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Prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites in native mammals from northern Australia.

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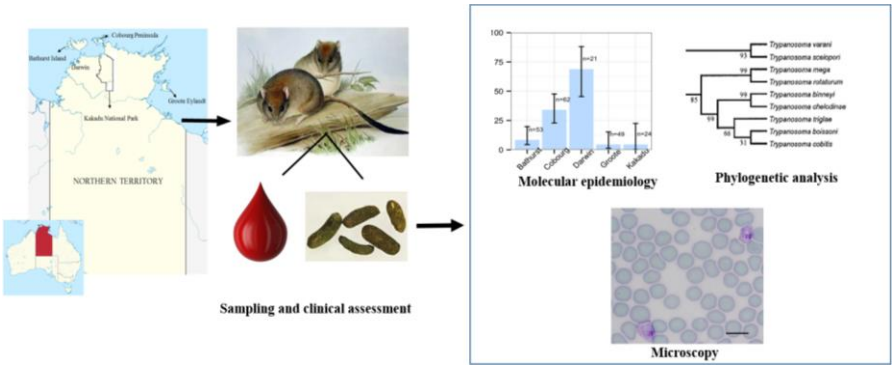
Graphical abstract

Prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites in native mammals from northern Australia.



Highlights

- First molecular survey of protozoans in native mammals from northern Australia
- Five genera of protozoans characterised
- High prevalence of *Trypanosoma* spp. detected in bandicoots and possums
- Association between *Trypanosoma* infection and signs of anaemia in bandicoots
- Baseline information for wildlife conservation and zoonotic disease programs

Abstract

A molecular survey was conducted to provide baseline information on the prevalence, genetic diversity and potential clinical impacts of blood-borne and enteric protozoans in native wild mammals from the Northern Territory (NT). A total of 209 blood and 167 faecal samples were collected from four target species; the northern brown bandicoot *(Isoodon macrourus*), common brushtail possum *(Trichosurus vulpecula*), northern quoll (*Dasyurus hallucatus*) and brush-tailed rabbit-rat (*Conilurus penicillatus*). Blood samples were screened by PCR at the 18S rRNA gene for trypanosomes, piroplasms and haemogregarines, with faecal samples tested for *Cryptosporidium* spp. at the 18S rRNA locus, and for *Giardia* spp.

at the glutamate dehydrogenase (*gdh*) and 18S rRNA loci. The potential clinical impact was investigated by associating clinical, haematological and biochemical parameters with presence or absence of infection. Overall, 22.5% (95% CI: 17.0-28.8%) of the animals tested were positive for haemoprotozoans. Trypanosomes were found in 26.6% (95% CI: 18.7-35.7%) of the bandicoots and were identified as *Trypanosoma vegrandis* G6, except for one unique genotype, most similar to *T. vegrandis* G3 (genetic distance = 7%). The prevalence of trypanosomes in possums was 23.7% (95% CI: 11.4-40.2%), and the genotypes identified clustered within the *T. noyesi* clade. The presence of *Babesia* sp. and *Hepatozoon* sp. was confirmed in bandicoots only, both at a prevalence of 5.3% (95% CI: 2.7-9.3%). The total prevalence of intestinal protozoan parasites observed was relatively low (3%; 95% CI: 1.0-6.9%). No evidence of clinical disease associated with protozoan parasitic infection was observed, however bandicoots positive for *Trypanosoma* exhibited a significantly lower packed cell volume (PCV) compared to negative bandicoots (p=0.046). To the authors' knowledge, this is the first research conducted in the NT to characterise protozoan parasites in threatened native mammals using both molecular and morphological tools; and to assess the potential clinical impacts of these agents. The absence of clear signs of major morbidity in infected animals seems to exclude a direct association between infections with these agents and possible population decline events in northern Australian native mammals. However until the cause(s) of population decline are ascertained for each individual mammal species, further studies are required. The outcome of the present investigation may be used to inform wildlife conservation and zoonotic disease programs.

Keywords: protozoan parasites; molecular typing; marsupials; wildlife; epidemiology

1. Introduction

Evidence from biodiversity surveys across the tropical north or "Top End" of the Northern Territory (NT) in Australia strongly suggests that many small to medium sized mammal species are in rapid and broad-scale decline, even in protected areas such as Kakadu National Park (Woinarski et al., 2010; Woinarski et al., 2011; Ziembicki et al., 2015). The declines in both diversity and abundance have probably been occurring for over 20 years, and infectious disease may be a potential contributor, acting in synergy with other factors such as introduced predators and changed fire regimes (Woinarski et al., 2015; Ziembicki et al., 2015).

Relatively few molecular epidemiological surveys have been conducted on protozoan parasites in Australian threatened wildlife species, particularly across the NT. Trypanosomes are widespread blood-borne protozoan which can infect a wide range of vertebrates including humans. Ten species of *Trypanosoma* have been described in native mammals in Australia to date (Thompson et al., 2014a; Barbosa et al., 2016a; Botero et al., 2016); there are also reports of the association of *Trypanosoma* spp. with the potential extinction of bulldog rats (*Rattus nativitatis*) and Macleay's rats (*Rattus macleari)* on Christmas Island (Pickering and Norris, 1996; Wyatt et al., 2008); anaemia and increased mortality in koalas (*Phascolarctos cinereus*) (McInnes et al., 2011); woylie or brush tailed bettong (*Bettongia penicillata*) population decline (Botero et al., 2013; Thompson et al., 2014b); and more recently, with severe clinical disease in an Australian little red flying fox (*Pteropus scapulatus*) (Barbosa et al., 2016a; Mackie et al., 2016).

Piroplasms are intraerythrocytic vector-borne protozoans belonging to the order Piroplasmida (phylum Apicomplexa), which include the genera *Theileria*¸*Babesia* and *Cytauxzoon.* Australian native mammals are believed to host multiple species of piroplasms, that have previously been identified as members of the genus *Babesia* or *Theileria* (Priestley 1915; Backhouse and Bolliger, 1959; Mackerras 1959; Barker et al. 1978; Collins et al. 1986;

Bangs and Purnomo 1996; O'Donoghue and Adlard 2000; Clark and Spencer 2007; Lee et al. 2009; Paparini et al. 2012a; Rong et al. 2012; Dawood et al. 2013; Kessell et al. 2014; Donahoe et al. 2015; Paparini et al. 2015). Although infections can be asymptomatic (Clark 2004; Vaughan et al. 2009; Paparini et al. 2012a; Rong et al. 2012; Portas et al. 2014), sporadic exceptions have been reported (Backhouse and Bolliger 1957, 78; Barker et al. 1978; Dawood et al. 2013; Kessell et al. 2014; Donahoe et al. 2015).

Other potential blood-borne pathogens such as *Hepatozoon* spp. have been identified in quolls, possums and bandicoots in Australia (Mackerras, 1959; Bettiol et al., 1996; Wicks et al., 2006). Nevertheless, there is still limited knowledge about the clinical significance of *Hepatozoon* spp. in Australian mammals.

Cryptosporidium and *Giardia* are intestinal parasites which can cause diarrhoeal illness in animals and humans worldwide. In Australia, *C. fayeri* and *C. macropodum* are the most common species reported in a range of marsupials; however, kangaroo genotype 1, brushtail possum genotype 1 and zoonotic species such as *C. cuniculus, C. meleagridis, C. muris, C. ubiquitum, C. hominis* and *C. parvum* have been reported in the eastern grey kangaroo (*Macropus giganteus*), western grey kangaroo (*Macropus fuliginosus*), southern brown bandicoot (*Isoodon obesulus*), common brushtail possum (*Trichosurus vulpecula*), common wombat (*Vombatus ursinus*) and brush-tailed rock-wallaby (*Petrogale penicillata*) (Koehler et al., 2016a; Zahedi et al., 2016a; Zahedi et al., 2016b). *Cryptosporidium* species including *C. tyzzeri, C. parvum,* mouse genotype II and rat genotypes have also been detected in exotic rodents in Australia such as wild house mice (*Mus domesticus*) and black rats (*Rattus rattus*) (Morgan et al., 1999; Foo et al., 2007; Paparini et al., 2012b). Whilst the majority of *Giardia* found in free-ranging terrestrial mammals have been identified as assemblages of *G. duodenalis* (Thompson et al., 2009), a unique species, *G. peramelis*, which appears to be host-adapted, has recently been described in the quenda, southwest Western

Australia (WA) subspecies of southern brown bandicoot (*Isoodon obesulus fusciventer*) (Adams et al., 2004; Hillman et al., 2016). The impacts of enteric parasites on wildlife health are not yet well elucidated.

The present study was part of a broader two-year collaborative project, conducted in the NT, to investigate the potential role of disease in the decline of native mammal species (Reiss et al., 2015). It was specifically aimed at identifying and characterising blood-borne and enteric protozoan parasites in these species using morphological and molecular analyses. This research also sought to investigate the potential clinical impact these protozoan parasitic infections may have upon their hosts.

2. Materials and methods

2.1 Sampling

The sampling was conducted from June 2013 to December 2014, across five different locations within the NT (Fig 1): peri-urban areas around Darwin, Kakadu National Park, Bathurst Island, Groote Eylandt and Cobourg Peninsula (for simplicity, these locations will be referred to as Darwin, Kakadu, Bathurst, Groote and Cobourg hereafter). These sites were pre-selected areas which formed parts of mammal survey programs conducted by the Department of Environment and Natural Resources (DENR) in the NT. Sampling areas in Darwin were selected by DENR as representative of relatively disturbed environments proximal to human settlement, whereas Cobourg, Bathurst and Groote represented relatively remote and less disturbed areas. Kakadu has intermediate proximity to human settlements and is relatively more disturbed than the islands, due to the presence of feral herbivores and pigs, a pre-park history of grazing, and significantly altered fire regimes. Kakadu is also where north Australian mammal declines have been most documented (Woinarski et al., 2010). Small cage and Elliot traps were used at sampling sites, in a variety of layouts depending on location, however most commonly in either a line of traps arranged as a grid, or a 50m by

50m square with 16 Elliot and 8 small cage traps distributed evenly. Traps were open for 3-5 nights, and cleared at dawn each morning.

A total of 209 blood and 167 faecal samples were collected from animals belonging to four target mammal species: northern brown bandicoot (*Isoodon macrourus*), common brushtail possum (*Trichosurus vulpecula*), northern quoll (*Dasyurus hallucatus*) and brush-tailed rabbit-rat (*Conilurus penicillatus*). These species were selected as they are representative of each major taxonomic group that have undergone decline in the NT: Phalangeridae (possums), Peramelidae (bandicoots), Dasyuridae (carnivorous marsupials) and Muridae (rodents). In addition, logistics dictated a focus on species of relatively large body size that were most likely to be trapped in reasonable numbers, to acquire sufficient specimen amounts and sample size. The numbers of samples collected from each target species across the five sampling sites are presented in Table 1.

All trapped animals suitable for the study were anaesthetised using a portable inhalational anaesthetic machine with a precision vaporizer ("The Stinger", Advanced Anaesthetic Specialists, Gladesville, NSW, Australia) delivering isoflurane (Delvet Isoflurane, Delvet Pty Ltd, Seven Hills, NSW, Australia) in 100% medical-grade oxygen. Animals trapped in Darwin were anaesthetised at the Flora and Fauna Unit of DENR. In remote areas, anaesthesia was performed using a field laboratory set up.

Blood samples of no more than 0.5% of the individual's body weight were collected from suitable superficial veins such as the femoral vein in bandicoots, the lateral tail and medial saphenous veins in possums, the jugular vein in quolls and the ventral tail or femoral vein in brush-tailed rabbit-rats. Whenever available, faecal samples were collected from inside the trap or transport bag and stored at -20ºC within eight hours of collection, until processed.

All aspects of the fieldwork were covered by Murdoch University Animal Ethics Committee (permit number RW2591/13) and Charles Darwin University-NT Animal Ethics Committee (permit numbers A11027 and A13026). Additionally, a permit for access to biological resources under part 8A of the Environment Protection and Biodiversity Conservation Regulations (2000) was obtained for sample collection within Kakadu (permit number RK846).

2.2 Clinical assessment

During the fieldwork, all animals were submitted to a full physical examination conducted by an experienced wildlife veterinarian or veterinary nurse. A range of standard data and assessments such as species, sex, age, and body and health condition were recorded.

Age class was assessed subjectively as adult, sub-adult or juvenile, based on the individual's body size, morphometric measurements and evidence or otherwise of reproductive activity. Molar tooth wear was assessed subjectively in all species other than rodents, and classified on a 4 point scale (nil, mild, moderate or significant wear) to assist in ageing of adults.

Health condition was also assessed subjectively as either normal or abnormal, based on a range of criteria including demeanour (quiet, agitated, highly agitated), state of alertness (unresponsive, reduced response, responsive), condition of the skin and pelage, assessed body condition and the presence of obvious wounds or disease. Abnormal health status was further classified as mild, moderate or severe and acute or chronic in nature.

2.3 Haematology and biochemistry

Blood samples were sent to laboratories in Darwin (BVL) and Adelaide (Gribbles Veterinary Laboratory, Wayville SA, Australia) for full haematological analyses, except packed cell volume (PCV) and total plasma protein (TPP) readings, which were performed in the field using a microcentrifuge and a hand-held refractometer, within 6 hours of sample

collection. In the laboratories, total plasma protein (TPP), total red cell count (TRCC), total white cell count (TWCC), red blood distribution, mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin content (MCHC) and platelet count were determined.

Serum biochemical parameters were determined on an automated analyser (Konelab 20, Thermo Electron, Victoria, Australia). The following variables were measured: sodium, potassium, chloride, urea, creatinine, glucose, total bilirubin, aspartate amino transferase (AST), alanine transaminase (ALT), gamma glutamyl transferase (GGT), alkaline phosphatase (ALP), globulin (Glob), albumin (Alb), albumin/globulin ratio, calcium, phosphate, magnesium, iron and creatine kinase (CK). Both haematological and biochemical tests conducted in the laboratories were performed within 72 hours of sample collection.

2.4 Microscopy and morphometric analysis

Thin blood smears were made from a drop of peripheral blood, air-dried and then fixed with methanol within four hours of sample collection. In the laboratory at Murdoch University, the smears were stained with Wright-Giemsa (Hematek® Stain Pak) using a Hema-Tek Slide Stainer (Ames Company Division, Miles Laboratories Pty Ltd, Springvale Victoria, Australia) and then coverslip mounted using DePeX mounting medium Gurr (Merck Pty. Limited, Kilsyth, Victoria, Australia).

Stained blood films were systematically examined by light microscopy for the presence of blood-borne parasites. Digital light micrograph images of any haemoprotozoans detected were taken at 1000 x magnification using Image-Pro Express software (Media Cybernetics, Inc., Bethesda, Maryland, U.S.A.) and then imported into software Image J (Abramoff et al., 2004) for morphometric analysis. Morphological features were compared with previously reported morphological measurements of corresponding haemoprotozoans infecting Australian wild mammals.

2.5 DNA Extraction

Genomic DNA was extracted from 200 µl of whole blood using a MasterPure™ DNA Purification Kit (EPICENTRE® Biotechnologies, Madison, Wisconsin, U.S.A.), according to the manufacturer's instructions. DNA was eluted in 30 µl of TE buffer provided in the kit and stored at -20ºC until use.

DNA was also extracted from 250 mg of each faecal sample, using a Power Soil DNA Kit (MolBio, Carlsbad, California). The DNA samples were stored frozen (-20 ºC) until processed. A DNA extraction blank (no sample added) was included in each batch of DNA extractions of both blood and faecal samples.

2.6 PCR amplification and sequencing

Initially, five genera of potential protozoan pathogens to Australian wildlife were targeted in the molecular investigation: *Trypanosoma*, *Babesia*, *Theileria*, *Cryptosporidium* and *Giardia*. DNA samples extracted from blood were screened for *Trypanosoma* at the 18S rRNA locus using a nested set of trypanosome-universal primers (SLF/S762R and S823F/S662R) as previously described (Maslov et al., 1996; McInnes et al., 2009). Universal piroplasm primers were used to amplify an approximately 850 bp fragment of the 18S rRNA gene, in a nested PCR performed as described by Jefferies et al. (2007). During the present study, the piroplasm primers also amplified nucleotide sequences from the haemogregarine *Hepatozoon* sp. However, these sequences did not align with previously reported *Hepatozoon* isolates from quendas, due to different primer sets used and thereby amplification of divergent regions of the 18S rRNA. Therefore, a PCR assay specific for haemogregarines was used to produce new sequences suitable for a complete phylogenetic analysis comprising all relevant species. An approximately 900 bp fragment of the *Hepatozoon* sp. 18S rDNA was amplified using a single round haemogregarine specific PCR, as previously described (Perkins and Keller, 2001; Wicks et al., 2006).

DNA extracted from faecal samples was screened by nested PCR for *Cryptosporidium* 18S rDNA as previously described (Ryan et al., 2003). The samples were also tested by nested PCR for *Giardia* spp. at the glutamate dehydrogenase (*gdh*) locus, using external and internal forward primers (GDHeF and GDHiF) designed by Read et al. (2004) and external and internal reverse primers (GDH2 and GDH4) sourced from Caccio et al. (2008), in an assay as previously described (Hijjawi et al., 2016). *Giardia*-positive samples at the *gdh* locus were also screened at the 18S rRNA gene using the external primers RH11/RH4LM and internal primers GiAR18SeR/GiAR18SiR as described by Hopkins et al. (1997) and Read et al. (2004).

The PCRs were performed in 25µl reaction volumes and then run on a 2% agarose gel containing SYBR Safe Gel Stain (Invitrogen, USA). Gel bands were visualized with a dark reader trans-illuminator (Clare Chemical Research, USA) and those corresponding to the expected length were excised and purified using an in-house filter tip method as described (Yang et al., 2013). DNA extraction blanks and positive and negative controls from each PCR batch produced appropriate results.

All purified PCR products were sequenced using corresponding internal reverse primers at a concentration of 3.2 picomoles with an ABI PrismTM Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA) on an Applied Biosystem 3730 DNA Analyzer.

Nucleotide sequences obtained in this investigation were deposited in GenBank under the accession numbers KX361174-84 and KX369229-32.

2.7 Phylogenetic analysis

Phylogenetic analyses were conducted for each group of parasites which produced clean partial nucleotide sequences at the 18S rRNA and/or *gdh* loci. To avoid duplicates, the sequences belonging to the same taxa were firstly aligned among each other by MUSCLE

(Edgar, 2004) using the software in MEGA 6 (Tamura et al., 2013). In cases where two or more sequences were 100% identical, and sourced from the same host species, the longest one containing less ambiguous bases was selected as the group representative isolate for evolutionary inferences. For each phylogenetic analysis, DNA sequences were imported into Geneious R7 and combined with other sequences retrieved from GenBank. Accession numbers for all sequences included in the analyses are provided on the left of each isolate name in the phylogenetic trees. The sequences were aligned by MUSCLE and then exported into MEGA 6 for the selection of the most appropriate nucleotide substitution model, using the dedicated function.

The evolutionary history of new trypanosome isolates with other *Trypanosoma* spp. was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) based on the Kimura 2-parameter model (Kimura, 1980). For the other parasites (*Babesia* spp., *Hepatozoon* spp., *Cryptosporidium* spp. and *Giardia* spp.), the evolutionary histories were inferred by the Maximum Likelihood method, based on the Tamura 3-parameter model (Tamura, 1992).

All positions with less than 95% site coverage were removed. The bootstrap method (500 replicates) was used to assess internal branch reliability and corresponding support values (>60%) were indicated at each node. Estimates of genetic divergence between sequences were generated in MEGA 6 based on the same model used to infer the phylogenetic relationships.

2.8 Statistical analysis

Prevalences (%) were calculated as n^{o} positives/ n^{o} samples x 100. Confidence intervals (95%) were calculated in Microsoft Excel version 2013 using the exact binomial method. Charts representing prevalence and error bars were also produced in Excel version 2013.

Univariate analyses were performed in "R" version 3.0.2 (R Core Development Team, 2013), where the dependent variables were the presence or absence of protozoan parasites. Measures of association were examined for nominal data (location, species, sex, season) and ordinal data (age class, body condition, tooth wear, ectoparasite burden) using the Odds Ratio (OR) with 95% CI, and p–values using the chi-square test (all categories sample sizes >5) or Fisher's exact test (any category sample size <5), with significance set at the level of 0.05.

Continuous data such as PCV, TPP, Alb, Glob, TWCC and body weight were checked for normality using the Shapiro-Wilk test and grouped by infecting parasite genus. Differences for normal data (all variables) were tested for significance using ANOVA. All measures of association and difference were analysed by location, species, and then location and species, where samples sizes permitted. Continuous data could only be evaluated within species (and sexes or age classes) to account for differences between these groups.

3. Results

3.1 Prevalence and molecular characterization

3.1.1 Blood-borne protozoan parasites

Overall, 22.5% (95% CI: 17.0-28.8%) of the animals tested were positive for haemoprotozoans. Molecular analysis based on a trypanosome universal assay revealed the presence of *Trypanosoma* spp. in 39 samples (18.6%; 95% CI: 13.6-24.6%), whereas *Babesia* sp. and *Hepatozoon* sp. were detected in 11 samples each (5.3%; 95% CI: 2.7-9.2%). The prevalence of *Hepatozoon* sp. was also confirmed by a haemogregarine-specific PCR. Overall prevalence estimates of *Trypanosoma* spp., *Babesia* sp., and *Hepatozoon* sp. are represented in Fig 2 (A).

Prevalence of each group of blood-borne protozoans across different host species is shown in Fig 2 (B). The presence of trypanosomes was confirmed in nine out of 38 possums (23.7%; 95% CI: 11.4-40.2%) and in 30 out of 113 bandicoots (26.6%; 95% CI: 18.7-35.7%);

while *Babesia* sp., and *Hepatozoon* sp. were found in bandicoots only, at a prevalence of 9.7% (95% CI: 2.7-9.2%) each. PCR and sequence analyses also revealed that 5% (95% CI: 1.8-10.8%) of the bandicoots were co-infected with *Trypanosoma* sp. and *Hepatozoon* sp. whilst 7% (95% CI: 3.1-13.4%) were co-infected with *Trypanosoma* sp. and *Babesia* sp. Northern quolls and brush-tailed rabbit-rats were negative for blood-borne protozoan parasites (95% CI: 0-7% and 0-41%, respectively).

Prevalence estimates (with 95% CI) of *Trypanosoma* spp., *Babesia* sp. and *Hepatozoon* sp. were also calculated across the five geographical sampling sites (Fig 2-C). Trypanosomes were found in animals from all sites and prevalence was significantly higher in Darwin (68.8%; 95% CI: 45.5-86.9%) and Cobourg (34.1%; 95% CI: 22.5-47.2%), compared to Bathurst (8.4%; 95% CI: 2.6-19.3%), Kakadu (4.6%; 95% CI: 0.2-21.8%) and Groote (4.5%; 95% CI: 0.6-14.6%). This site difference remained when examining the effect within bandicoots only, where the odds of trypanosome infection was significantly higher in bandicoots from Darwin (OR= 13.7, 95% CI: 3.1-59.7, p=0.0004) and Cobourg (OR= 2.9, 95% CI: 1.0-8.1, p=0.04) compared to all other sites. Bandicoots positive for *Babesia* sp. were from three different locations (Darwin, Cobourg and Bathurst), whereas *Hepatozoon* sp. was identified in bandicoots from all locations except Groote. The differences between prevalence of *Babesia* sp. and *Hepatozoon* sp. across locations were not statistically significant.

3.1.2 Enteric protozoan parasites

Molecular investigation revealed that five out of 167 (3.0%; 95% CI: 1.0-6.8%) faecal DNA samples were positive for enteric protozoan parasites. PCR analysis at the 18S rRNA gene confirmed the presence of *Cryptosporidium* sp. in one individual only (0. 6%; 95% CI: 0-3.3%), while four faecal DNA samples (2.4%; 95% CI: 0.7-6.0%) were positive for *Giardia* sp. at the *gdh* locus.

The samples positive for *Giardia* DNA at the *gdh* locus were also tested at the 18S rRNA locus; this confirmed the species to be *G. peramelis,* as previously described in quendas (Hillman et al., 2016). Table 2 shows all enteric protozoan species detected in the present investigation across different host species and locations. Prevalence estimates of *G. peramelis* per host species are presented in Table 3.

3.2 Phylogenetic analysis

3.2.1 Blood-borne protozoan parasites

All trypanosome sequences sourced from bandicoots clustered within the *T. vegrandis* clade of which 24 were 100% identical to each other (represented in the tree as *T. vegrandis* NTB42) and also identical to the previously described *T. vegrandis* genotype 6 (G6) (Fig 3). Another group of five identical sequences, represented by *T. vegrandis* NTB72, also clustered with *T. vegrandis* G6, exhibiting 99% genetic similarity to this isolate (one single nucleotide polymorphism - SNP). Although the phylogenetic position of the remaining isolate (NTB84) within a broader clade comprising *T. copemani, T. gilletti and T. vegrandis* was notable (bootstrap value = 97%), its association with the *T. vegrandis* genotypes was poorly supported (bootstrap values < 60%). In addition, the isolate exhibited 10 SNPs and a relatively larger genetic distance (7%) from its most closely related genotype (*T. vegrandis* genotype 3) at the 18S rRNA gene, suggesting it is a unique genotype (Fig 3).

Phylogenetic analysis also revealed that *Trypanosoma* 18S rDNA partial sequences identified in possums grouped within the *T. noyesi* clade, which is closely related to the *T. cruzi* clade of trypanosomes and comprises isolates sourced from a range of Australian marsupial species. Nine sequences identical to each other and represented by the isolate NTB15 were identical to *T. noyesi* H25 and *T. noyesi* AP-2011a-64 in this analysis. The remaining isolate NTB58 exhibited four SNPs and a small genetic divergence (2%) from NTB15, H25 and AP-2011a-64.

The partial 18S rDNA sequences identified as *Babesia* sp. (n=11) were identical to each other and associated with the *B. macropus* clade (Fig 4). The new reported sequences were genetically distinct from all previously reported isolates and exhibited 99% similarity to *B. macropus* from eastern grey kangaroos (eight nucleotide differences), 98% similarity to a *Babesia* sp. sourced from a woylie (15 nucleotide differences) and 97% similarity to a wallaby-derived isolate characterised as *B. macropus* (21 nucleotide differences). In the present analysis, the kangaroo-derived *B. macropus* diverged 1.2% from its woylie-derived sister species and 2% from wallaby-derived *B. macropus*.

During preliminary analysis, the *Hepatozoon* sp. sequences obtained using piroplasm primers did not align with previously reported sequences sourced from Western Australian marsupials (data not shown). Hence, another set of primers was used to obtain new *Hepatozoon* 18S rDNA sequences, which were then compared to a more representative group of *Hepatozoon* spp. Phylogenetic reconstructions revealed four distinct isolates, either identical or very closely related to previously reported *Hepatozoon* sp. from quendas from WA (Fig 5). A group of eight sequences identical to each other and represented by *Hepatozoon* sp. NT72 in the tree, shared 100% identity with *Hepatozoon* sp. isolates B3, B11 and B12, while sequences NT30 and NT59 showed high similarity to the same three southern brown bandicoot-derived isolates (two SNPs; genetic distance = 0.27%). The *Hepatozoon* sp. NT31 also clustered within the "Australian marsupials" clade and was most closely related to *Hepatozoon* sp. NT30 (three SNPs; genetic distance = 0.4%) and *Hepatozoon* sp. B27 (five SNPs; genetic distance = 0.7%).

3.2.2 Enteric protozoan parasites

Phylogenetic analysis revealed that the *Cryptosporidium* sp. isolate from a possum from Bathurst was 99% similar (one SNP) to *Cryptosporidium* sp. isolate BTP1, previously

reported in common brushtail possums from New South Wales (NSW) (Hill et al., 2008) (data not shown).

Four partial *Giardia* DNA sequences were amplified at the *gdh* locus. Of these, two sequences sourced from possums (NTF 136 and NTF 137) showed high similarity to each other (2 SNP's). For this reason and the relatively short length of isolate NTF137 (340 bp), this isolate was removed from the final phylogeny dataset. Phylogenetic reconstructions placed the sequences in a separate and strongly bootstrap supported clade (100%), basal to all *G. duodenalis* assemblages (Fig 6). A distance similarity matrix confirmed the new sequences to be distinct from all assemblages within the G. *duodenalis* species cluster, with a minimum genetic distance of 12% from *G. duodenalis* assemblage C (from a dog). Unfortunately, *gdh* sequences from the recently described *G. peramelis* from quendas (Hillman et al., 2016) were not available in GenBank. Therefore, partial 18S rRNA gene sequences were generated and phylogenetic reconstructions at this locus revealed that the newly reported *Giardia* from northern Australian mammals grouped with the unique *G. peramelis* in a well-supported clade (bootstrap value = 82%) (data not shown) and exhibited 1% genetic distance from *G. peramelis*. This finding suggests the isolates belong to the same species, as in this analysis the minimum genetic distance between two distinct species was 4% (between *G. microti* and *G. duodenalis* D). At the 18S locus, the *G. peramelis* isolates were 85% similar to their most closely related species (*G. duodenalis* assemblage C).

3.3 Microscopy and morphometric analysis

Light microscopy confirmed the presence of *Hepatozoon* sp. in two out of 11 animals positive for this parasite by PCR (Fig 7). The gamonts (final development stage within the vertebrate host) observed were oval, relatively large, and easily detected by microscopy analysis of Giemsa stained blood films. Both intra and extra-erythrocytic forms were

observed, all of them exhibiting a sub-terminal granular nucleus, slightly narrower than the gamonts' width.

Eleven organisms were measured in total. The parasites' lengths ranged from 7.5 – 9.3 μm (mean =8.47 μm ± 0.15) and the observed range for width was 3.02 – 4.54 μm (mean = 3.61 μm ± 0.11).

The organisms were visually detected in blood films from bandicoots (NT2 and NT30), which provided *Hepatozoon* DNA sequences identical to each other and 99.6% similar to *Hepatozoon* sp. isolates B8, B13, B27 and B28 from Western Australian bandicoots. All blood films analysed were negative for other blood-borne protozoan parasites.

3.4 Potential clinical impact of protozoan parasites

Of individual animals examined during sample collection, the majority (91.8%) were assessed to be in good health and body condition. There were no obvious clinical associations between the presence or absence of protozoan parasites of any type and the individual's clinically assessed health status ($p>0.05$).

Bandicoots positive for trypanosomes had significantly lower PCV ($p=0.046$) than negative bandicoots. Animals with lower tooth wear scores (0, 1) were more likely to be trypanosome positive than those with higher tooth wear scores (2, 3) (OR=2.74, 95% CI 1.3-5.7, $p=0.006$). For both possums and bandicoots, no significant associations were found for trypanosome detections when examined against TPP, Glob, Alb, TWCC, age class or sex ($p>0.05$). No seasonal association was found in haemoparasite presence in this study.

There was no detectable association with abnormal health parameters associated with *Babesia* sp., *Hepatozoon* sp., *Cryptosporidium* sp. and *Giardia* sp.

4. Discussion

To the authors' knowledge, this is the first identification of protozoan parasites from the genera *Trypanosoma, Babesia, Hepatozoon, Cryptosporidium* and *Giardia* in native mammals from northern Australia, using molecular methods.

Despite not being visualized in any of the blood films, trypanosomes were the most prevalent haemoprotozoa among the animals tested according to the molecular assays performed, particularly amongst bandicoots and possums. *Trypanosoma vegrandis* has been reported previously in a range of marsupials from WA, NSW and Queensland (Qld) (Paparini et al., 2011; Botero et al., 2013; Barbosa et al., 2016b). This study reports, for the first time, the occurrence of *T. vegrandis* in the NT and its host range extension to include the northern brown bandicoot, providing further evidence that *T. vegrandis* is ubiquitous in the Australian continent.

Phylogenetic reconstructions also revealed a novel *Trypanosoma* genotype (NTB84) infecting bandicoots, which exhibited a relatively large genetic divergence from *T. vegrandis* genotype G6 at the 18S locus. This fact, combined with the poor association of NTB84 with the *T. vegrandis* clade, suggests that it could potentially be a novel species clustering in a monophyletic assemblage with *T. copemani*, *T. gilletti* and *T. vegrandis*. Additional molecular characterization is necessary to confirm this hypothesis as reliance on 18S rDNA data alone is inadequate for species delimitation in trypanosomes (Hamilton and Stevens, 2011).

Despite recent research and greatly improved knowledge of its taxonomy and distribution, there remains limited data on the impact of *T. vegrandis* on the health of Australian marsupials. A previous study reported pathological changes in woylies co-infected with *T. vegrandis*, *T. noyesi* H25 and *T. copemani* (Botero et al., 2013). However, a significantly higher prevalence of *T. vegrandis* (either in single or mixed infections) in healthy woylie populations has led to the hypothesis that *T. vegrandis* could potentially

moderate the pathogenicity of *T. copemani* in this host species (Botero et al., 2013; Thompson et al., 2014b).

Although no association between physical signs of disease and infection with trypanosomes was observed, analyses of blood parameters revealed that bandicoots positive for *Trypanosoma* spp. were more likely to have a lower PCV value. This finding is consistent with the results from McInnes et al. (2011), who associated the presence of *T. gilletti* with lower PCV values in koalas with signs of concomitant diseases. Lower PCV values are indicative of anaemia, an abnormality associated with trypanosomiasis in animals and humans (Chisi et al., 2004; Mackie et al., 2015). Another interesting finding is that individuals with lower tooth wear scores (and likely younger) were more likely to be positive for *Trypanosoma* spp. This could be explained by the potential lower level of immunocompetence in younger animals compared to adults.

The phylogenetic position of the two new possum-derived *T. noyesi* genotypes is consistent with previous studies showing intraspecific diversity within *T. noyesi*, and its genetic proximity to *T. cruzi*, the causative agent of human Chagas disease (Noyes et al., 1999; Stevens et al., 1999; Hamilton et al., 2007; Paparini et al., 2011; Botero et al., 2013; Botero et al., 2016). The finding of *T. noyesi* in possums from the NT provides evidence of a wider geographical distribution, as this trypanosome species had only been reported in marsupials from Victoria and WA to date (Noyes et al., 1999; Paparini et al., 2011; Botero et al., 2013).

Phylogenetic analysis revealed that the *Babesia* sequences from the present study formed a separate group closely associated with *B. macropus* from eastern grey kangaroos (Dawood et al., 2013); yet the genetic distance of 1% (10 nucleotide differences) is smaller than the distance amongst other named species in the present analysis, and therefore not sufficient to define the new group as a distinct species. The paucity of morphological

observation also hinders the definition of the isolates reported, as they could potentially be *B. thylacis*, for which no genetic data is available (Mackerras, 1959; Bangs and Pumomo, 1996). Moreover, no clinical signs of disease were associated with infection in bandicoots, whereas *B. macropus* appears to be highly pathogenic to its marsupial hosts (Dawood et al., 2013; Donahoe et al., 2015). Morphology associated with phylogenetic analyses based on a more variable genetic marker would help to better determine the evolutionary relationships amongst the marsupial-derived *Babesia* spp.

The molecular prevalence of *Hepatozoon* sp. in northern brown bandicoots from the NT was lower than previous prevalence estimates reported in southern brown bandicoots from WA (51%) (Wicks et al., 2006). Gametocytes of *Hepatozoon* sp. were only observed by microscopy in two out of 11 samples that were positive by PCR. This observation is consistent with other reports that light microscopy is less sensitive than PCR-based methods for protozoan parasite detection, especially where infection levels are low (Morgan et al., 1998; Wicks et al., 2006; Pizarro et al., 2007; Tavares et al., 2011). Hence, we recommend the use of molecular analysis as the most effective screening method of blood samples for *Hepatozoon* sp.

This is the second published study that has employed molecular methods for the detection of *Hepatozoon* spp. in Australian marsupials. Phylogenetic analysis revealed that all bandicoot-derived isolates grouped together, exhibiting low genetic distances from each other. Although isolates NT30 and NT31 were placed in relatively more distant positions compared to NT59 and NT72, the high genetic similarity between them and their most closely related bandicoot-derived isolate (99.6% and 99.7% respectively) suggests that all genotypes belonging to the "Australian marsupials" clade, represent small variations of the same un-named species.

The morphological features and measurements of *Hepatozoon* observed in the present study were consistent with previous descriptions of *H. peramelis,* for which no DNA sequences are available (Welsh and Dalyell, 1909; Mackerras, 1959) and subsequent isolates from other Australian marsupials (Bettiol et al., 1996; Wicks et al., 2006). Based on this and the fact that the new reported organisms did not genetically differ from the parasites described by Wicks et al. (2006), we believe that they are most likely to be *H. peramelis*. If all *Hepatozoon* sp. found in bandicoots to date are in fact *H. peramelis*, then our study provides supporting evidence that this parasite species has a wide distribution, occurring in NSW, Qld, Tasmania, WA and NT.

The prevalence of the *Cryptosporidium* sp. BTP1 in possums from the NT (0.6%) was lower than the prevalence of genotypes BTP1 and BTP2 in possums from eastern Australia (5.6-11.3%) (Hill et al., 2008). In addition, previously reported prevalence estimates of a range of *Cryptosporidium* spp. and genotypes in Australian marsupials (1.9%-19.3%) were higher compared to the present results (Power et al., 2003; Power et al., 2004; Cox et al., 2005; Power et al., 2005; McCarthy et al., 2008; Ng et al., 2011; Yang et al., 2011; Ryan and Power, 2012; Dowle et al., 2013; Nolan et al., 2013; Koehler et al., 2014; Vermeulen et al., 2015; Koehler et al., 2016b; Zahedi et al., 2016a; Zahedi et al., 2016b). The absence of clinical signs of disease associated with *Cryptosporidium* sp. BTP1 indicates a stable host-parasite relationship typical of host-adapted *Cryptosporidium* species (Hill et al., 2008; Power, 2010). Our study also corroborates the hypothesis that the BTP1 genotype may be host-specific and provides evidence of its wider geographic distribution. Although *Cryptosporidium* isolates have been found in exotic rodents (*Rattus rattus*) in the NT, raising concern as to whether they could pose any harm to native wildlife (Paparini et al., 2012b), no rodent genotypes were detected in the animals examined, including native rodents (brush-tailed rabbit-rats).

The overall low prevalence of *Giardia* sp. found in native mammals from the NT is in line with the previous observation that Giardia is a remarkably rare parasite in native wildlife in WA (Thompson et al., 2010). The confirmation of *G. peramelis* in the present study was based on sequence analysis at the 18S rRNA gene, as *gdh* nucleotide sequences for *G. peramelis* were not available (Hillman et al., 2016). The present study provides the first genetic sequences for *G. peramelis* at the *gdh* locus and phylogenetic analysis further clarifies its taxonomic position, supporting its species status and extending its host range to include common brushtail possums, northern brown bandicoots and brush-tailed rabbit-rats, contradicting previous assumptions that *G. peramelis* was host-specific (Adams et al., 2004; Thompson et al., 2010; Hillman et al., 2016).

In the present study, animals positive for protozoan parasites were detected at all sampling sites but in general there was no significant difference in prevalence between degraded and less degraded environments. For example, the prevalence of trypanosomes was significantly higher in Darwin compared to Kakadu, Bathurst and Groote ($p=0.05$); however not significantly different from Cobourg, which is considered to have a more intact small mammal assemblage than other areas. Factors such as vector distribution and host preferences presumably play a role in the geographic distribution of these protozoans in native mammals from the NT, and therefore needs to be investigated. Temporal and spatial analyses involving a larger sample size are also required to determine whether there is a link between specific pathogens and population declines.

Overall, the health of individuals examined did not appear to be adversely affected by infection with protozoan parasites. However, these findings do not exclude potential risk from infection, as even host-adapted parasites can pose a threat to wildlife in the presence of ecological changes and other stress factors. In addition, a lack of associated clinical signs alone is not sufficient to exclude parasitic diseases from the factors that may be contributing

to the decline events in the NT. For instance, trypanosomes have been implicated in reduced fitness and increased susceptibility to predators in woylies (Thompson et al., 2014b). In this respect, the removal of parasitised and therefore compromised individuals from the population (e.g. by predation or other secondary mechanisms) could bias the results of prevalence surveys towards individuals with greater immunocompetence (and hence lower parasitism).

In conclusion, this study provides valuable baseline data on the prevalence and genetic diversity of blood-borne and enteric protozoan parasites infecting native mammal species in the NT. The knowledge generated provides a benchmark for future investigation of the spatial and temporal dynamics of parasitic infection in the NT; work which is essential to determine whether these organisms are contributing to the decline of native mammal populations. Furthermore, ongoing studies are recommended as exotic pathogenic parasites could enter Australia from the northern part of the continent and have devastating effects on highly susceptible and naïve populations.

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Fig. 1 Geographic representation of **s**urvey areas within the NT and location of the territory in the Australian continent (insert).

Fig. 2 Prevalence of *Trypanosoma* spp., *Babesia* sp. *and Hepatozoon* sp. in native mammal species from the NT, by PCR. (A) Overall prevalence. (B) Prevalence per host species (C) Prevalence across surveyed locations. Sample sizes are indicated in the figure's legends. Error bars represent 95% confidence interval. Within each graph (A, B and C), values represented by different letters are statistically distinct ($p<0.05$).

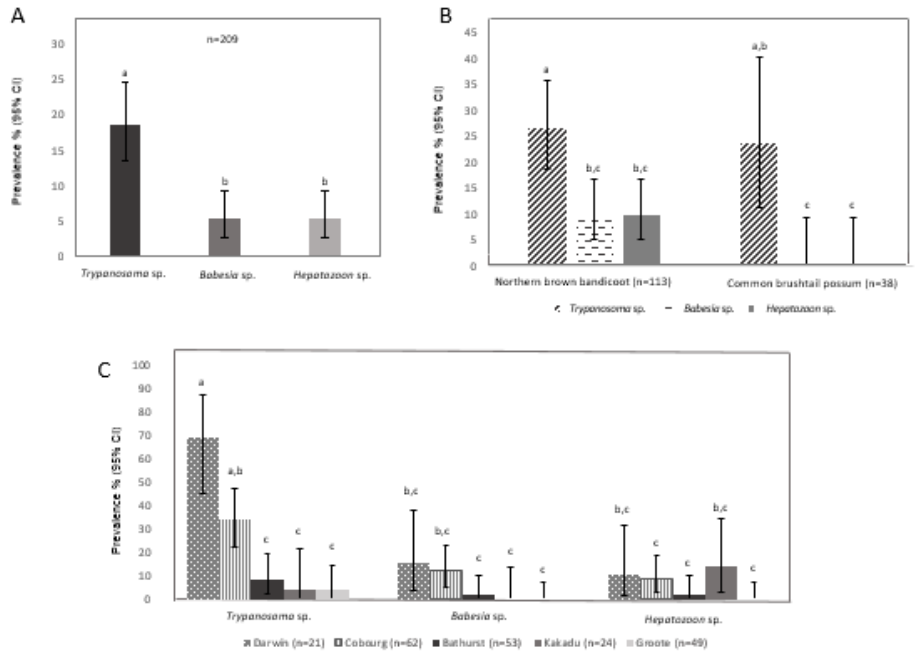

Fig. 3 Evolutionary relationships of new reported marsupial-derived trypanosome isolates with other *Trypanosoma* spp., based on 18S rDNA partial sequences (~350bp). Evolutionary history was inferred using the Neighbor-Joining method, based on the Kimura 2-parameter method (Kimura, 1980). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.

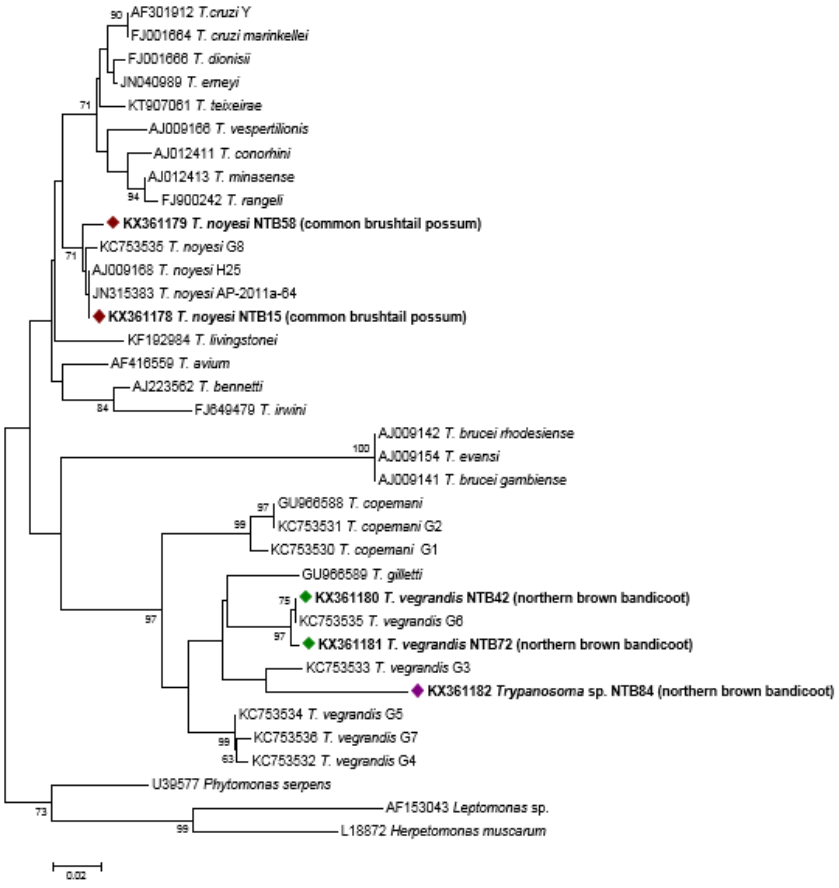

Fig. 4 Phylogenetic analysis of *Babesia* spp. with the bandicoot-derived *Babesia* sp., based on 18S rDNA partial sequences (~650bp). Evolutionary history was inferred using the Maximum Likelihood method, based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.

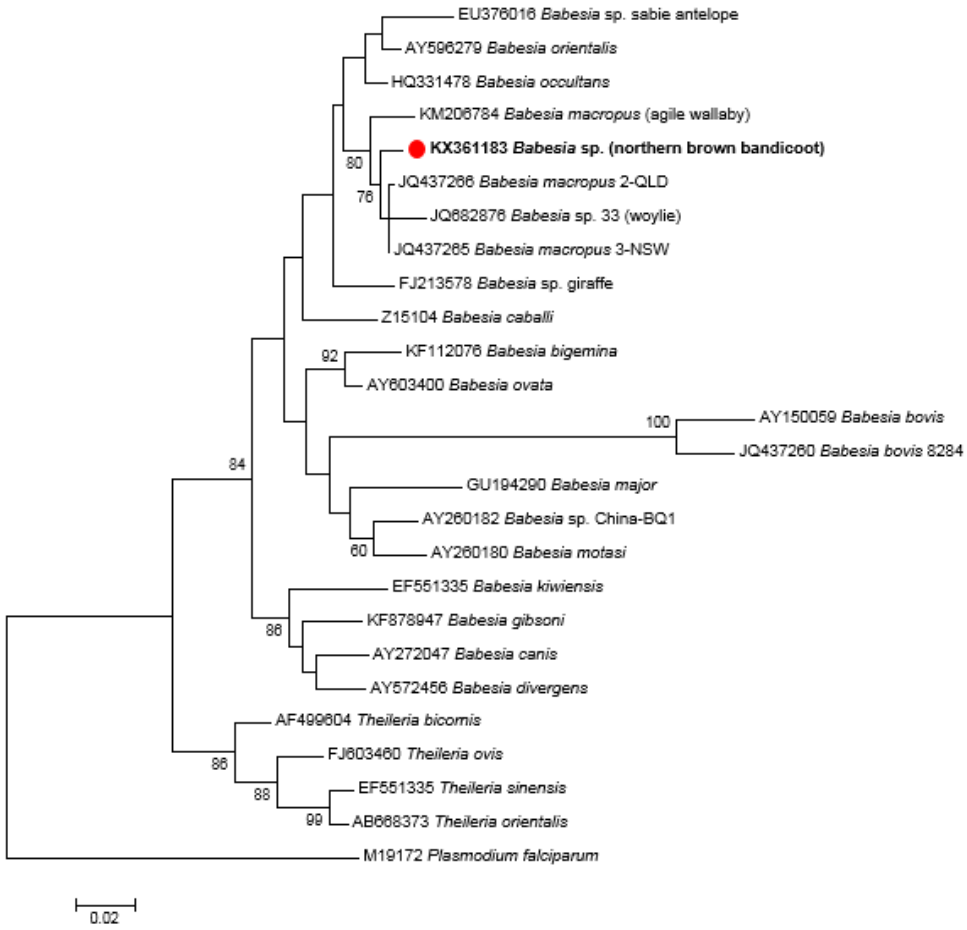

Fig. 5 Evolutionary relationships of new reported bandicoot-derived isolates of *Hepatozoon* sp. with other *Hepatozoon* spp., based on 18S rDNA partial sequences (~800bp). Evolutionary history was inferred using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap values (>60%) based on 500 replicates are indicated at the left of each supported node. The scale bar is the proportion of base substitutions per site.

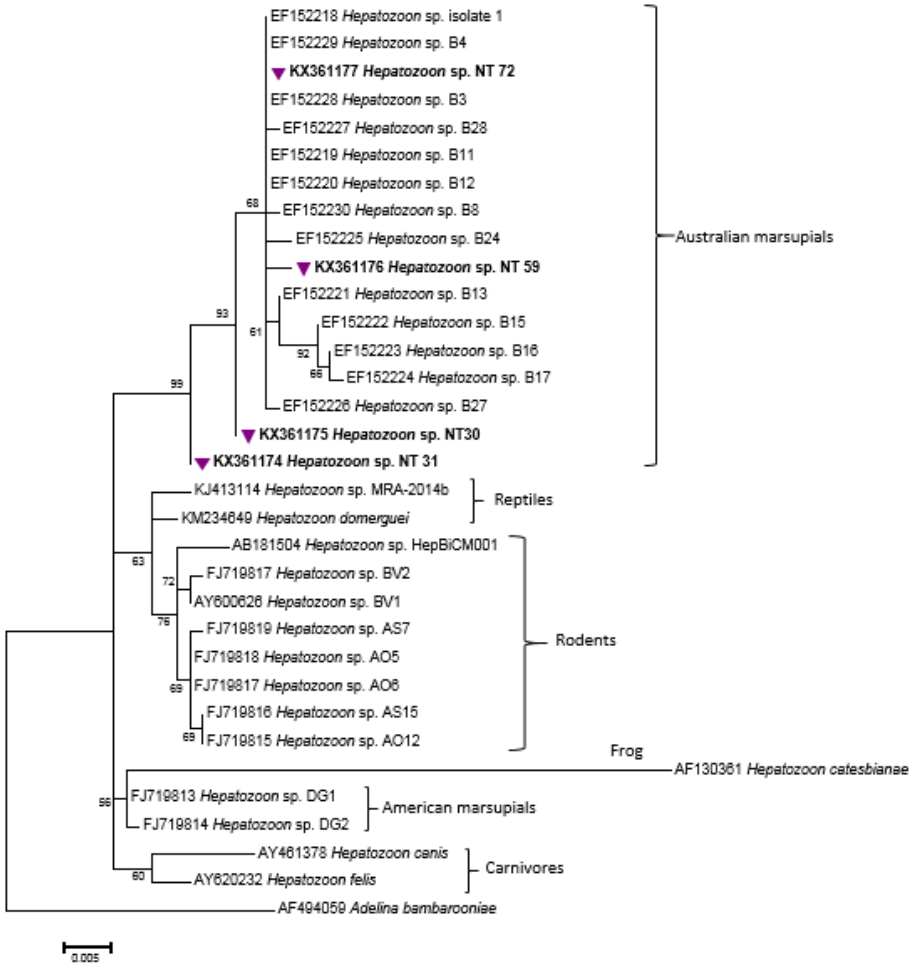

Fig. 6 Phylogenetic analysis of *Giardia peramelis* and *Giardia duodenalis* assemblages, based on GDH partial sequences (~400 bp). Evolutionary history was inferred using the Maximum Likelihood method, based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap support (>60%) is indicated at the left of each node. The scale bar is the proportion of base substitutions per site.

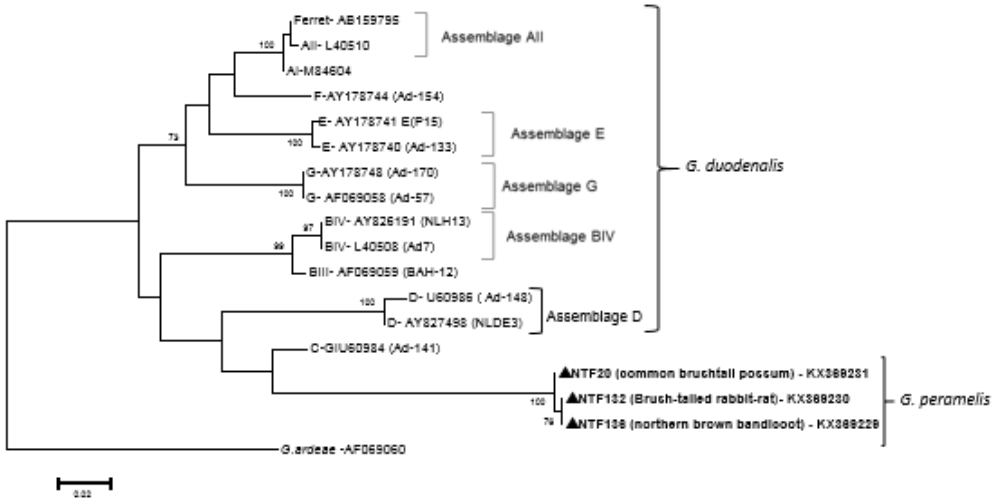

Fig. 7 (a, b) Microscopic detection of *Hepatozoon* sp. in Giemsa stained blood films from a northern brown bandicoot (*Isoodon macrourus*). Scale bars represent 10 µm.

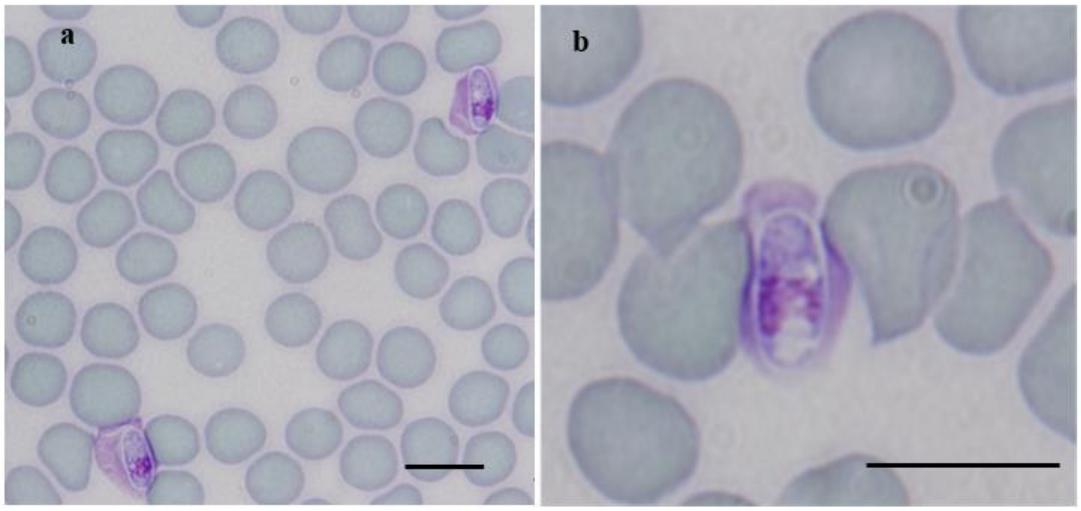

Table 1 Number of blood and faecal samples collected from four target mammal species across five locations in the Northern Territory of Australia

	Blood						Faeces					
Mammal species	D	K	B	G	C	Total	D	K	B	G	C	Total
NB bandicoot	12	8	26	13	54	113	2	6	10	0	44	62
Brushtail possum	9	1	26	0	2	38	12	1	43	0	2	58
Northern quoll	0	15	0	36	0	51	0	10	0	0	0	10
BT rabbit-rat	0	0	1	0	6	7	0	0	0	0	37	37
Total	21	24	53	49	62	209	14	17	53	0	83	167

*D = Peri-urban areas around Darwin/ K = Kakadu National Park/ B = Bathurst Island/ G = Groote Eylandt/ C = Cobourg Peninsula/ NB bandicoot = northern brown bandicoot/ BT rabbit-rat = brush-tailed rabbit-rat

Table 2 Enteric protozoan parasites isolated from three native mammal species from the Northern Territory of Australia

Sample code	Parasite species	Host species	Location
NTF 20	*Giardia peramelis*	Northern-brown bandicoot	Bathurst
NTF 26	*Cryptosporidium* brush-tail possum genotype	Brush-tail possum	Bathurst
NTF 132	*Giardia peramelis*	Brush-tail rabbit-rat	Darwin
NTF 136	*Giardia peramelis*	Brush-tail possum	Darwin
NTF 137	*Giardia peramelis*	Brush-tail possum	Darwin

Table 3 Prevalence of *Giardia peramelis* in native mammals from northern Australia

Host species	Nº positives/ tested	Prevalence (%)	95% CI
Northern brown bandicoot	1/62	1.61	0-8.7
Common brushtail possum	2/58	3.45	0.4-11.9
Northern quoll	0/10	0	0-30.8
Brush-tail rabbit-rat	1/37	2.7	0.1-14.2
Total	4/167	2.4	0.7-6.0