwini*, *T. gilletti*
		Brush-tailed possum	*Trichosurus vulpecula*	*T.* sp. H25, *T. copemani*
		Boodie (Burrowing bettong)	*Bettongia lesueur*	*T.* sp., T. sp. H25
		Woylie (Brush-tailed bettong)	*Bettongia penicillata*	*T. copemani*, *T. vegrandis*, *T.* sp. H25
	Order: Diprotodontia (Diprotodont Marsupials)	Gilbert's potoroo	*Potorous gilbertii*	*T. copemani*
		Banded hare wallaby	*Lagostrophus fasciatus*	*T.* sp. H25
		Swamp Wallaby	*Wallabia bicolor*	*T.* sp.
		Brush-tailed rock wallaby	*Petrogale penicillata*	*T.* sp.
		Tammar wallaby	*Macropus eugenii*	*T. vegrandis*
		Western grey kangaroo	*Macropus fuliginosus*	*T. vegrandis*
		Eastern grey kangaroo	*Macropus giganteus*	*T.* sp. H25
		Quokka	*Setonix brachyurus*	*T. copemani*
Eutherians(Placental mammals)	Order: Chiroptera (Bats)	Grey-headed flying fox	*Pteropus poliocephalus*	*T. pteropi*
		Dusky horseshoe bat	*Hipposideros ater*	*T. hipposideri*
	Order: Rodentia (Rodents)	Water rat	*Hydromys chrysogaster*	*T. lewisi*
		Bush rat	*Rattus fuscipes*	*T. lewisi*, *T.* sp.
		Djoongari (Shark Bay mouse)	*Pseudomys fieldi*	*T.* sp.

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2. Materials and methods

2.1. Isolates/animal sources

As part of a wider study into platypus health and behaviour, samples were obtained from wild platypuses captured in the Inglis River, in north-west Tasmania, Australia (−40.982134, 145.726426). The study was approved by the Animal Ethics Committee of Murdoch University, Western Australia (Permit Number RW 2422/11), Department of Primary Industries, Parks, Water and Environment (DPIPWE), Tasmania (Permit to take Wildlife for Scientific Purposes Numbers FA 11131 and FA 12165), and the Inland Fisheries Service, Tasmania (Exemption Permit Number 2011-10). All animals were released after examination and the procedures described below.

2.2. Blood samples and ectoparasites

Venous blood samples ($n = 28$) were collected from the bill sinus of anaesthetised platypuses, captured between September and December 2012, as previously described (Whittington and Grant, 1995). Blood was collected from 28 platypuses into potassium EDTA-treated microtubes (Sarstedt Australia, Technology Park, SA, Australia), refrigerated at 4 °C, until dispatch to Murdoch University, where it was stored frozen at −20 °C, until processed (Table 2). Whilst anaesthetised, the skin and hair coat of each platypus was inspected for ectoparasites and any that were observed were removed, stored in 70% ethanol, and sent to Murdoch University for identification and molecular analyses (Table 2). One engorged adult tick was kept alive in a humidified tube until it produced eggs. Adult ticks ($n = 10$; from 6 animals), nymphs ($n = 5$; from 2 animals), a leech ($n = 1$), and tick eggs ($n > 500$ in two batches; from an engorged tick, collected from one animal), were collected, and morphologically identified (when possible) using a stereo dissecting microscope Olympus SZ61 I (Olympus, Tokyo, Japan), with reference to a standard key (Roberts, 1970). Genomic DNA, extracted from the blood and the ectoparasites, was screened for the presence of the trypanosomatid-specific DNA, using molecular approaches as described below.

2.3. Blood film analysis

A single drop of peripheral blood was used to make thin blood smears, which were stained on arrival at Murdoch University with a modified Wright's stain using an Ames Hema-Tek slide stainer (Bayer, Leverkusen, Germany). Stained blood films were systematically examined at ×400 magnification for the presence of trypomastigotes using an Olympus BX50 microscope with screen views generated by a DP Controller v3.2.1.276 (Olympus). If parasites were observed, their morphology was noted.

2.4. DNA extraction

Ectoparasites were washed twice in sterile, molecular grade water and sliced on sterile Petri dishes, using sterile scalpel blades. Total genomic DNA was isolated using the QIAamp DNeasy Animal Tissue Spin-Column Protocol (Qiagen, Valencia, CA, USA). Lysis was achieved by overnight digestion with Proteinase K (Qiagen), followed by bead beating on a benchtop Vortex Genie 2 vortex (5 min, maximum speed) (MO BIO Laboratories, Carlsbad, CA, USA). DNA was eluted in 60 μL of TE buffer. All nymphs collected from the same animal host were pooled, and processed in the same way; two pools of nymphs in total (namely PTN50 and PTN104.5, from platypuses 50 and 104, respectively) were analysed (Table 2). Based on several preliminary trials carried out prior to this study, pooling appears desirable to increase the amplification success rate, from this difficult biological matrix. DNA from two batches of tick eggs laid by one gravid female tick (PT104.4), collected from one animal, was extracted in the same way, except that no scalpel blade was used (PTE samples). For whole blood/EDTA (200 μL), total genomic DNA was isolated, according to the manufacturer's instructions, using a MasterPure Purification Kit (Epicentre Biotechnologies, Madison WI, USA) and resuspended in 50 μL of TE buffer.

Mock extractions were carried out from sterile molecular-grade water, to exclude DNA contamination from reagents and consumables. All DNA preparations were checked for purity by agarose gel electrophoresis, and quantified by spectrophotometric absorbance using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA).

2.5. Molecular analyses

A nested PCR protocol, targeting a variable region of the trypanosomatid-specific small ribosomal subunit RNA gene (18S rDNA), was performed for initial sample screening, as previously described (McInnes et al., 2011). Briefly, two fragments of the 18S rDNA were amplified by two nested PCR assays, performed using one common primer set for the primary (i.e. external) amplification, and two sets for the secondary (i.e., internal) amplification. The secondary fragments partially overlap and cover an approximately 1.5 kb-long fragment of the target locus. Common external primers were: SLF (5′-gcttgtttcaaggacttagc-3′) and S762 (5′-gacttttgcttcctctaatg-3′); internal primers were: S825F (5′-accgtttcggcttttgttgg-3′) and SLIR (5′-ac- attgtagtgcgcgtgtc-3′), for one nested PCR (approximately 959 bp), and S823 (5′-cgaacaactgccctatcagc-3′) and S662 (5′-gactacaatggtctctaatc-3′), for the other (approximately 904 bp). Thermal cycling conditions consisted of a pre-PCR step with 95 °C for 5 min, 50 °C for 2 min and an extension of 72 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s and an extension of 72 °C for 2 min and 20 s, plus a final extension step of 72 °C for 7 min.

DNA preparations found positive at the 18S locus, were also amplified, by hemi-nested PCR, at the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene, as previously described (Hamilton et al., 2004; McInnes et al., 2009). Briefly, an approximately 880 bp-long fragment was amplified using primary primers GAPDHF (5′-ctymtcggnamkgagatygayg-3′) and GAPDHR (5′-grtksgartadccccactcg-3′), and secondary primers GAPDHF and G4a (5′-gttytgcagsgtcgccttgg-3′). The PCR conditions consisted of a pre-PCR step with 95 °C for 5 min, 50 °C for 2 min and an extension of 72 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s (primary PCR) or 52 °C for 30 s (secondary PCR) with an extension of 72 °C for 2 min and 20 s, and finishing with an extension of 72 °C for 7 min. All amplifications performed included negative and positive controls, consisting of sterile molecular-grade water, and genomic DNA preparations from trypanosomatid-infected animals identified (and sequenced) during previous analyses, respectively.

PCR products were run on a 1% agarose gel containing SYBR Safe Gel Stain (Invitrogen, Carlsbad, CA, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, Dolores, CO, USA). PCR products corresponding to the expected length were excised, purified using a MO BIO UltraClean DNA purification kit, and sequenced, bi-directionally (i.e. forward and reverse primers), using an ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), on an Applied Biosystem 3730 DNA Analyzer.

2.6. Phylogenetic analysis

Phylogenetic analyses were conducted on the sequences obtained during the present study and additional sequences

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Table 2
Summary of platypus and ectoparasite samples analysed and results.

Sample no.	Sample/batch ID*	Source species	Molecular analysis			Microscopy	
			Sample type	*Trypanosoma* 18S rDNA	gGAPDH	Sample type	Results
1	PB 9	Platypus (*Ornithorhynchus anatinus*)	Blood	Positive	Positive	Peripheral blood smear	Positive
2	PB 52			Positive	Positive		Positive
3	PB 70			Positive	Positive		Positive
4	PB 72			Positive	Positive		Positive
5	PB 73			Positive	Positive		Positive
6	PB 74			Positive	Positive		N/A
7	PB 75			Positive	Positive		N/A
8	PB 76			Positive	Positive		Positive
9	PB 77			Positive	Positive		Positive
10	PB 78			Positive	Positive		Positive
11	PB 79			Positive	Positive		Positive
12	PB 80			Positive	Positive		Positive
13	PB 81			Positive	Positive		Positive
14	PB 82			Positive	Positive		Positive
15	PB 83			Neg.	Neg.		Positive
16	PB 84			Positive	Neg.		Positive
17	PB 85			Positive	Positive		Positive
18	PB 87			Positive	Neg.		Positive
19	PB 88			Positive	Positive		Positive
20	PB 89			Positive	Positive		Positive
21	PB 90			Positive	Positive		Positive
22	PB 91			Positive	Positive		Positive
23	PB 93			Positive	Neg.		Positive
24	PB 94			Positive	Positive		Positive
25	PB 95			Neg.	Neg.		Positive
26	PB 96			Neg.	Neg.		Positive
27	PB 97			Neg.	Neg.		Positive
28	PB 104			Positive	Neg.		Positive
Positives (subtotal)				24	20		26
1	PT 11	*Ixodes ornithorhynchi* Lucas, 1846	Adult ticks	Neg.	Neg.	n.a.	n.a.
2	PT 20			Neg.	Neg.		
3	PT 24.1			Neg.	Neg.		
4	PT 24.2			Neg.	Neg.		
5	PT 34			Neg.	Neg.		
6	PT 38			Neg.	Neg.		
7	PT 104.1			Neg.	Neg.		
8	PT 104.2			Neg.	Neg.		
9	PT 104.3			Neg.	Neg.		
10	PT 104.4			Neg.	Neg.		
Positives (subtotal)				0	0		
1	PTN 50	*Ixodes ornithorhynchi* Lucas, 1846	Tick nymphs	Neg.	Neg.	n.a.	n.a.
2	PTN 104			Neg.	Neg.		
Positives (subtotal)				0	0		
1	PTL 57	?	Leech	Positive	Positive	n.a.	n.a.
Positives (subtotal)				1	1		
1	PTE 104.1	*Ixodes ornithorhynchi* Lucas, 1845	Tick eggs	Neg.	Neg.	n.a.	n.a.
2	PTE 104.2			Neg.	Neg.		
Positives (subtotal)				0	0		

Abbreviations: Neg. = no amplification or No viable product/sequence obtained; N/A = not available; n.a. = not applicable; ? = unknown.
* *Note:* Numbers refer to the ID of the animal host. Multiple ectoparasites from the same animal hosts are distinguished by the appended numerical digits. Tick nymphs and tick eggs were pooled in batches.

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retrieved from GenBank (GenBank accession numbers available from authors upon request). Of the six *T. binneyi* 18S rDNA sequences currently available from GenBank (AF297087, AF073878, AJ620567, AJ620566, AJ620565, AJ132351; retrieved September 2013), four sequences (underlined) were only between 514 and 741-bp-long, and/or only partially overlapped with the 18S alignment generated during the present study. For these reasons, they were not included in the analysis.

Sequence chromatogram files were analysed by FinchTV 1.4 (http://www.geospiza.com/Products/finchtv.shtml), and imported into Geneious Pro V. 7.1.5 (Biomatters, Auckland, NZ) and MEGA6 (Tamura et al., 2011), for manipulations and alignments. Degenerate bases were bioinformatically assigned to polymorphic positions, using the proper plugin in Geneious Pro V. 7.1.5 (Biomatters, Auckland, NZ). Alignments obtained by MAFFT v7.017 (Katoh et al., 2002) and refined by MUSCLE (Edgar, 2004) were tested for the most appropriate nucleotide substitution models by MEGA6 (partial deletion option, with 95% threshold) (Tamura et al., 2011) (Akaike Information Criterion), prior to reconstructing the phylogeny by PhyML (Guindon et al., 2010), through the T-REX server (Boc et al., 2012).

Including gaps, the final 18S rDNA alignment used for the analysis (36 sequences) included 1442 characters, and 230 (16%) parsimony-informative sites. For gGAPDH (32 sequences), there were 713 characters, and 194 (27%) parsimony-informative sites. A third 18S/gGAPDH concatenated alignment was also generated and used for phylogenetic reconstructions (31 sequences; 450/2,153 parsimony-informative sites).

For the 18S, the following PhyML settings were used: Model: TN93 + G (Tamura and Nei, 1993); Statistical test: bootstrap:

500; Number of categories: 4; Gamma: fixed to 0.28; Invariable sites: fixed to 0.00. For gGAPDH, the following PhyML settings were used: Model: GTR + G (Nei and Kumar, 2000); Statistical test: bootstrap: 500; Number of categories: 4; Gamma: fixed to 0.34; Invariable sites: fixed to 0.00. Finally, the concatenated analysis, involved the following settings: Model: GTR + G (Nei and Kumar, 2000); Statistical test: bootstrap: 500; Number of categories: 4; Gamma: fixed to 0.26; Invariable sites: fixed to 0.00.

Maximum parsimony (MP), and neighbour-joining (NJ) trees were constructed using MEGA6 (Tamura et al., 2011) (500 bootstrap replicates; partial deletion option, with 95% threshold). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm, with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). For NJ, the evolutionary distances were computed using the p-distance method.

Estimates of evolutionary divergence between sequences (p-distance) were computed using MEGA6 (Tamura et al., 2011); all positions with less than 95% site coverage were eliminated. Compared to the pairwise deletion option, this is a more stringent criterion, because fewer than 5% alignment gaps, missing data, and ambiguous bases are allowed at any position.

Sequences obtained during the present study were submitted to GenBank, under accession numbers KJ867127 to KJ867170.

3. Results

3.1. Peripheral blood smear analysis and ectoparasites identification

Peripheral blood smears were available for examination from 26 platypuses examined (Table 2). Most individuals (24/28) were adults, and were determined clinically to be in reasonable or good health. Significant morphological abnormalities were not observed within the blood cell lines of these platypuses, with the exception of intra-erythrocytic inclusions, consistent with piroplasms (e.g. *Theileria* spp.) that were observed in all blood smears examined. Large, deeply basophilic-stained trypomastigotes, with an undulating membrane extending the length of the organism and a free flagellum, were observed in all available (26/26) blood films (Fig. 1). These were consistent with previous morphological descriptions of *T. binneyi* Mackerras, 1959 (McMillan and Bancroft, 1974). It was noted that trypomastigote morphology varied according to their relative position within the blood smear (Fig. 1): trypomastigotes within the monolayer and feather regions of the blood smear were wider, with clearer cytoplasmic detail (Fig. 1a) in contrast to organisms in the proximal, thick parts of the blood smear, which were more slender and appeared to be folded longitudinally (Fig. 1b).

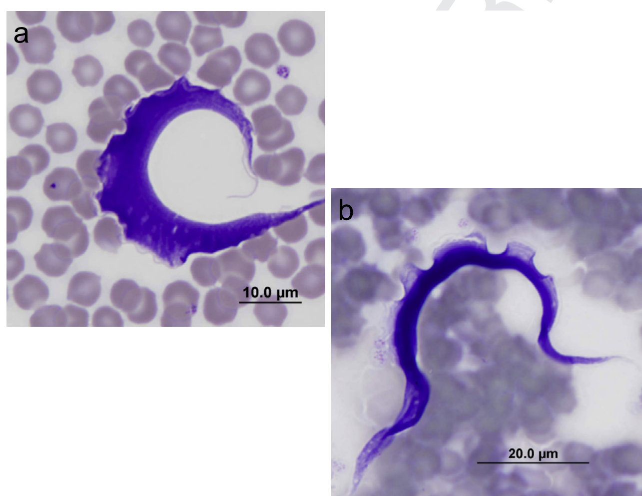

Fig. 1. Trypomastigotes of *Trypanosoma binneyi* at the proximal (thick) end (Fig. 1a), and the distal (feather) end (Fig. 1b) of a peripheral blood smear from a platypus. Note the morphological variation of the trypomastigote between locations. Modified Wrights stain, original magnification ×1000.

Adult and nymphal ticks collected from the animals (PT and PTN samples, respectively; Table 2) were identified as *Ixodes ornithorhynchi* Lucas, 1846. With one exception (PT104.1), the adult ticks were either unengorged, or partially engorged. Prior to processing, a fully engorged female tick removed from a platypus (PT104.4) was kept temporarily alive, and produced >500 gold-coloured eggs.

3.2. Molecular detection of trypanosomes

Overall, 24 blood samples (85.7%) and one leech, screened by PCR, were positive for trypanosome 18S rDNA (Table 2). To the best of our knowledge, this is the first report of the identification of *T. binneyi*-like DNA in a leech. For one isolate (PB73) only one primer provided a viable sequencing product, and the shorter sequence obtained from this sample was omitted from the final alignment. For the phylogenetic reconstructions, 23 consensus 18S rDNA sequences (i.e., forward- and reverse-sequencing) in total were generated from all positive samples ($n = 25$).

Trypanosomatid infections were also confirmed by PCR/sequencing of the gGAPDH gene. After discarding a lower quality consensus sequence from PB87, the range of sequence lengths was 749–856 bp. For this locus, 21 consensus sequences were obtained, from 20 blood samples and one leech. Thus, this PCR assay targeting the gGAPDH locus appeared relatively less efficient than that for the 18S rDNA, because for four samples (PB84, PB87, PB93, PB104) viable sequences were not obtained or were of lower quality (Table 2).

3.3. Phylogenetic analyses

Four genotypes were identified within the platypus-derived 18S sequences obtained during the present study, all of which grouped loosely with the two known *T. binneyi* isolates (AAW and H29; GenBank accession numbers AJ620565 and AJ132351, respectively) (Hamilton et al., 2005), in a distinct clade with up to 98% bootstrap value (Fig. 2).

Of the four novel variants, PB93 was basal to the clade and diverged from AAW by 11 differences (over a 1,442 character-long gapped alignment). All the remaining platypus-derived genotypes grouped in clade with 69–96% bootstrap support (Fig. 2 inset). Isolate PB79 was almost identical to (and equally distant from) *T. binneyi* isolates AAW and H29 (6 differences from each); PB88 and PB91 (respectively, 5 and 8 differences from AAW) clustered together, at the base of the remaining 19 sequences (6 or 7 differences from AAW). This latter group, representing the bulk of the 18S sequences from this study formed a clade but with non-meaningful support (<50%) for all reconstructions methods. Genetic distances (p-distance) calculated based on the highly-gapped 18S alignment (gap treatment option: partial deletion), showed that PB93, the seemingly most unique variant, was only 0.3% distant from AAW and 0.5% from H29. These figures confirm the high degree of similarity between the genotypes from the present study and *T. binneyi*, at the 18S locus.

For validation, a second phylogenetic reconstruction was conducted including all the 18S *T. binneyi* sequences available from GenBank (AF297087, AF073878, AJ620567, AJ620566, AJ620565, AJ132351; retrieved September 2013), apart from one (AF297087), which spans a downstream region of the gene, and only partially overlaps with the other sequences in the alignment.

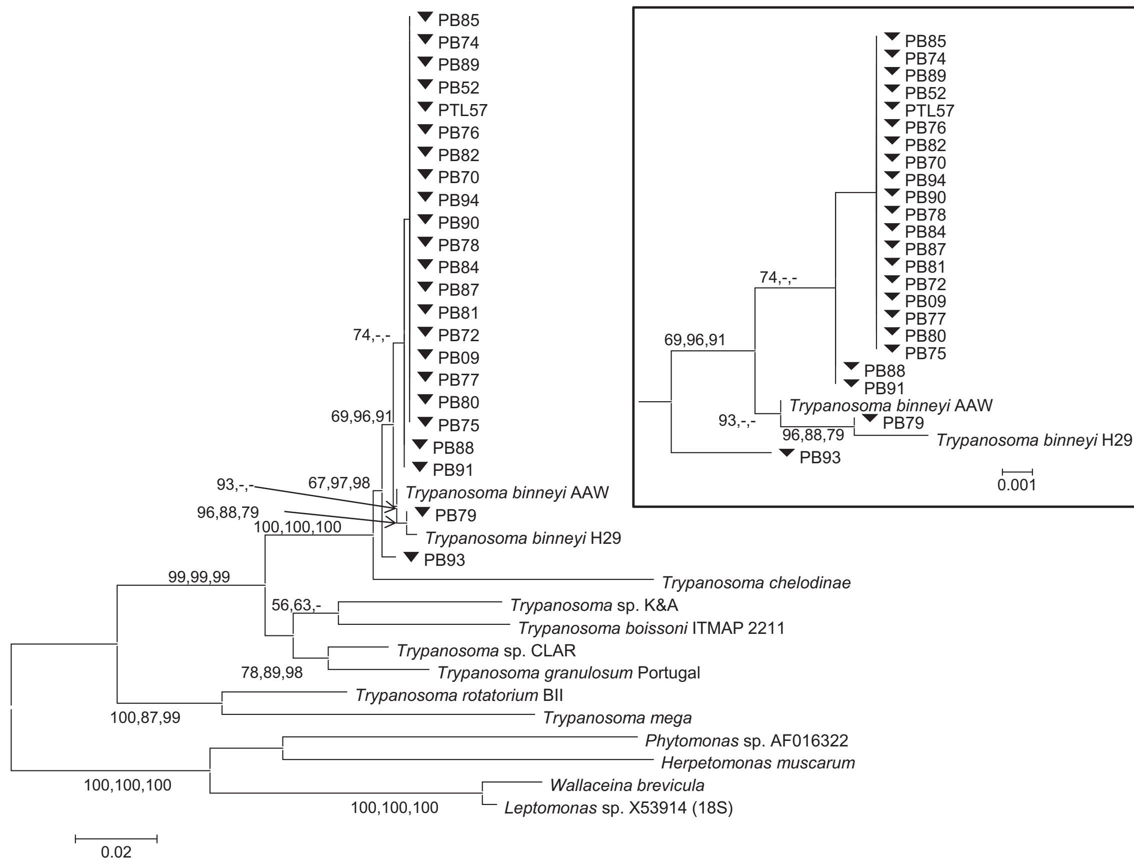

Fig. 2. Phylogenetic analysis of the *Trypanosoma* spp., based on partial sequences of the 18S rDNA locus. Evolutionary history was inferred using the Maximum Likelihood method. Branch support/bootstrap values (⩾50% only shown) for Maximum Likelihood, Maximum Parsimony and Neighbour Joining analyses respectively, are indicated by each node. *Abbreviation:* – branch support/bootstrap value <50%.

This analysis, based on a shorter dataset, confirmed the topology of the *T. binneyi*/*Trypanosoma chelodinae* clade obtained with the longer alignment, as well as the novelty of the genotypes obtained during the present study.

The viable sequences obtained for gGAPDH (Table 2) formed three main groups of *T. binneyi*-like genotypes, with genetic distances ranging from 0.1% to 0.4%, from the known isolate AAW (Fig. 3). PB75 and PB79 were virtually identical to each other (2 differences, over a 713 character-long gapped alignment), basal to the whole clade, and showed only 4–5 differences from *T. binneyi* AAW. This latter isolate, the only for which gGAPDH sequences are currently available in GenBank, loosely grouped with the remaining 19 sequences and showed only 5–6 differences with PB80, PB91, and the other bulk of 17 sequences. This large clade comprising most of the sequences (including the leech) appeared separated from *T. binneyi* AAW (80% bootstrap support), but only according to the ML reconstruction method. For this locus, no viable sequences were obtained from PB93.

A third reconstruction was finally conducted, starting from a concatenated 18S/gGAPDH alignment ($n = 31$ sequences). This was 2,153 character-long, with 62.1% identical sites, 92.4% pairwise identity, and sequence length ranging from 2035 to 2100 bp. The tree topology obtained, clearly compatible with the 18S and gGAPDH trees, further confirmed the identification of novel variants of *T. binneyi*, yet showing very high similarity of the known genotype from GenBank. This observation was further supported by the presence of low (i.e., >50%) bootstrap values associated with each branch within the *T. binneyi* clade (Fig. 4).

4. Discussion

As was been reported decades ago (Mackerras, 1959; McMillan and Bancroft, 1974; Owen undated, ca. 1934–5), these apparently asymptomatic trypanosome infections appear to be very prevalent in free-living platypuses and can be observed even in small numbers of individuals, as was the case of the present study. The present study provides further support for the relatively high rate of infections with trypanosomes in the platypus. The morphology of the trypomastigotes were consistent with previous descriptions of *T. binneyi* and the variations in the characteristics of some of these organisms described previously (and in Fig. 1) are most likely explained by artefact; a similar observation was noted by previous authors (McMillan and Bancroft, 1974).

The three reconstruction methods implemented (ML, MP, NJ) provided trees with somewhat consistent topology, for both loci. Overall, these results confirmed the identification of previously unidentified genotypes of *T. binneyi*, showing, however, very limited differences with sequences already available from this species. It is well known that alignment quality can have a profound impact on distance values and downstream analyses (Schloss, 2010). Despite the greater length (1442 vs. 713 characters, for 18S and gGAPDH, respectively), the 18S alignment only had 16% parsimony-informative sites, against 27% for gGAPDH. Possibly even more importantly, the different genetic constraints associated with the protein-coding locus gGAPDH, and the 18S rDNA hypervariable regions of the loops, clearly affected the features of the alignment obtained. Thus, the frequency and length of gaps (due to insertion

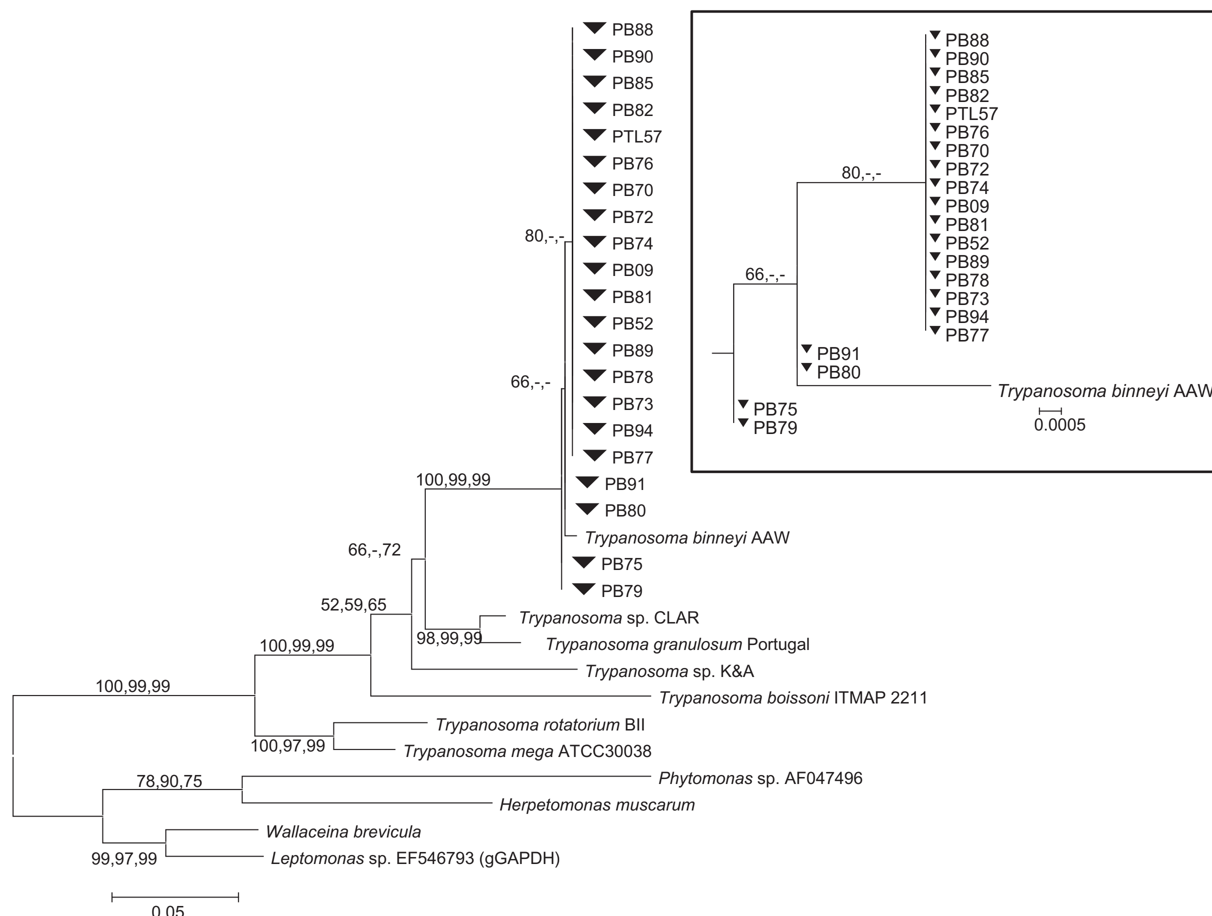

Fig. 3. Phylogenetic analysis of the *Trypanosoma* spp., based on partial sequences of the gGAPDH gene. Evolutionary history was inferred using the Maximum Likelihood method. Branch support/bootstrap values (⩾50% only shown) for Maximum Likelihood, Maximum Parsimony and Neighbour Joining analyses respectively, are indicated by each node. *Abbreviation:* – branch support/bootstrap value <50%.

or the deletion of bases – INDELs), was remarkably different between the two alignments. These gaps can be treated in different ways, with profound effects not only on bootstrap values and topologies during phylogenetic reconstructions (Talavera and Castresana, 2007), but also, importantly, on genetic distance calculations, and preliminary analyses conducted in our hands with a larger dataset, clearly confirm this effect (data not shown).

Unlike other taxa (e.g., *Theileria* spp. and *Babesia* spp. (Schnittger et al., 2003)), there are no established molecular criteria for delimiting species within the genus *Trypanosoma*. Another problem is that sufficient details of the implemented bioinformatics methods are often unavailable. In describing *Trypanosoma irwini* n. sp., McInnes et al. (2009) argued that, in comparison to the genetic distances between accepted species of trypanosomes, values of 0.9% and 10.7%, for the 18S and gGAPDH loci, respectively, should warrant separate species status (McInnes et al., 2009). Using the rather stringent partial–deletion option, the largest minimum genetic distance values from AAW observed during the present study were 0.3–0.4% at the 18S and gGAPDH loci, respectively. All pairwise genetic distances varied slightly, but proportionally, for all pairs of taxa, if alternative gap treatment options were chosen (data not shown).

Despite not being sufficiently large to warrant separate species status, the genetic distances, measured for the novel platypus-derived sequences (from *T. binneyi* AAW and H29), are still considerable, when compared to those separating other operational taxonomic units (OTUs) in the tree. For instance, based on two preliminary alignments generated, the p-distance values calculated between the valid (i.e., named) species *Trypanosoma corvi* and *Trypanosoma culicavium* isolate PAS109, and between *Trypanosoma lewisi* Af and *Trypanosoma microti* TRL 132, were 0.6% and 0.4%, respectively, while between *Trypanosoma rangeli* TryCC 643 and *Trypanosoma grayi* BAN1 there was only 0.2% genetic distance (18S rDNA). At gGAPDH, *T. lewisi* L32 and *T. microti* TRL132 displayed a genetic distance of 1.5% (data not shown).

The final selection of sequences used for the analysis, included only high-quality chromatograms carefully trimmed, checked, and manually edited. Double peaks, observed irrespective of the locus, occasionally occurred within stretches of evenly-spaced and strong peaks, lacking any baseline noise (degenerate bases were bioinformatically assigned to these positions). This evidence suggests the occurrence of possible mixed infections, associated with multiple genotypes harboured by the same host. Alternatively, these positions could represent genuine polymorphisms due to heterozygosity of the multi-copy 18S rDNA.

The vector of *T. binneyi* is currently unknown, but it has been suggested that aquatic leeches carrying fish trypanosomes first infected the platypus with this parasite (Hamilton et al., 2004). Not only is this consistent with the fact that most vectors within the aquatic clade are leeches, but it would also explain the morphological resemblance of *T. binneyi* with the other fish-derived trypanosomes, and its molecular systematics within the aquatic clade. During the present study a single leech collected from a platypus (PTL57) provided positive molecular amplification at the two loci tested (leeches are not commonly found on the platypuses currently under study – JM personal communication). Unfortu-

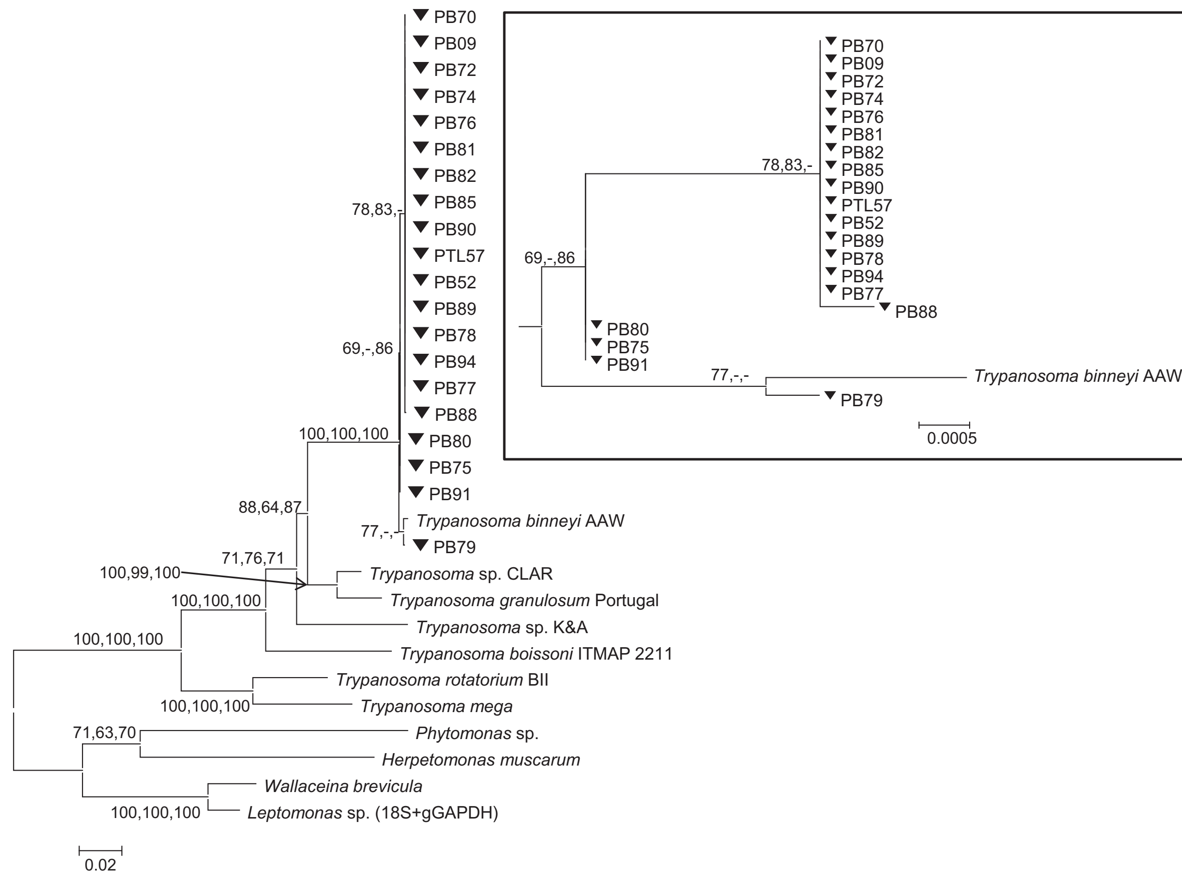

Fig. 4. Concatenated phylogenetic analysis of the *Trypanosoma* spp., based on partial sequences of the 18S rDNA and gGAPDH genes. Evolutionary history was inferred using the Maximum Likelihood method. Branch support/bootstrap values (⩾50% only shown) for Maximum Likelihood, Maximum Parsimony and Neighbour Joining analyses respectively, are indicated by each node. *Abbreviation:* – branch support/bootstrap value <50%.

nately, due to the poor state of the tissues in the sample upon arrival at Murdoch University, morphological identification of the leech species was not possible. Moreover, despite the outstanding quality and length (>1000 bp) of a 18S sequence obtained from the leech-extracted DNA, molecular identification of this leech was not possible with confidence, using the primer set adopted (data not shown). Most likely this disappointing result is due to the low level of divergence, within the amplified region, between the various species of hirudinean ectoparasites. In fact, for our leech-derived 18S sequence, BLAST returned hits for a number of different known leech species (Glossiphoniidae and others), all with strikingly-similar *E*-values, % identity, and% query cover. This high-level of homology between the query and the hits thwarted the confident identification of the leech from the platypus. Clearly, further investigations will have to consider alternative primer sets, possibly targeting a different locus (Siddall and Burreson, 1998), and/or after identification of possibly misidentified sequences in GenBank.

Moreover, despite having two fixed, stained blood smears from the platypus from which the leech was collected, a whole blood sample was not available for DNA studies. Based on the phylogenetic reconstructions, however, the leech was clearly harbouring DNA similar to *T. binneyi*, and was virtually identical (at the two markers studied) to that isolated from several infected platypuses (Figs. 2 and 3). Thus, it is plausible that, at least based on the two DNA regions studied, the leech was carrying the same species/genotype of parasite, harboured by the infected individuals. Given its haematophagous habit, together with the relatively high prevalence of trypanosome infection in the platypuses studied, it was not unexpected to detect trypanosomatid DNA in this leech. However, the finding does raise the interesting possibility that leeches may vector *T. binneyi* (and genotypes) as discussed above for other aquatic vertebrate hosts.

Surprisingly, considering the high prevalence of trypanosome infections and tick infestations (JM personal observation), no trypanosomatid-like DNA was detected within the tissues of any of the ticks screened molecularly. This may be explained by the fact that the ticks analysed were mostly either unengorged or only partially engorged, suggesting that blood feeding had not commenced, or was incomplete. In addition, compared to blood, spectrophotometric and electrophoretic assessments of the ticks and eggs showed a generally lower yield/quality associated with the DNA preparations (data not shown); this, and the possible presence of PCR inhibitors, may have produced false negative results, and an underestimation of the number of positive ectoparasites.

Vectors within the aquatic clade of the genus *Trypanosoma* appear to be restricted to aquatic invertebrates only (leeches), and for some parasite species they are unknown (Haag et al., 1998; Hamilton et al., 2007). The role for ixodid ticks as vector of some terrestrial trypanosome species has long been known, and many studies worldwide have previously detected trypanosomes in the haemolymph and gut of a variety of tick species (El Kady, 1998; Latif et al., 2004; Mackerras, 1959; Morzaria et al., 1986; Shastri and Deshpande, 1981; Thekisoe et al., 2007). More recently, a study of native Australian mammals identified *Ixodes australiensis* as the vector for *Trypanosoma copemani* (Austen et al., 2011). Phylogenetic associations between aquatic trypanosomes and naturally-infected terrestrial invertebrate hosts were shown in previous reports (Bartlett-Healy et al., 2009; Ferreira et al., 2008).

Experimental insect transmission studies (with aquatic trypanosomes) also demonstrated that mosquitoes (*Culex territans*) are capable of transmitting *Trypanosoma rotatorium* (Desser et al., 1973), a sand fly (*Phlebotomus vexator*) transmitted a trypanosome from a toad (*Bufo boreas*) (Anderson and Ayala, 1968), and a midge (*Corethrella wirthi*) one from a treefrog (*Hyla cinerea*) (Johnson et al., 1993) (reviewed by (Hamilton et al., 2004)). At least in principle, however, it is possible that terrestrial invertebrate vectors like ticks are capable of transmitting some aquatic trypanosomes, as already suggested (Munday et al., 1998); thus, until further studies are conducted, this debated theory cannot be refuted.

In conclusion, the present study detected a high prevalence of apparently asymptomatic infections by trypanosomes (consisting of 3–4 genotypes, depending on the locus), in platypuses living in north-west Tasmania, Australia. Albeit not identical, the parasites were highly genetically similar (⩾99%) to the known species *T. binneyi*. More importantly, the molecular identification of trypanosomes in a leech removed from a platypus supports the hypothesis that these hirudineans are a potential vector for *T. binneyi* (and closely related genotypes) (Hamilton et al., 2007). Further studies are required to confirm this, and to characterize the leech to species level, using a more informative primer set.

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