# *Theileria gilberti* n. sp. (Apicomplexa: - Babesiidae) in the Gilbert's Potoroo (*Potorous gilbertii*)

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ABSTRACT

The morphology and genetic characterisation of a new species of piroplasm identified in the blood of the Gilbert's potoroo (*Potorous gilbertii*) from the Two Peoples Bay Nature Reserve near Albany, Western Australia, is described from blood and tissue samples from 16 Gilbert's potoroos. Microscopy of blood showed these parasites are highly pleomorphic with a mean length of 1.8 µm and mean width of 0.85 µm. Phylogenetic analysis of 18S rRNA sequence data identified the piroplasm as a new species of *Theileria* that is closely related to other Australian marsupial piroplasm species. Based on biological and molecular data, it is proposed that the parasite from Gilbert's potoroo be given the name *Theileria gilberti* n. sp.

**Key words**. Australia, haemoparasite, marsupial isolate.

The term ‘piroplasm’ is a collective name for protozoan parasites of similar phenotype that infect mammalian erythrocytes in their life cycle and encompass two main genera; *Theileria* and *Babesia*. Information on the piroplasms of native Australian wildlife is sparse and most records are confined to individual case reports and incidental findings (O’Donoghue 1997). The first official documentation of a piroplasm in a native Australian animal was made by Priestley (1915) who observed and named *Theileria tachyglossi* in peripheral blood smears made from the short-beaked echidna, a monotreme, and since then a variety of piroplasms has been recorded from ten marsupial and two monotreme species in Australia (Fig. 1). Most recently 3 species of *Theileria* were named from three distinct host species from within the Macropodoidea: *Theileria brachyuri* from the quokka (*Setonix brachyurus*); *Theileria fuliginosa* from a western grey kangaroo (*Macropus fuliginosus*); and *Theileria penicillata* from a brush-tailed bettong (*Bettongia penicillata*) (Clark and Spencer 2007).

Gilbert’s potoroo (*Potorous gilbertii*) is a critically endangered small marsupial (Order: Diprotodontia, Family: Potoroidea) that was rediscovered in November 1994 after being presumed to be extinct for almost 100 years (Sinclair, Danks, and Wayne 1996). It is now known to inhabit only a small area of Two Peoples Bay Nature Reserve near Albany, Western Australia (Courtenay and Friend 2004). The present study describes the morphological and genetic characterisation of a small piroplasm identified in the blood of Gilbert’s potoroo and its relationship with a morphologically similar parasite of the long-nosed potoroo (*Potorous tridactylus*) and with other piroplasm species of the Macropodoidea. We consider that the piroplasm infecting Gilbert’s potoroo is a new species and propose the name *Theileria gilberti* n. sp.

## MATERIALS AND METHODS

### Isolates.

*Gilbert's potoroo*. Sixteen blood and seven mixed tissue samples were collected from 16 trapped or deceased Gilbert's potoroos of various ages from Two Peoples Bay Nature Reserve near Albany, Western Australia (Table 1). Tissue samples were 1cm$^3$ and were preserved in 2 ml 70% ethanol while blood samples were stored in 1.3 ml potassium EDTA microtubes (SARSTEDT, Hildesheim, Germany) to prevent clotting. Tissue and blood samples were stored at room temperature and -20°C respectively.

*Long-nosed potoroo*. Liver samples were collected from five wild-caught, now deceased, long-nosed potoroos from Victoria, eastern Australia, previously identified as being piroplasm-positive by microscopy (Table 2).

*Ectoparasites*. Seven adult ticks, two nymphal ticks, and one adult flea, concurrently collected with blood samples from live Gilbert's potoroos, were identified and screened for the presence of the piroplasm using PCR analysis of the 18S rDNA gene (Table 3).

**Microscopy.** A single drop of peripheral blood obtained from the lateral caudal vein of *P. gilbertii* was used to make thin blood smears, which were stained with a modified Wright's stain using an Ames Hema-Tek® slide stainer (Bayer, Leverkusen, Germany). Fifteen blood smears were made from 4 wild Gilbert's potoroos (Table 1). Smears were examined for intraerythrocytic parasites in the peripheral regions of the film and 1,000 erythrocytes were counted using a 100x objective and the number of infected cells noted for the calculation of parasitemia in each blood smear (% of 1,000 erythrocytes infected).

**DNA extraction.** DNA was isolated from ticks and 200 µl of whole blood using QIAamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and QIAamp® DNA Mini Kit (QIAGEN, Hilden, Germany) respectively, according to the manufacturer's instructions.

**PCR amplification.** A nested set of universal piroplasm primers was used to amplify an 850-bp fragment of the 18S rDNA gene as described by Jefferies, Ryan, and Irwin (2007). For the

primers BTF-1 and BTR-1, 1 µl of extracted DNA was added to a 24 µl reaction mixture comprising 0.6875 units of *Tth Plus* DNA Polymerase (Fisher Biotech, West Perth, Australia), 200µM of each dNTP (Fisher Biotech), 12.5 pmoles of each primer (Invitrogen, California, USA), 2.5 µl 10X PCR buffer (Fisher Biotech), and 1.5 µl of 25 mM $MgCl_2$ (Fisher Biotech). Amplification was performed using a GeneAmp PCR thermal cycler (Perkin Elmer, Foster City, California) beginning with an activation step of 94 °C for 3 min, 58 °C for 1 min, and 72 °C for 2 min followed by 45 cycles of amplification (94 °C for 30 sec, 58 °C for 20 sec, and 72 °C for 30 sec), and a final extension phase at 72 °C for 7 min. PCR reagent concentrations, and cycling conditions for BTF-2 and BTR-2 were similar to that described above except 1 µl of primary phase PCR product was used as template in the secondary reaction and the annealing temperature was increased to 62 °C. Amplified DNA was electrophoresed at 80V for 60 min in a 1.5% agarose gel (Promega Corporation, Madison, USA) stained with 10 mg/ml ethidium bromide (Amresco, Ohio, USA).

**DNA sequencing.** DNA was sequenced using an ABI Prism™ Dye Terminator Cycle Sequencing Kit (Applied Biosystems (ABI), Foster City, California) according to the manufacturer's instructions.

**DNA sequence and phylogenetic analysis.** The sequenced products were analysed using the computer program SeqEd v.1.0.3 (ABI) and were aligned using the software package CLUSTAL W (Chenna et al. 2003). Phylogenetic analyses were conducted on the sequences obtained from Gilbert's potoroos from Western Australia, long-nosed potoroos from Victoria, ectoparasites collected from Gilbert's potoroos, and a number of additional sequences obtained from GenBank including those of *Theileria brachyuri* (DQ437684, DQ437685), *Theileria fuliginosa* (DQ437686), and *Theileria penicillata* (DQ437687). Sequences obtained during this study have been deposited in GenBank under the accession numbers EF554394 (Gilbert's potoroo) and EF554395 (long-nosed potoroo).

jModeltest 0.1.1 (David Posata - http://darwin.uvigo.es/) was used to select an appropriate evolutionary model. Distance analyses were conducted using TREECON v1.3b (Van de Peer and De Wachter 1993) using Kimura's 2 parameter distance and parsimony analysis was carried out using MEGA v2.1 (Kumar et al. 2001). *Plasmodium falciparum* (accession number M19172) was used as an outgroup. Maximum Likelihood (ML) analyses were used to validate the phylogenetic relationship inferred by the neighbor joining analyses. ML analysis was performed by PAUP* (Version 4.0b2) using an heuristic search, conducted using the following settings: K80 model settings with 2 substitution types; transition/transversion ratio estimated by ML; empirical base frequencies used; starting branch lengths obtained using Rogers-Swofford method; branch-length optimisation by one dimensional Newton–Raphson with pass limit=20; starting trees obtained by step wise addition; addition sequence = as-is; branch swapping algorithm=TBR. Bootstrap analyses for distance and parsimony methods were conducted using 1,000 replicates or 108 replicates (ML analysis) to assess the reliability of inferred tree topologies.

RESULTS

**Morphology.** *Theileria gilberti* n. sp. appeared as dark, basophilic bodies surrounded by pale cytoplasm with a fine limiting membrane. The organisms were highly pleomorphic and occurred mostly singly within an erythrocyte, but occasional pairs and multiple parasites were noted (Figs. 2--5). The piroplasms ranged in length from 1--2.5 μm with a mean of 1.8±0.51 μm, and in width from 0.5--1.2 μm with a mean of 0.85±0.21 μm (n = 100). A parasitaemia of approximately 10--15% was observed in each of the blood smears examined.

**Genetic analysis.** All 16 blood and 7 tissue samples from the 16 Gilbert's potoroos were positive for *T. gilberti* n. sp. by PCR. Genetic sequences from all these samples and the ectoparasites tested in this study were sequenced and showed 100% similarity to one another. All five long-nosed potoroo samples were positive by PCR and sequences were obtained for two of

these isolates; these showed 100% similarity to one another and 98.1% similarity to the sequences obtained from the Gilbert's potoroo.

Kimura's 2-parameter distance, parsimony, and ML analysis analyses of 18S rRNA sequence alignments of *Theileria*, *Cytauxzoon*, unclassified Piroplasmida isolates, and some clinically important *Babesia* species demonstrated that the isolates derived from Gilbert's potoroo and the long-nosed potoroo were genetically distinct (Fig. 7, Neighbor-joining (NJ) tree illustrated). Two GenBank sequences were obtained for *T. brachyuri*: isolate PSC5 (DQ437684) and isolate PSC12 (DQ437685). However, it appears that these sequences from the same host are genetically distinct, being 3.4% divergent from each other. Isolate PSC5 grouped closely with the isolates from Gilbert's potoroo reported in this paper at 0.7% genetic distance (Table 4). Isolate PSC12 grouped most closely with *T. fuliginosa*, but was 4.6% divergent from Isolate PSC5 and 3.8% divergent from the isolates from Gilbert's potoroo (Table 4). The isolates from Gilbert's potoroo and the long-nosed potoroo exhibited a 1.9% genetic distance from each other. Our analysis further demonstrated that the isolate from the long-nosed potoroo is genetically very closely related to *T. penicillata*, exhibiting only 0.2% genetic distance from it (Fig. 6).

## DISCUSSION

We have described the morphology and genetic characterisation a new species of *Theileria* - *Theileria gilberti* n. sp. infecting Gilbert's potoroo. The small size of the organism initially indicated that it was most likely a member of the order Piroplasmida, within the phylum Apicomplexa, and could be either a "small" *Babesia* or a species of *Theileria* or *Cytauxzoon*. It is similar in size to *T. brachyuri* (0.6--3.1 µm x 0.6--1.9 µm) (Clark and Spencer 2007). Piroplasms are morphologically similar with sizes typically ranging from 1--2.5 µm (Mahoney 1977) and 1--2 µm (Mehlhorn and Shein 1984). Therefore, genetic analysis is required to more accurately identify the species. Sequence and phylogenetic analysis using distance, parsimony, and ML analysis showed that *T. gilberti* n. sp. is genetically distinct but closely related to *T. brachyuri* isolate PSC5.

It has been proposed that the genetic distance at the 18S rRNA locus 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). As the genetic distance reported here between *T. gilberti* n.sp. and *T. brachyuri* isolate PSC5 is 0.7%, this indicates that they are separate species. Similarly as the genetic distance between *T. brachyuri* isolates PSC5 and PSC12 is 3.4%, we suggest that these two isolates actually represent two genetically distinct species of *Theileria* and not the one species (*T. brachyuri*) as described by Clark and Spencer (2007). No discussion is made of the genetic differences between *T. brachyuri* isolates PSC5 and PSC12 by these authors although the two isolates are clearly different in the phylogenetic tree provided (Clark and Spencer 2007). Furthermore, since *Theileria penicillata* and our isolate from the long-nosed potoroo exhibit only 0.2% genetic distance from each other, then using the 0.7% criterion for species differentiation (Schnittger et al. 2003), the isolate from the long-nosed potoroo described here should be considered conspecific with *T. penicillata.*

We chose the 18S rRNA gene for the analyses reported here since it has been shown to be highly conserved (Appels and Honeycutt 1986; Hillis and Dixon 1991; Mindell and Honeycutt 1990). *Theileria gilberti* n. sp. and the isolates from the long-nosed potoroo reported, together with the three other *Theileria* species recently identified (Clark and Spencer 2007), represent the first genetically classified piroplasm species of Australian marsupials.

The original description of a piroplasm in the long-nosed potoroo was made by Mackerras, Mackerras, and Sanders (1953) in samples collected from the Mount Nebo area, near Brisbane, S. Queensland. Despite its being morphologically different, this isolate was named *Theileria peramelis* because it was thought to be the same species of piroplasm that Mackerras et al. (1953) had previously identified in the long-nosed bandicoot, *Perameles nasuta*. In order to establish the true identity of the parasite in the long-nosed potoroo, the organism would need to be genetically characterised from both the original long-nosed potoroo and long-nosed bandicoot, a procedure that

is probably not possible unless original archival material still exists from the animals studied by Mackerras et al. (1953).

Microscopy and PCR were equally effective in the detection of *Theileria* in our samples. These results differ from a number of reports in which it was concluded that PCR was much more effective in detecting piroplasms than microscopy (Almeria et al. 2001; Figueroa et al. 1996; Homer et al. 2000). In situations where circulating parasite levels are extremely low it can be very difficult to visually detect piroplasms in erythrocytes. Diagnosis can be particularly problematic with the "small *Babesia*" and *Theileria* species. We assume that microscopy was so effective in the detection of *Theileria* infection in our study of the Gilbert's potoroo because the marsupials had relatively high parasitaemias.

Prior to this report of *T. gilberti* n. sp. in Gilbert's potoroo, piroplasm infections in native Australian mammals were documented in 11 host species: two species of monotreme - the short-beaked echidna and platypus; and nine species of marsupial - the long-nosed potoroo, Proserpine rock-wallaby, southern and northern brown bandicoots, long-nosed bandicoot, brown antechinus, woylie, western grey kangaroo and the quokka (O'Donoghue and Adlard 2000). As some native Australian mammals may be endangered, parasite-host interactions should be studied to determine the role that piroplasms might play in influencing population dynamics.

Finally, the presence of *T. gilberti* n. sp. observed in the blood from all of the Gilbert's potoroos reported here indicates that theileriosis is possibly endemic in the species in the Two Peoples Bay area. However, its significance at the individual and population levels is unknown at the present time.

The genetic and biological data discussed above indicate that the differences between the new species of *Theileria* infecting Gilbert's potoroo and other *Theileria* spp. are comparable to those between established species. Therefore, we recommend naming the *Theileria* from Gilbert's potoroo *T. gilberti* n. sp. after the host in which it was found.

Phylum Apicomplexa

Order Piroplasmida

Family Theileriidae

*Theileria gilberti* n. sp. (Figs. 2--5)

**Description.** *Theileria gilberti* n. sp. appear as dark, basophilic bodies surrounded by pale cytoplasm with a fine limiting membrane. The organisms are highly pleomorphic and occur mostly singly within an erythrocyte, but occasional pairs and multiple parasites are observed. This piroplasm ranges in length from 1--2.5 μm with a mean of 1.8±0.51 μm, and in width from 0.5--1.2 μm with a mean of 0.85±0.21 μm (n = 100).

**Type host**. Gilbert's Potoroo (*Potorous gilbertii)*

**Other hosts**. Unknown

**Type locality**. Two Peoples Bay Nature Reserve near Albany, Western Australia (Latitude 34.94133º S, Longitude 118.1651º E).

**Other localities**. Unknown.

**Location in host**. Intraerythrocytic.

**Prepatent period**. Unknown.

**Patent period**. Unknown.

**Etymology**. This species is named *Theileria gilberti* n. sp. to reflect its host species.

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| Symbol | Host Species | Piroplasm sp. | References |
|---|---|---|---|
| ■ | *Ornithorhynchus anatinus* (Platypus) | *Theileria ornithorhynchi* | (Mackerras 1958) |
| ■ | *Tachyglossus aculeatus* (Short-beaked echidna) | *Theileria tachyglossi* | (Mackerras 1959; Priestley 1915; Seddon 1952; Seddon and Albiston 1966) |
| ● | *Isoodon obesulus* (Southern brown bandicoot) | *Theileria peramelis* | (Mackerras 1959; Munday 1978; 1988) |
| ◆ | *Perameles nasuta* (Long-nosed bandicoot) | *Theileria peramelis* | (Mackerras 1959) |
| ▲ | *Potorous tridactylus* (Long-nosed potoroo) | *Theileria peramelis* | (Mackerras 1959) |
| ◣ | *Tachyglossus aculeatus* (Short-beaked echidna) | *Babesia tachyglossi* | (Backhouse and Bolliger 1959; Ristic and Lewis 1977) |
| ▰ | *Isoodon obesulus* (Southern brown bandicoot) | *Babesia thylacis* | (Mackerras 1959) |
| ▼ | *Ornithorhynchus anatinus* (Platypus) | *Theileria sp.* | (Mackerras 1958) |
| ⬟ | *Perameles nasuta* (Long-nosed bandicoot) | *Theileria sp.* | (Mackerras 1958; Munday 1978; 1988) |
| ⬢ | *Isoodon macrourus* (Northern brown bandicoot) | *Theileria sp.* | (Seddon and Albiston 1966) |
| ✚ | *Potorous tridactylus* (Long-nosed potoroo) | *Theileria sp.* | (Mackerras et al. 1953; Mackerras 1958; Munday 1978; Speare et al. 1989) |
| ⬣ | *Tachyglossus aculeatus* (Short-beaked echidna) | *Babesia sp.* | (Backhouse and Bolliger 1957; Mackerras 1959) |
| ◖ | *Antechinus stuartii* (Brown antechinus) | *Babesia sp.* | (Arundel, Barker and Beveridge 1977) |
| ◗ | *Petrogale persephone* (Proserpine rock wallaby) | *Babesia sp.* | (O'Donoghue 1997) |
| ✦ | *Setonix brachyurus* (Quokka) | *Theileria brachyuri* | (Clark & Spencer 2007) |
| ★ | *Macropus fuliginosus (Western Grey Kangaroo)* | *Theileria fuliginosa* | (Clark & Spencer 2007) |
| ✹ | *Bettongia penicillata (Brush-tailed bettong)* | *Theileria penicillata* | (Clark & Spencer 2007) |
| ✹ | *Potorous gilbertii (Gilbert's potoroo)* | *Theileria gilberti n. sp* | This study |

Fig. **1**. Geographic distribution of recorded marsupial and monotreme hosts and piroplasms in Australia. 1: North-east drainage, 2: South-east drainage, 3: Tasmanian drainage, 4: Murray-Darling drainage, 5: South Australian drainage, 6: South-west drainage, 7: Far-west drainage, 8: North-west drainage, 9: Carpentarian drainage, 10: Lake Eyre drainage, 11: Bulloo-Bancannia drainage, and 12: Western plateau drainage (modified from O'Donoghue and Adlard 2000).

Fig. **2--5**. Infected erythrocytes from *Potorous gilbertii*. Arrows indicate the typical appearance of intraerythrocytic *Theileria gilberti* n. sp.

Fig. **6**. Phylogeny of *Theileria* and Piroplasmida species inferred by Neighbour-Joining analysis using Kimura's 2-parameter distance. Percentage bootstrap support (>50%) from 1,000 replicate

samples (analyzed by Neighbor Joining, ML and parsimony methods respectively) or 108 replicates (ML analysis) is indicated at the left of the supported node. ns = node not supported by method.