1 **Running title:** Molecular identification of the *Trypanosoma (Herpetosoma) lewisi* clade in black rats (*Rattus*
2 *rattus*) from Australia.

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22 **Keywords:**

23 *Trypanosoma lewisi*; *Rattus rattus*; Australia; black rats; ship rats

26 Abstract

27 Invasive rodent species are known hosts for a diverse range of infectious microorganisms and have long been
28 associated with the spread of disease globally. The present study describes molecular evidence for the
29 presence of a *Trypanosoma* sp. from black rats (*Rattus rattus*) in northern Sydney, Australia. Sequences of
30 the 18S ribosomal RNA (rRNA) locus were obtained in two out of eleven (18%) blood samples with
31 subsequent phylogenetic analysis confirming the identity within the *Trypanosoma lewisi* clade.

33 Introduction

34 Black rats (*Rattus rattus*) are distributed throughout the world and considered one of the most significant
35 invasive species. In Australia, black rats became established alongside European settlement during the
36 1770's, although the precise date of their first arrival on the continent is unclear (Banks and Hughes 2012).
37 Black rats can act as amplifying hosts for a diverse range of pathogens that can affect humans, wildlife and
38 domestic animals and a recent review of black rats in Europe identified at least 20 zoonotic infectious agents
39 associated with the species (Strand and Lundkvist 2019). However, despite the global recognition of these
40 rodents as hosts of pathogens, there is a relatively limited understanding of the range of infectious agents
41 present in Australian populations of black rats (Banks and Hughes 2012).

43 Trypanosomes are a group of flagellate protozoan parasites, the vast majority of which are transmitted by
44 blood-feeding invertebrates. Worldwide at least 44 trypanosome species are known to infect rodents (Dybing
45 et al. 2016). In Australia, recent research has revealed the presence of several novel trypanosomes infecting
46 native Australian marsupials (Thompson et al. 2014), however investigation into the presence of
47 trypanosomes in Australian rodents, either native or introduced, has been lacking in recent years.

49 The results shared in this short communication form part of a broader ongoing investigation into vector-
50 borne microorganisms present in Australia. To the authors' knowledge, this study provides the first molecular
51 identification of *Trypanosoma lewisi*-like organisms from black rats on mainland Australia.

53 **Methods**

54 Small mammal trapping was conducted during April and May 2019 at two sites in northern Sydney,
 55 Australia; Irrawong Reserve and Warriewood Wetlands, Warriewood (-31.69°, 151.28°) and North Head,
 56 Manly (-33.81°, 151.29°). Two transects of 20 trap stations were set up at each site, with each station
 57 including one Elliot type B trap (46 x 15.5 x 15 cm) and one medium sized cage trap (72 x 32 x 31 cm) to
 58 target small and medium sized mammals. Traps were baited with peanut butter and oat balls and set for 3
 59 consecutive nights. The sampling was conducted with approval of the Animal Ethics Committees of
 60 Murdoch University (Permit number R3026/18) and the University of Sydney (Permit number 2018/1429).
 61 Venous blood was collected into 1mL EDTA tubes for the detection of haemoparasites. Thin blood smears
 62 were prepared and stained with modified Wright-Giemsa. Blood films were inspected by light microscopy
 63 (Olympus BX51) for the presence of trypanosomes at x 400 magnification and under oil immersion (x 1000).
 64 Total genomic DNA was extracted from 200 µl of blood using a MasterPure DNA purification kit
 65 (Epicentre® Biotechnologies, Madison, Wisconsin, U.S.A) following the manufacturer's recommendations.
 66 Where 200 µl of blood was not available, PBS was used to make samples up to 200 µl. DNA was eluted in
 67 30 µl of TE buffer and stored at -20°C.
 68
 69 Blood samples were screened for the presence of *Trypanosoma* spp. using a nested PCR approach targeting a
 70 ~550 bp product of the 18S ribosomal RNA (rRNA) gene with external primers TRY927F/TRY927R and
 71 internal primers SSU561F/SSU561R, as previously described (Noyes et al. 1999). Reactions were carried out
 72 in 25 µl volumes, 2 µl of gDNA was added to the primary PCR and 1 µl of the primary product was used as a
 73 template for the secondary assay. PCR products were electrophoresed on a 1% agarose gel stained with
 74 SYBR safe (Invitrogen, USA), and amplicons of the correct size were excised and purified using previously
 75 described methods (Yang et al. 2013). Sanger sequencing was carried out using internal primer sets in both
 76 directions and sequencing was performed at the Australian Genome Research Facility (Perth, Australia).
 77 Samples that returned a positive identification for *Trypanosoma lewisi*-like were further investigated. A near
 78 full-length fragment of the 18S rRNA locus was obtained using two nested PCR assays. Reactions were
 79 carried out in 25 µl volumes using external primers SLF/S762 and internal primer sets S823/S662 and S825/
 80 SLIR as described (McInnes et al. 2009). Gel electrophoresis and Sanger sequencing using internal primers

in both directions were carried out as above. No-template and extraction controls were included throughout the laboratory processes. Extractions, pre-PCR and post-PCR procedures were performed in laboratories physically separated from each other in order to minimise the risk of contamination. In addition, no *T. lewisi* species have been previously isolated or amplified in the specific laboratories used.

Nucleotide sequences from *Trypanosoma* species were retrieved from GenBank (Benson et al. 2017) and aligned with sequences obtained in the present study using MUSCLE (Edgar 2004), gaps were removed using Gblocks (Castresana 2000) with less stringent parameters. The final alignments were imported into MEGA 7 (Kumar et al. 2016), and the most appropriate nucleotide selection model was selected using the dedicated feature based on the Bayesian Information Criterion (BIC). Bayesian phylogenetic reconstruction was conducted in MrBayes v3.2.6 (Ronquist et al. 2012) using a MCMC length of 1,100,00, burn in of 10,000 and sub-sampling every 200 iterations. Genetic distances were calculated using the Kimura model, positions containing gaps and missing data were eliminated.

Results and Discussion

In total, 11 black rat blood samples were collected for analysis from Warriewood Wetlands (n=4), and North Head (n=7). Two rat samples from North Head were positive for *Trypanosoma* species by molecular methods, and of these a blood smear was only available in one case. Unfortunately, no trypomastigote stages were observed by light microscopy despite prolonged searching of the cell layer. Black rat samples that were negative for molecular evidence of trypanosomes were also screened by microscopy, however this also did not return any positive observations. The absence of a morphological identification in this report is disappointing, however it is not unexpected when parasites reside in their natural host. Mackerras (1959) reported that rats (*R. rattus*) experimentally infected with *T. lewisi* go through an acute phase where parasites multiply rapidly, followed by a chronic phase, during which parasite numbers progressively diminish and disappear from circulation.

Initial screening produced ~550 bp product of the 18S rRNA gene in samples BR042 and BR048, these sequences were 100% identical to each other. A near full length 18S rRNA sequence (1,928 bp) was obtained

from both samples also confirming that the sequences were 100% identical and a representative sequence of the 18S rRNA gene from sample BR042 was used for phylogenetic purposes (GenBank accession MN512227).

Phylogenetic analysis of the shorter (326 bp) 18S rRNA gene alignment was used in order to include a wider variety of reference sequences, in particular for the context of the present study to include the only other *T. lewisi*-like sequences from Australia (Averis et al. 2009). Figure 1 shows the phylogeny of the *Trypanosoma* genus (Fig 1a) and the resolution within the *T. lewisi* clade (Fig 1b). As demonstrated by the polytomy present in Fig 1b, this short region of the 18S rRNA gene is insufficient in the differentiation of members within the *T. lewisi* clade. Due to the speed at which the 18S rRNA locus has evolved, short regions of this locus have been reported as being unsuitable for inference of evolutionary relationships between *Trypanosoma* species (Hamilton and Stevens, 2011).

Reconstruction of phylogenetic relationships over a longer region (1,627 bp) of the 18S rRNA gene exhibited superior resolution within the *T. lewisi* clade (Fig 2). In this phylogeny, sequences obtained from Australian black rats in the present study did not fall within the *T. lewisi* sensu stricto clade; instead they formed a distinct group that branched separately from other reference sequences. Pairwise distance analysis over a 1,627 bp alignment of the 18S rRNA gene demonstrated sequences from the black rat were 99.5% similar to *Trypanosoma microti* (AJ009158). The next most similar sequences were *Trypanosoma* sequences from voles in Japan (AB242275, AB242276) and a flea from Czech Republic (KF054111), all of which were 99.4% similar. Members of the *T. lewisi* sensu stricto clade, as shown in Fig 2., were all 100% identical to each other over the 1,627 bp alignment. These were the third most similar sequences (99.3%) to the *Trypanosoma* sp. identified in the present study. The phylogeny in the present study supports previous research by Hamilton et al. (2005) showing that the *T. lewisi* clade can be divided into two subclades, consisting of *T. lewisi*, *T. musculi*, *T. rabinowitschae*, *T. blanchardi* and *T. grosi* in subclade one and *T. nabiasi*, *T. microti*, *T. otospermophili* in subclade two. Sequences obtained in the present study from Australian black rats fall within subclade two of the *T. lewisi* clade.

136

137 Morphological identification of rodent trypanosomes in Australia, attributed to *T. lewisi*, was first made by T.
 138 L. Bancroft in 1888 from black rats in Brisbane (Mackerras 1959), with subsequent records by various
 139 scientists who confirmed the presence of this parasite in; Brisbane by Pound (1905), in Perth by Cleland
 140 (1906, 1908), and in Sydney by Johnston (1909) (cited by Mackerras 1959). *Trypanosoma lewisi* was first
 141 identified in native Australian fauna by Mackerras (1958). Morphological detection of the parasite has been
 142 made from the bush rat (*Rattus fuscipes*; Queensland) and the water rat (*Hydromys chrysogaster*;
 143 Queensland) (Mackerras 1958, 1959). More recently, molecular reports of *Trypanosoma* species from the *T.*
 144 *lewisi* clade have been made from native wildlife in Western Australia, including two bush rats (*Rattus*
 145 *fuscipes*), a dibbler (*Parantechinus apicalis*) and an ash-grey mouse (*Pseudomys albocinereus*) (Averis et al.
 146 2009). Interestingly, despite sampling from 371 native mammals, 19 different species and 14 sites, detection
 147 of *T. lewisi*-like species was confined only to mammals from Fitzgerald River in the south-west of Australia.
 148 The identification of *T. lewisi*-like spp. by Averis et al. (2009) was limited by the short size of the 18S rRNA
 149 locus analysed (444 bp). As demonstrated in the present study, across a short region of the 18S rRNA locus,
 150 trypanosomes within the *T. lewisi* clade can share a high sequence similarity (Fig 1b), however upon more
 151 robust analysis of a longer fragment it is evident that sequences within the *T. lewisi* clade form distinct
 152 groups. Additional genetic information (e.g. glycosomal glyceraldehyde-3-phosphate dehydrogenase
 153 (*gGAPDH*)) also assists in determining the phylogenetic relationships of these closely related species. The
 154 rabbit trypanosome (*Trypanosoma nabiasi*), which also falls within the *T. lewisi*-clade, has been identified
 155 from Australian rabbits and their associated fleas (*Spilopsyllus cuniculi*) in New South Wales and Victoria
 156 (Hamilton et al. 2005).

157

158 Christmas Island is an external Australian Territory located in the Indian Ocean, south of Indonesia and was
 159 once home to endemic populations of *Rattus macleari* and *Rattus nativitatis*. The introduction of black rats
 160 and their associated trypanosomes to regions previously free of these species has long been considered
 161 responsible for the extinction of two native rat species, a hypothesis that dates back to the time of the
 162 extinction events in the early 1900's by parasitologist H.E. Durham (Durham 1908). Recent research has
 163 confirmed Durham's initial reports and concluded that the rapid decline and extinction of the two endemic

rat species was correctly attributed to infections with *T. lewisi* (Wyatt et al. 2008). A review of historical records demonstrated a rapid extinction event following the arrival of black rats on the island in September 1900 and an absence of native rat sightings by October 1904 (Green 2014). While there is strong support for the placement of the trypanosome species responsible within the *T. lewisi* clade, the nature of the ancient DNA study by Wyatt et al. (2008) using museum specimens meant that only a short fragment of the 18S rRNA gene was amplified. As such, differentiation within the *T. lewisi* clade is difficult in this case. A recent study by Dybing et al. (2016) investigated the presence of *Trypanosoma* and *Leishmania* spp. from feral cats (*Felis catus*) and black rats (*R. rattus*) on Christmas Island. Through molecular analysis of spleen samples, the study did not detect any *Trypanosoma* or *Leishmania* species. In addition, the same study reported an absence of these parasites from feral cat samples from Dirk Hartog Island and sites from south-west Western Australia.

North Head is situated on the northern side of Sydney harbour and is dominated by Eastern Suburbs banksia scrub, a declared endangered ecological community (Environment Protection and Biodiversity Conservation Act 1999). In addition to being home to endangered populations of long-nosed bandicoots (*Perameles nasuta*) and little penguins (*Eudyptula minor*), reintroductions of native fauna species, such as bush rats (*Rattus fuscipes*), eastern pygmy possums (*Cercartetus nanus*) and brown antechinus (*Antechinus stuartii*), have also been carried out at North Head by the Australian Wildlife Conservancy. While there is no evidence of spill-over of trypanosomes within the *T. lewisi* clade to native species to-date, ongoing monitoring of populations is encouraged given the historical significance of this parasite with respect to native animal declines (Wyatt et al. 2008; Green 2014).

In addition to trypanosomes, black rats may act as reservoirs for many other sources of infectious agents (Banks and Hughes 2012). Additional information regarding the presence, distribution and diversity of pathogens harboured by black rats in Australia is critical to understanding pathogen spill-over dynamics (Becker et al. 2019). Future research encompassing both morphological and molecular techniques is ongoing by the authors. Collection of ectoparasites, blood, and tissue samples from both native and introduced

191 wildlife will likely continue to shed light on the diversity and distribution of vector-borne microorganisms
192 impacting wildlife, domestic animals and humans.

193

194 **Conflict of interest statement**

195 On behalf of all authors, the corresponding author states that there is no conflict of interest

196 **Acknowledgements**

197 This study was part-funded by the Australian Research Council (LP160100200), Bayer HealthCare
198 (Germany) and Bayer Australia. S.L.E. is supported by an Australian Government Research Training
199 Program (RTP) Scholarship, C.L.T is supported by a scholarship from the Northern Beaches Council. This
200 project was also part supported by The Holsworth Wildlife Research Endowment & The Ecological Society
201 of Australia (awarded to S.L.E) and the Paddy Pallin Science Grant from The Royal Zoological Society
202 (awarded to C.L.T). We thank Jenna Bytheway, Dr Henry Lydecker and the Australian Wildlife Conservancy
203 ecologists Dr. Viyanna Leo and Mareshell Wauchope for their invaluable assistance in the field.

204 **Ethical statement**

205 The sampling was conducted under Murdoch University Animal Ethics Committee permit number R3026/18
206 and University of Sydney Animal Ethics Committee Permit number 2018/1429.

207

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209 Figure Captions

210 **Fig 1.** Bayesian phylogenetic reconstruction of *Trypanosoma* based on a 326 bp fragment of the 18S rRNA
 211 locus using the HKY + G substitution model. All positions containing gaps and missing data were
 212 eliminated. Phylogeny of the *Trypanosoma* genus (a) and insert tree (b) to show resolution of the *T. lewisi*-
 213 clade based on a short 326 bp to include a larger set of reference sequences, sequences obtained from
 214 Australia are shaded. All posterior probabilities at branch nodes >0.8 (see colour key on figure) unless
 215 indicated, number of substitutions per nucleotide position is represented by the scale bar. Collapsed nodes
 216 represented with triangular branches. New sequence from the present study is designated in bold.

217
 218 **Fig 2.** Bayesian phylogenetic reconstruction of *Trypanosoma lewisi* clade using 1,627 bp fragment of 18S
 219 rRNA locus based on HKY + G substitution model. Node labels represent posterior probabilities, number of
 220 substitutions per nucleotide position are represented by the scale bar. *Trypanosoma cruzi* (AJ009150) was
 221 used as group out. New sequence from the present study is designated in bold. Isolate hosts: European mole
 222 (*Talpa europaea*), squirrel flea (*Ceratophyllus (Monopsyllus) sciurorum*), black rat (*Rattus rattus*),
 223 Columbian ground squirrel (*Urocyon columbianus*), Japanese grass vole (*Microtus montebelli*),
 224 Richardson's ground squirrel (*Spermophilus richardsonii*), Siberian flying squirrel (*Pteromys volans*) field
 225 vole (*Microtus agrestis*), Japanese/Anderson's red-backed vole (*Myodes andersoni*), striped field mouse
 226 (*Apodemus agrarius*), house mouse (*Mus musculus*), European hamster (*Cricetus cricetus*), garden dormouse
 227 (*Eliomys quercinus*), Chinese white-bellied rat (*Niviventer confusianus*), greater bandicoot rat (*Bandicota*
 228 *indica*), brown rat (*Rattus norvegicus*), brown howler monkey (*Alouatta guariba*), common marmoset
 229 (*Callithrix jacchus*), night monkey (*Aotus* sp.). Country abbreviations; Australia (AU), Brazil (BR), Canada
 230 (CA), China (CN), Czech Republic (CZ), France (FR), Indonesia (ID), Japan (JP), United Kingdom (UK),
 231 United States of America (US), unknown (unk).

(a)

(b)

0.004