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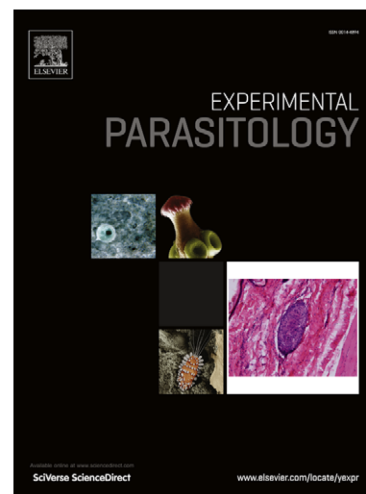

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Andrea Paparini, Una M Ryan, Kris Warren, Linda M McInnes, Paul de Tores, Peter J Irwin

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Identification of novel *Babesia* and *Theileria* genotypes in the endangered marsupials, the woylie (*Bettongiapenicillataogilbyi*) and boodie (*Bettongialesueur*).

Andrea Paparini[a], Una M Ryan[a*], Kris Warren[a], Linda M McInnes[a], Paul de Tores[b]and Peter J Irwin[a].

[a]Division of Health Sciences, Murdoch University, Murdoch, Western Australia, 6150, Australia; [b]Department of Environment and Conservation, Western Australian Wildlife Research Centre, Perth, Western Australia, 6946, Australia.

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[*]Corresponding author. Mailing address: Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, 90 South Street, Murdoch, Western Australia, 6150, Australia. Phone: 61 8 9360 2482. Fax: 61 893104144. E-mail: Una.Ryan@murdoch.edu.au

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ABSTRACT

Piroplasms, which include the genera *Theileria* and *Babesia*, are blood-borne parasites transmitted mainly by tick vectors. Relatively little is known about their prevalence and clinical impact in Australian marsupials. In the present study the occurrence and molecular phylogeny of these parasites were studied in both wild and captive marsupials from Western Australia (WA) and Queensland (QLD).

Blood samples were screened by microscopy and molecular methods, using PCR and DNA sequencing of the 18S ribosomal RNA gene (18S rDNA). Overall, 7.1% of the blood samples (8/113) were positive for piroplasm 18S rDNA. *Theileria*and *Babesia* rDNA was detected in 0.9% (1/113) and 6.2% (7/113) of the animals respectively. The single *Theileria* positive was identified in one of three boodies(*Bettongialesueur*) screenedfrom a wildlife rehabilitation centre in WA,while all seven *Babesia* positives were detected in WA in wild captured woylies(*Bettongiapenicillataogilbyi*). Small intraerythrocytic inclusions were observed in blood films made from 6 of these individuals.This is the first report of a *Babesia*sp. in woylies, and *Theileria*sp. in boodies.

Phylogenetic analysis indicated that the woylie-derived *Babesia* was genetically distinct and most closely related to *B. occultans*, the causative agent of a benign form of cattle babesiosis(genetic similarity 98.4%). The *Theileria* identified was most closely related to the marsupial-derived species *T. penicillata* from the woylie, *T. brachyuri*from the quokka (*Setonixbrachyurus*), and *Theileria* sp. fromthe long-nosed potoroo (*Potoroustridactylus*).

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Keywords: Piroplasms;marsupials; wildlife;*Babesia*;*Theileria*;molecular phylogeny.

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1. Introduction

Piroplasm is a generic term to describe haemo-protozoan parasites, belonging to the order Piroplasmida (phylum Apicomplexa). This *taxon* includes the genera *Babesia* and *Theileria* which are intra-erythrocytic parasites,mainly transmitted by tick vectors, causing significant economic losses for their high pathogenicityin domesticated animals like cattle, sheep, horses, and goats, and significant clinical disease in companion animals (Irwin, 2010). Little is known about the prevalence and clinical impact of piroplasms on marsupials.

There are at least seven named *Theileria* spp. in Australian native mammals: *T. tachyglossi* in the echidna (family Tachyglossidae)(Priestley, 1915); *T. peramelis* in the southern brown bandicoot (*Isoodonobesulus*), *T. ornithorhynchi* in the platypus (*Ornithorhynchus anatinus*)(Mackerras, 1959); *T. gilberti* in Gilbert's potoroo (*Potorousgilbertii*) (Lee, et al., 2009); *T. brachyuri* in the quokka(*Setonixbrachyurus*), *T. fuliginosa* in the western grey kangaroo (*Macropusfuliginosus*), and *T. penicillata* in the woylie (*Bettongiapenicillataogilbyi*)(Clark and Spencer, 2007). Unnamed piroplasm species (presumed *Theileria* spp.) have been found in the northern brown bandicoot (*Isoodonmacrourus*), long-nosed bandicoot (genus *Perameles*) and long-nosed potoroo (*Potoroustridactylus*) (Clark, 2004). There are at least two named *Babesia* spp. in Australian marsupials and monotremes: *B. tachyglossi* in the echidna (Mackerras, 1959), *B. thylacis* in the bandicoot (order Peramelemorphia) and quoll (genus *Dasyurus*) (Bangs and Purnomo, 1996, Mackerras, 1959), plus a number of un-named piroplasms (presumed *Babesia* spp.) in the agile antechinus(*Antechinus agilis*), echidna, and Proserpine rock-wallaby (*Petrogalepersephone*)(Clark, 2004)

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and eastern grey kangaroo(*Macropusgiganteus*)(Ladds, 2009). In addition, several *Babesia* spp. have been reported in South American opossums (family Didelphidae)(da Serra Freire, 1979, Herrera and Urdaneta-Morales, 1991).

In the present study, we examined the occurrence and molecular phylogeny of *Babesia*spp. and *Theileria*spp., in both wild and captive Australian marsupials in Western Australia (WA) and Queensland (QLD).

2. Materials and Methods

2.1 Animal sources

A total of 113 blood samples were collected from wild and captive marsupials, and screened by molecular methods and microscopy for piroplasm presence (Table 1).

Wild animals were trapped with approvalof the Murdoch University Animal Ethics Committee (Permit W2284/09), using standard procedures. Net weight, body condition, presence and condition of joeys in the pouch, and evidence of illness or injury were recorded. Of the 113 collected blood samples, 61 originated from 4 species of wild Australian marsupials trapped in 2009, in the Jarrah Forest region, south-east of Dwellingup, WA (32°42'51.91"S; 116° 3'43.07"E). This group consisted of: 18 chuditch (*Dasyurusgeoffroyii*), 24 common brushtail possums (*Trichosurusvulpecula*), 4 southern brown bandicoots and 15 woylies.

Blood samples (n=33) were also collected from 8 marsupials species sheltered at the Kanyana Wildlife Rehabilitation Centre in Perth, WA, and included: 3 boodies (*Bettongialesueur*), 5 juvenile western red kangaroos (*Macropusrufus*), 4 juvenile western grey kangaroos, 2 wallaroos (family Macropodidae), 8 woylies, 7 bilbies

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(*Macrotislagotis*), 3 western barred bandicoots (*Perameles bougainville*), and 1 chuditch.

Additional blood samples were collected from wild northern quolls (*Dasyurushallucatus*) (n=6) captured in regions surrounding Cairns, QLD, and from wild western ringtail possums (*Pseudocheirusoccidentalis*) (n=13), collected from Locke Nature Reserve Busselton, WA (33°38'59.80"S; 115°20'40.60"E).

Blood samples were collected using the following sites for the different marsupials:i) Lateral tail vein: common brushtail possums, woylies, boodies, kangaroos, wallaroos, bilbies, western ringtail possums;ii) Jugular or Femoral vein: southern brown bandicoots, chuditch, western barred bandicoots, northern quolls. Blood was collected in potassium EDTA-treated microtubes (Sarstedt, Germany), and stored frozen at -20°C until processing.

2.2 Microscopy

A single drop of peripheral blood was used to make thin blood films which were stained with a modified Wright's stain using an Ames Hema-Tek slide stainer (Bayer, Germany). Stained blood films were systematically examined using a BX50 microscope (Olympus, Japan) with screen views generated by a DP Controller (version 3.2.1.276, Olympus, Japan). A total of 200 fields of the central monolayer and the feather regions of each blood film were initially examined for intra-erythrocytic inclusions at x400 magnification. If inclusions were observed, these were further evaluated at x1000 magnification and if confirmed as piroplasms,the morphology noted.The film was then evaluated by examining a further 200 high power (x1000) fields.

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2.3 Molecular analyses

Total genomic DNA was isolated, according to the manufacturer's instructions, from whole blood/EDTA (100 µL), using the MasterPure Purification Kit (Epicentre Biotechnologies, USA). Mock extractions were carried out from an equal volume of sterile molecular-grade water, to exclude DNA contaminations in reagents and consumables.

A nested set of universal piroplasm primers was used to amplify an 850bp fragment of the 18S ribosomal RNA gene (18S rDNA) as previously described(Jefferies, et al., 2007).All amplifications performed included negative and positive controls, consisting of sterile molecular-grade water, and genomic DNA preparations from piroplasm-infected animals identified (and sequenced) during previous analyses, respectively. All positives were also amplified using the primers: BT18SF1 (5'-ACCTGGTTGATCCTGCCAGTAG-3') and BT18SR1(5'-GCAGGTTCACCTACGGAAACC-3'), followed by BT18SF2 (5'-TTGTAGGGCTAATACAYGYTCG-3') and BT18SR2 (5'-CACGGTCCGAATAATTCACC-3') to produce a 1,466bp fragment as previously described (Di Placido et al., unpublished).

PCR products were run on a 1% agarose gel containingSYBR Safe Gel Stain (Invitrogen, USA), and visualized with a dark reader trans-illuminator (Clare Chemical Research, USA). Bands corresponding to the expected length were excised, purified using a MO BIO UltraClean DNA purification kit (MOBIO Laboratories, USA), and sequenced using an ABI Prism Terminator Cycle Sequencing kit (Applied Biosystems, USA), on an Applied Biosystem 3730 DNA Analyzer. Sequences

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obtained during the present study were submitted to GenBank (accession numbers JQ682872 to JQ682879)

2.4 Phylogenetic analysis

Phylogenetic analyses were conducted on the sequences obtained during the present study and additional sequences retrieved from GenBank. Sequencing chromatogram files were analysed by FinchTV 1.4 (http://www.geospiza.com/Products/finchtv.shtml), and imported into Bioedit Sequence Alignment Editor (Hall, 1999), and MEGA 5 (Tamura, et al., 2011), for manipulations and alignment by CLUSTAL W (Larkin, et al., 2007). After selecting the most appropriate evolutionary model by MEGA 5, maximum likelihood (ML) based on the Tamura 3-parameter model (Tamura, 1992), maximum parsimony (MP), and neighbor-joining (NJ) trees were constructed using MEGA 5. Models with the lowest BIC scores (Bayesian Information Criterion) were selected. Estimates of evolutionary divergence between sequences were calculated using MEGA 5, using the Tamura 3-parameter model. Evolutionary rate differences among sites were modelled using a discrete Gamma distribution (number of categories = 6). The rate variation model allowed for some sites to be evolutionarily invariable, and in all analyses performed (including distance computations) all positions containing gaps and missing data were eliminated (i.e., “complete deletion” option).

3. Results

3.1 Piroplasmdetection

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Over the entire study, 7.1% (8/113) of the blood samples were positive for piroplasm 18S rDNA.*Theileria*spp. and *Babesia* spp. rDNA was found, respectively, in 0.9% (1/113) and 6.2% (7/113), of all the blood samples screened by molecular methods (Table 1). The single *Theileria* positive was identified in one of the three screened boodies from Kanyana: a detection rate of 3% (1/33), in this sample source. *Babesia* sp. 18S rDNA was found in 11.5% (7/61) of the screened animals from Dwellingup, and 46.7% (7/15) of the woylies captured in this location (Table 1).*Babesia* organisms were not detected in the woylies from the Kanyana Wildlife Rehabilitation Centre.Of the woylies that were positive for *Babesia*, the body condition was fair to good. Some individuals were suffering from alopecia, but this was attributed to the capture process, and no other clinical information was available.

3.2 Microscopy

Blood films were available for review from 50 individuals from Dwellingup and 3 boodies from Kanyana (Table 1).Of the 7 samples positive for *Babesia*by PCR, blood smears were available for 6 individuals, and piroplasms were observed in 5 of these (Figure 1).The single boodie that was positive for *Theileria* by PCR also had small numbers of piroplasms detected in the blood films (Figure 2).

In all cases where piroplasms were observed, the organisms were single intraerythrocytic inclusions, small in size (measuring 0.8-2μm in diameter) and few in number; parasitaemias in all cases were <0.01%.No morphological differences were discernible between the piroplasms observed in the blood of the woylies (*Babesia* spp.) and in the boodie (*Theileria* sp.), although the total number of organisms

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detected in the latter sample was low.Furthermore, no inclusions were observed in any lymphocytes or other white blood cells of the boodie.

3.3 Sequence analysis and phylogeny

Seven partial *Babesia* sp. 18S rDNA sequences,with lengths ranging from about 1,000bp to 1,375bp, were obtained from an equal number of woylies. Thesesequences were 100% identical. The boodie-derived *Theileria* sp. 18S rDNA sequence was 1,443bp long. A set of shorter (615-852bp) *Theileria* sp. sequences available from GenBank, which showed high similarity with the boodie-derived genotype were included in the alignment. Analysis involved 49 nucleotide sequences, and after removal of gaps and missing data, there were a total of 527bpin the final dataset.For comparison, analysis of a subset of longer sequences (n=42; ≥1,303bp), was also conducted.

Comparison of the trees constructed using the shorter alignment, and the ML, MP, and NJ methods, showed analogous topology. The woylie-derived *Babesia* sp. genotype formed a unique clade, strongly supported by all methods (bootstrap values99%) (Figure 3).This new genotype displayed ≤2.0% genetic distance from a group of sequences previously found in cattle, and maximum similarity (98.4%) with *B. occultans*(Gray and de Vos, 1981), and two un-named *Babesia* spp. (genotypes bovine and Kashi 2).Compared to *B. occultans*, there were 8 single nucleotide polymorphisms (SNPs), over the527bp-long processed alignment, or 19 SNPs, when the longer alignment was considered (1,147positions in the final processed dataset).

The *Theileria* sp. from the boodie clustered with a selection of short sequences previously isolated from various marsupials, such as*T. brachyuri* from the quokka

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(Clark and Spencer, 2007), *T. penicillata* from the woylie(Clark and Spencer, 2007), *T. gilberti* from the Gilbert's potoroo(Lee, et al., 2009), *T. fuliginosa* from the western grey kangaroo (Clark and Spencer, 2007), and *Theileria* sp. from the long-nosed potoroo (*Potoroustridactylus*)(Lee, et al., 2009). The group of marsupial-derived *Theileria* genotypes including the isolate from the boodie, showed bootstrap values ranging from 62% (ML) to 84% (NJ) (Figure 1). The genetic distance between the *Theileria* sp. from the boodie, and the quokka- woylie- and long-nosed potoroo-derived genotypes was 0.2% (1 SNP in the final processed dataset).

4. Discussion

In the present study, two marsupial species out of the twelve screened were found positive for piroplasm-specific DNA, and the overall detection rate of piroplasm-infected animals was7.1% (8/113). Few studies have examined the prevalence of piroplasms in marsupials. One study reported a prevalence of 100% (16/16) for *T. gilberti* from the critically endangered Gilbert's potoroo(Lee, et al., 2009), and anotherrecent studyreported aprevalence of 80.4%for*T. penicillata* in woylies (Rong et al., unpublished). In the present study, none of the 23 woylies examined from Dwellingup (n=15) and Kanyana (n=8), was positive for *T.penicillata*, or any other *Theileria* species.*Babesia* was however identified in the woylies at a prevalence of 6.2%, but only in the wild-captured woylies from the Dwellingup forest. No *Babesia*spp. infections weredetectedby molecular or microscopic examinations in the woylies sheltered inKanyana.

Microscope analysis of blood smears indicated the presence of piroplasms in 6 out of 8 PCR positive samples.The size range of these organisms approximates to

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those referred to as "small" piroplasms with sizes typically ranging from 1-2.5 µm (Mahoney, 1977) and 1-2 µm (Mehlhorn and Schein, 1985), consistent with *Theileria* and the 'small' *Babesia* spp., as confirmed by the results of our phylogenetic analysis. One of the taxonomic characteristics of the genus *Theileria* is an exo-erythrocyticlife-cycle stage in leucocytes, but no inclusions were observed in the lymphocytes or any other type of white blood cells in the blood films examined from the *Theileria*-positive boodie.This study confirms that phenotypic characteristics, as determined by microscopy, are unreliable for speciation and therefore genetic analysis is required to more accurately identify the species.

Phylogenetic analyses indicated that the seven woylie-derived *Babesia* isolates were identical, and most closely related,but genetically distinct, to genotypes and named species from ungulates, in particularto *B. occultans* whichis a low or non-pathogenic bovine *Babesia*(Ros-Garcia, et al., 2011).

This is the first report of *Babesia* in the woylie and it is interesting since another report (Clark and Spencer, 2007)and unpublished observations (Rong et al., unpublished, Basile et al., unpublished) of piroplasms in this host living in Western Australia have been of *T.penicillata*, a parasite that seems to have minimal pathogenic effect. The woylies in Dwellingup have not been previously screened for *Babesia* or *Theileria*, but a recent study identified a novel trypanosome genotype in these animals(Paparini, et al., 2011), indicating that there may be different transmission dynamics operating in this group.

The finding of a *Babesia* sp. is potentially significant as the woylie has recently undergone a dramatic reduction in abundance, despite no apparent increase in the number or type of predators in the region, and no apparent decrease in natural resources(DEC, 2008, Groom, 2010). However, there is substantial data to support the

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alternate, but not mutually exclusive hypothesis, thatmesopredator release of the feral cat (*Feliscatus*) has resulted in increased predation on woylies (de Tores, unpublished data).

Babesiosis is an important economic disease in livestock and can be potentially fatal with symptoms includingfever, anaemia, icterus, haemoglobinuria and emaciation (Rodriguez, et al., 1994). Little is known about the clinical effects of piroplasm infections in marsupials. High parasitaemias appear to be well tolerated and have little pathological effect in some species (e.g., Gilbert's potoroo) (Lee, et al., 2009), whereas in other marsupials such as the brown antechinus (*Antechinusstuartii*),heavy parasitaemia by *Babesia* sp. was highly prevalent in moribund animals undergoing semelparity(Barker, et al., 1978). In the present study, very little clinical information was available and it was therefore not possible to determine the impact of *Babesia* on these woylies. However, given the decline of the woylie, it would be important to conduct a larger study that included in depth clinical analysis to examine the impact of *Babesia* on woylies.

It has been proposed that the genetic distance at the 18S rDNA required for a piroplasm to be classified as a distinct species is 0.7% and 3.4% for the genera *Theileria* and *Babesia*, respectively (Schnittger, et al., 2003). By these criteria, the novel *Babesia* identified in the woylie is not a separate species as it was <2% divergent from its closest named relative (*B. occultans*).However, thedistance value is difficult to interpret as the authors providedlimited information as to how the genetic distances were calculated,and by this criterion, many established *Babesia* species would not be considered valid species. For example, by our analysis (527bp-long processed alignment), the genetic distance between *B. major* and *B. crassa*(bootstrap values ≥87%) was only 2.3%, and the genetic distance between *B. ovata*and *B.*

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bigemina(bootstrap values ≥57%) was only 2.1% (Figure 3). The values above were very similar also when the analysis was based on the 1,147bp-long processed alignment (data not shown). According to these findings, we conclude that further morphological and/or genetic analyses are required to determine the species status of the *Babesia* identified in the woylies.

A novel *Theileria* genotype was also identified in a boodie (also known as the burrowing bettong). This small, rat-like marsupial belongs to the family Potoroidae, which includes the rat-kangaroos, potoroos, and other bettongs. This is the first report of *Theileria* in a boodie, which is listed as a threatened species under the Western Australian *Wildlife Conservation Act 1950*. The novel sequence exhibited 0.2% genetic distance from *T. penicillata*, *T. brachyuri* and *Theileria*sp. ex long-nosed potoroo, and should be considered a novel genotype, rather than a new species. The clinical impact of this*Theileria*isolate on the boodie is unknown.

The consensus 18S rDNA tree produced (Figure 3) seem to suggest that *Theilerialestoquardi* is slightlymore related to *T. parva*, rather than to *T. annulata*, as expected (Schnittger, et al., 2003).This unanticipated result is likely caused by the gaps/missing data treatment option (complete deletion), which was selected for the final phylogenetic analysis presented in figure 3. A number of trials performed during this study confirmed the close genetic similarity between these three *taxa*, butalso revealed the effects of the implemented gaps/missing data treatment, on their relative clustering pattern. For instance, when all sites were retained during the analysis, the position of the woylie-derived *Babesia* sp. clade did not change, but *T. lestoquardi* grouped with *T. annulata* (data not shown).Optimal treatment of gaps (generally representing insertions or deletions) is debated, and a digression on theirinformative value is beyond the scope of this paper. However the presented analysis confirms that

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rRDNA alignments may require *ad hoc* strategies,different from protein-coding data sets (Talavera and Castresana, 2007).

In conclusion, the genetic analysis of the 18S rDNA confirms the identification of novel *Babesia*sp. and*Theileria*sp. genotypes in woylies and a single boodie respectively. No particular clinical signs have been observed in any of the infected animals, and the clinical significance of *Babesia* and *Theileria*is currently unknown, but further studies should be conducted to determine if *Babesia* is contributing to the decline of the woylie.

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Figure captions

Figure 1.Microscopic detection of *Babesia* sp.

Photomicrograph of a blood film from a woylie (*Bettongiapenicillataogilbyi*) (original magnification x1000) showing two intra-erythrocytic piroplasms (*Babesia* sp.).

Figure 2.Microscopic detection of *Theileria* sp.

Photomicrograph of a blood film from a boodie (*Bettongialesueur*)(original magnification x1000) showing an intra-erythrocytic piroplasm (*Theileria* sp.).

Figure 3.Protozoan phylogeny.

Phylogenetic analysis of the relationships of piroplasms including novel boodie- and woylie-derived isolates of piroplasms, based on 18S rDNA partial sequences. Evolutionary history was inferred using the Maximum Likelihood method. The bootstrap consensus tree inferred from 500 replicates, and branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Branch support/bootstrap values for Maximum Likelihood, Maximum Parsimony and Neighbor-Joining analyses respectively, are indicated at the left of each node. Woylie- and boodie-derived sequences obtained during the present study are indicated with a dot (●) and a triangle (▼) respectively. Marsupial-derived sequences are underlined.The number before the species designation refers to the GenBankGI number; information provided after the species designation refers to isolate codeor host species.

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Research Highlights

- Identification of novel *Babesia* and *Theileria* genotypes in endangered marsupials
- Detection by PCR and microscopy
- First report of *Babesia* in woylies
- First report of *Theileria* in a boodie
- Phylogenetic characterization.

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Table 1. Prevalence of *Theileria* and *Babesia* 18SrDNA in a range of marsupial hosts from different geographic locations in Australia.

SAMPLE GROUP		MICROSCOPY		MOLECULAR ANALYSIS			
Source	Species (Nr. of animals)	Blood smears (Nr.)	Observations	Blood samples (Nr.)	Theileria 18S PCR (Nr. positives)	Babesia 18S PCR (Nr. positives)	% prevalence (B/T)* (SE; 95% C.I. interval)
DWELLINGUP, WA	Chuditch (18)	14	n.p.o.	18			
	Common brushtail possum (24)	21	n.p.o.	24			
	Southern brown bandicoot (4)	3	n.p.o.	4			
	Woylie (15)	12	Small piroplasms (n=6)	15		7	46.7 (B) (0.129; 21.4-71.9)
Total	**61**	**50**		**61**		**7**	**11.5 (B) (0.041; 3.5-19.5)**
KANYANA, WA	Boodie (3)	3	Small piroplasms (n=1)	3	1		33.3 (T) (0.272; 0.0-86.7)
	Western red juvenile (5)	n.p.	n.a.	5			
	Western grey juvenile (4)	n.p.	n.a.	4			
	Wallaroo (2)	n.p.	n.a.	2			
	Woylie (8)	n.p.	n.a.	8			
	Bilby (7)	n.p.	n.a.	7			
	Western barred bandicoot (3)	n.p.	n.a.	3			
	Chuditch (1)	n.p.	n.a.	1			
Total	**33**	**3**		**33**	**1**		**3.0 (T) (0.030; 0.0-8.9)**
CAIRNS, QLD	Northern quoll (6)	n.p.	n.a.	6			
Total	**6**	**n.a.**		**6**			
BUSSELTON, WA	Western ringtail possum (13)	n.p.	n.a.	13			
Total	**13**	**n.a.**		13			
Grand total	**113**	**53**		**113**	**1**	**7**	**0.9 (T) (0.009; 0.0-2.6)** **6.2 (B) (0.023; 1.8-10.6)**

Abbreviations: n.p.o. = no parasites observed; n.a. = not applicable; n.p. = not provided. *Prevalence for *Babesia* or *Theileria* is indicated by the letter B or T, respectively.

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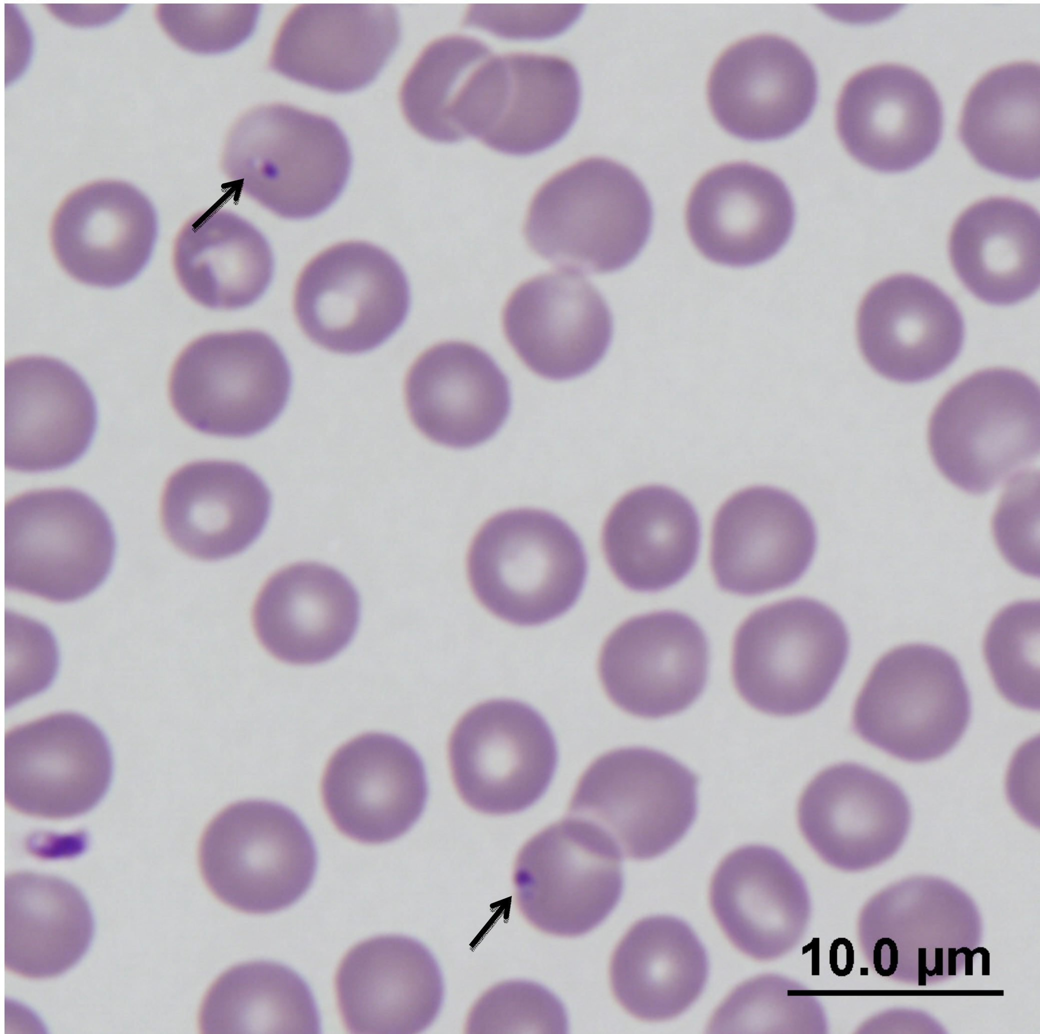

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Figure 1

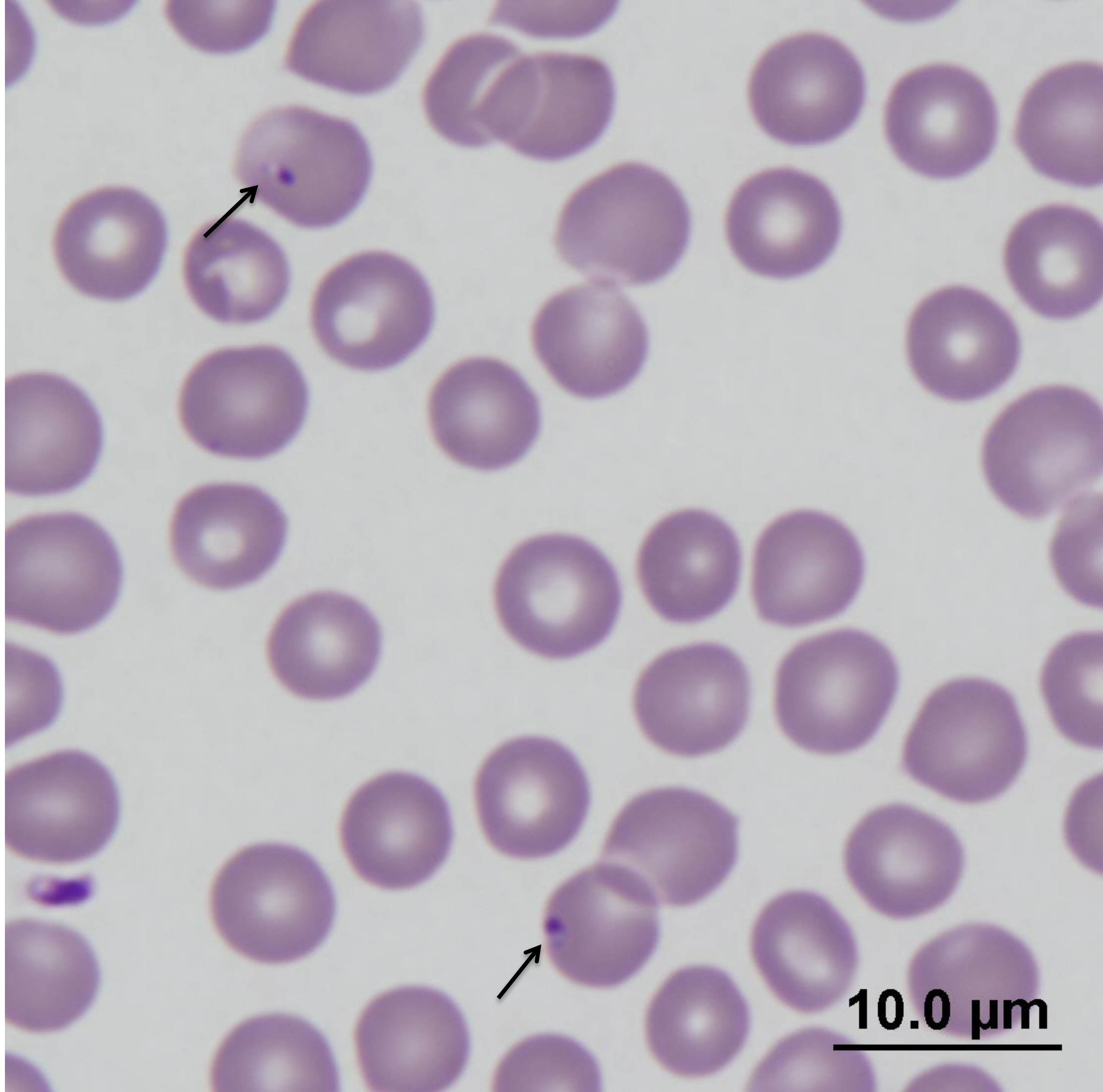

Figure 2

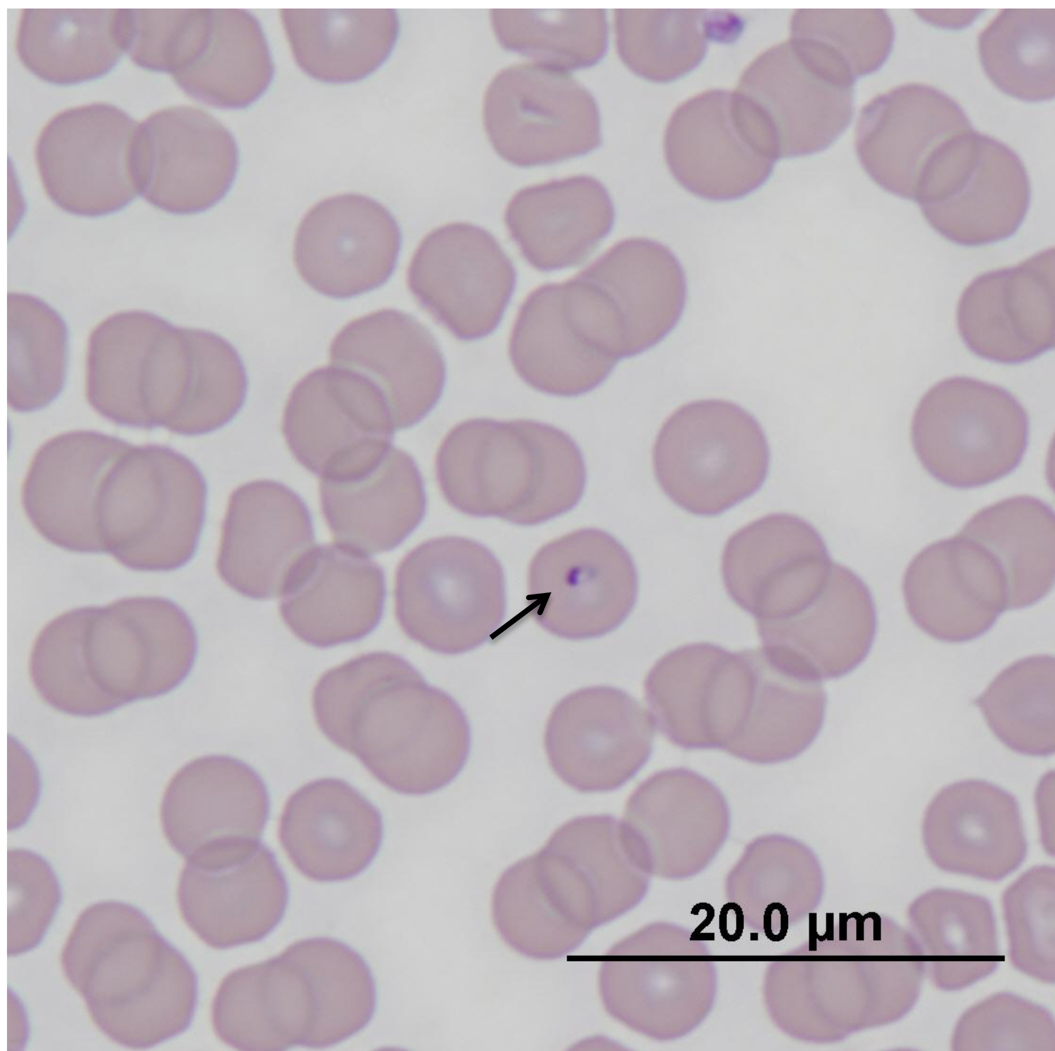

Figure 3