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Short communication

Mitochondrial genome analysis reveals intraspecific variation within Australian hard tick species☆

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ABSTRACT

Ticks rank second in the world as vectors of disease so it is paramount we understand their biology in order to advance disease control. Mitochondrial genome sequences of tick species have been used increasingly to resolve relationships of closely related species, correct taxonomic discrepancies and to understand intraspecific variation. Despite this, our understanding and advances in tick biology are obstructed by the lack of complete mitochondrial genomes available for most species, particularly amongst Ixodidae ticks that are highly prevalent and taxonomically diverse. Even fewer have more than one representative genome sequence meaning that answering questions over intra-species variation is rarely possible. Here, we present the adult tick mitochondrial genomes of two species which had not previously been sequenced, *H. bancrofti* and *I. tasmani*, as well as multiple representatives for both adult *I. holocyclus* and *I. tasmani*. Complete mitochondrial genomes were used to investigate the intraspecific variation within geographically dispersed *I. tasmani* ticks as well as *I. holocyclus* ticks parasitising different host species. Although sample sizes were limited, *I. tasmani* diversity appeared to be influenced by geography, while the genetic diversity observed in *I. holocyclus* was not influenced by host or geography. This genetic resource will support downstream studies into the population genetics of Australian hard ticks and inform efforts to expand this work to other Australian tick species. To build an appropriate repertoire, future analyses should include Australian tick species that are yet to be genome sequenced, particularly those that carry pathogens while including multiple representatives of each species.

1. Introduction

Ticks (Chelicerata: Anactinotrichida: Ixodida) are globally distributed ectoparasites of mammals, aves, reptiles and amphibians (Parola and Raoult, 2001). The capacity to parasitise a large range of host species marks ticks as one of the largest pathogen transmitters globally, second only to mosquitoes (de la Fuente et al., 2008). Because they are capable of transmitting a diverse range of microorganisms infecting both human and animal hosts, ticks have become important disease vectors and reservoirs (de la Fuente et al., 2017).

The tick suborder, Ixodida, has two major divisions: soft ticks (family Argasidae) and hard ticks (family Ixodidae). The Ixodidae family represents 702 of the 896 formally classified tick species and is comprised of two groups (Prostriata and Metastriata) based on morphological and genotypic differences. The Prostriata ticks consist of 243 species of the single genus *Ixodes*. Metastriata ticks consist of 459 species divided into 13 genera. Among the hard tick species, genus *Haemaphysalis* is the second largest for species diversity accounting for 166 species within the Metastriata group (Guglielmone et al., 2010).

Quality complete reference sequences such as nuclear and mitochondrial genomes, are necessary for advances in areas that are crucial for tick-borne disease control, particularly the study of tick–tick (Liu et al., 2013), tick–host (Mccoy et al., 2013) and tick–pathogen relationships (Gall et al., 2016). Mitochondrial DNA has proven to be a useful tool for understanding the evolutionary, geographical and pathogenic relationships of tick species (Shao and Barker, 2006; Burger et al., 2013). In more recent analyses, the examination of one or multiple relatively conserved protein coding genes has been favoured as they provide much higher resolution of genus and species level relationships over rRNA genes (Williams-Newkirk et al., 2015; Guo et al., 2016; Sui et al., 2017). However, to further distinguish closely related species, understand divergent evolution and measure intraspecies variation, multiple complete mitochondrial genomes are still necessary, as one or two protein-coding genes do not resolve these types of

☆ Protein-coding genes are abbreviated as *atp6* and *atp8* for ATP synthase subunits 6 and 8, *cox1–3*, for cytochrome c oxidase subunits 1–3, *cob* for cytochrome *b* and *nad1–6* and *4L* for NADH dehydrogenase subunits 1–6 and 4L

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relationships in all species, nor do they allow for assessment of whole-genome evolution (Kain et al., 1999; Shaw et al., 2002; Shao and Barker, 2006; Song et al., 2011; Burger et al., 2012; Liu et al., 2013; Sui et al., 2017). To date only 6 of 243 *Ixodes* and 22 of 459 Metastriata tick species have a complete mitochondrial genome sequenced. This has resulted in diminished accuracy of inferred taxonomic relationships within many Ixodidae species as mitochondrial or nuclear rRNA based phylogenies are used instead (Norris et al., 1999), or a complete exclusion of particular species from analyses, leaving evolutionary and intraspecies relationships unresolved.

Prevalent Australian tick species *Ixodes tasmani*, *Ixodes holocyclus* and *Haemaphysalis bancrofti* are widely dispersed across the continent and can be found parasitising a range of human and animal hosts (Barker and Walker, 2014; Burnard et al., 2017). All three tick species are important disease vectors in Australia with bacterial pathogens such as *Theileria orientalis*, *Rickettsia* and *Bartonella* species as well as *Rickettsia australis* transmitted by *H. bancrofti, I. tasmani* and the paralysis tick *I. holocyclus*, respectively (Barker and Walker, 2014). Both *I. tasmani* and *H. bancrofti* still lack a representative mitochondrial genome and *I. holocyclus* only has one representative sequence available. The intraspecific relationships of these Australian Ixodidae tick species remain unclear (Shaw et al., 2002; Song et al., 2011). Due to their prevalence, host selection and disease transmitting capacity it is important to understand these tick-tick and tick-host relationships.

In this study, we sequenced the mitochondrial genomes for one *Ixodes* tick species, *I. tasmani* and one Metastriata tick species, *H. bancrofti,* while expanding the available mitochondrial genome sequence information for *I. holocyclus*. Analysis of the genetic diversity within *I. tasmani* and *I. holocyclus* ticks was then performed in relation to geographical location and host species parasitised.

2. Materials and methods

2.1. Tick collection, identification and DNA extraction

Tick samples were collected by veterinarians and wildlife carers using forceps and stored in 70% ethanol as previously described (Burnard et al., 2017). Tick species were derived primarily from two sites in South-East Queensland (QLD) and/or Central New South Wales (NSW), regions along the eastern coast of Australia and separated by a geographic distance of approximately 800–1000 km. In total, eight tick pools consisting of three Ixodidae species, *I. tasmani* (n = 3), *I. holocyclus* (n = 4) and *H. bancrofti* (n = 1) were selected for sequencing (Table 1).

2.2. Genome sequencing

Previously collected and extracted DNA samples were utilised for metagenome sequencing following previously described sample preparation methods (Taylor-Brown et al., 2016). The Australian Genome Research facility (AGRF) performed shotgun library preparation and an Illumina HiSeq to produce 125 bp paired end reads.

2.3. Contig assembly and mitochondrial genome annotation

Ixodidae mitochondrial DNA was filtered from the metagenome using reference sequence mapping in BBMap v37.25 (Bushell, 2017). GenBank representatives *I. holocyclus* NC_005293 and *H. formosensis* JX573135 were used as reference genomes. The reads were assessed with FastQC (Andrews, 2010), then quality and adaptor trimmed using Trimmomatic v0.33 (Bolger et al., 2014). Trimmed reads were then subject to *de novo* assembly using SPAdes v3.1.1 (Bankevich et al., 2012) in metagenome mode using k-mer values obtained from KmerGenie (Chikhi and Medvedev, 2014). Mitochondrial Ixodida contigs were differentiated from other mitochondrial and bacterial contigs using BLASTn analyses and reference genome mapping in Geneious R9 (Kearse et al., 2012). The raw reads were then mapped back to the contigs using Geneious R9 to ensure coverage and completeness. The resulting consensus sequences were annotated using PROKKA (Seemann, 2014), with manual annotation curated in Artemis (Rutherford et al., 2000). The annotation of trRNA genes were verified with tRNAscan-SE (Lowe and Chan, 2016). Both 16S and 12S rRNA and control regions were annotated by BLASTn analyses and pairwise alignment with *I. holocyclus* and *H. flava* respectively. Bowtie2 v2.1.0 (Langmead and Salzberg, 2012) and SAMtools v1.4 (Li et al., 2009) were used to generate the average depth of coverage across each mitochondrial genome.

2.4. Phylogenetic relationships of Australian Ixodidae ticks

Nucleotide sequences consisting of the 13 protein coding genes of the mitochondrial genome from 30 hard tick species, including 28 GenBank representatives were aligned with ClustalW (Thompson et al., 1994) in Geneious 9.1.5. Soft tick species *Ornithodoros moubata* and *Carios capensis* were used as an out-group. The alignment was trimmed to a length of 11,429 bp and subject to a maximum likelihood phylogeny analysis of the 33 sequences constructed with PHYML and run to 1000 replicates (Guindon et al., 2010) under the TN93 + G evolution model determined by MEGA5 (Tamura et al., 2011).

2.5. Analysis of intraspecies variation within I. tasmani and I. holocyclus

Full-length complete mitochondrial nucleotide sequences of the three *I. tasmani* tick pools were aligned with ClustalW. Full-length complete mitochondrial nucleotide sequences of four *I. holocyclus* tick pools obtained in this study were also aligned with GenBank reference genome *I. holocyclus* in ClustalW. For each set of alignments, DnaSP was used to estimate the number of haplotypes present in each alignment, as well as the degree of this diversity by the support value Hd (Rozas and Rozas, 1995).

Table 1
Metadata of ticks used to generate mitochondrial genomes.

ID	Tick species	Pool (n =)	Location	Host	Genome length (bp)	GC content (%)	Average depth of coverage
C9b2	*Haemaphysalis bancrofti*	1	QLD	Eastern grey kangaroo *(Macropus giganteus)*	14,673	21.6	93X
A6	*Ixodes tasmani*	5	QLD	Koala *(Phascolarctos cinereus)*	15,219	22.1	548X
B6	*Ixodes tasmani*	5	NSW	koala	15,227	22.1	10X
C9	*Ixodes tasmani*	5	NSW	koala	15,163	22.0	3X
B9b2	*Ixodes holocyclus*	1	QLD	Brush tail possum *(Trichosurus vulpecula)*	15,007	22.6	251X
E5b2	*Ixodes holocyclus*	1	QLD	Spotted tail quoll *(Dasyurus maculatus)*	15,006	22.7	30X
H4	*Ixodes holocyclus*	5	QLD	koala	15,004	22.7	2939X
I7	*Ixodes holocyclus*	5	QLD	koala	15,010	22.7	293X

3. Results and discussion

3.1. Mitochondrial genome features of H. bancrofti, I. tasmani and I. holocyclus

The complete mitochondrial genomes of *H. bancrofti*, *I. tasmani* and *I. holocyclus* range in size from 14,673 bp to 15,227 bp. The mean coverage found for each of these mitochondrial genomes was between 10 – 2939x, with the exception of one sample (C9 with 3x coverage) (Table 1). All eight mitochondrial genomes contain the 13 protein-coding genes, two rRNA genes and 22 tRNA genes common to Metazoa, with two non-coding control regions both similar in size and sequence, positioned on the genome as previously described (Shao et al., 2005). The arrangement of genes is identical to the arrangement previously reported for Metastriata and *Ixodes* ticks, respectively (Black and Roehrdanz, 1998; Shao et al., 2005), providing quality reference sequence information for future analyses. *H. bancrofti* is a similar size to the representative genomes of the *Haemaphysalis* genus at 14,673 bp with a comparable GC content of 21.6% (Burger et al., 2013). The *I. holocyclus* genomes were almost identical in size and GC content to the representative *I. holocyclus* genome ranging from 15,004–15,010 bp and 22.0% GC, but were also comparable to that of the closely related *I. uriae* (Shao et al., 2005; Table 1). The *I. tasmani* genomes, sequenced for the first time in the present study, are also of an expected and similar size ranging from 15,163 to 15,227 bp with 22.0% GC content, comparable to the *I. holocyclus* genome (Shao et al., 2004).

3.2. Confirmation of the phylogenetic relationships of prevalent Australian tick species

To confirm the phylogenetic relationships and accuracy of the *H. bancrofti* and *I. tasmani* mitochondrial genomes, a maximum likelihood phylogenetic tree was built. Overall, the topology was consistent with that of previously described mitochondrial DNA (Williams-Newkirk et al., 2015) and rRNA phylogenies (Burger et al., 2012, 2013; Fig. 1). Within the Metastriata, support for *H. bancrofti* grouping with the *Haemaphysalis* genus was strong, with *H. bancrofti* branching as the earliest independent lineage (BS 100). As there are only a handful of *Haemaphysalis* mitochondrial genomes available, additional species would be required to determine sister species relationships. It is possible *H. bancrofti* would place in an Australasian *Haemaphysalis* clade similar to that seen within the *Ixodes* (Burger et al., 2012, 2013) however, due to the absence of mitochondrial genomes of the remaining eight *Haemaphysalis* tick species present in Australasia (Roberts, 1970), there is no evidence to support this type of relationship.

Within the *Ixodes* genus, the Australasian *Ixodes* and non-Australasian *Ixodes* clades are formed (BS 100) with strong support as expected (Burger et al., 2012; Fig. 1). Within the Australasian *Ixodes* clade, *I. tasmani* representative C9 has strong support for an independent lineage from *I. holocyclus* and *I. uriae* (BS 100) as described in previous rRNA phylogenies (Xu et al., 2003; Burger et al., 2012, 2013). The *I. holocyclus* representative I7 from this study clusters with the previously sequenced *I. holocyclus* collected via a flagging method in Sydney, New South Wales, Australia (BS 100) as sister species to *I. uriae* (BS 100) as expected (Xu et al., 2003; Burger et al., 2012, 2013).

3.3. Intraspecies variation within Ixodes tasmani and Ixodes holocyclus

To assess the intraspecies variation within *I. tasmani* and *I. holocyclus*, we examined the whole mitochondrial DNA sequences from each species in more detail. Sequence alignment among the three newly sequenced *I. tasmani* genomes revealed that each was unique, having < 1% nucleotide dissimilarity (101–224 SNPs) across the 15,235 bp full-length mitochondrial genome alignment. The diversity observed was confirmed with DnaSP as the three *I. tasmani* mitochondrial genomes were determined to be unique haplotypes with a strong level of variation (Hd: 1.0). This nucleotide variation was evenly distributed across each open reading frame across the mitochondrial genome with the exception of the gene *nad4L* which was 100% conserved across the three sequences (Supplementary Fig. 1). The variation observed within the three koala-parasitising *I. tasmani* species (Fig. 2a), two from Southern Australia (NSW) B6 and C9 and A6 from South East (QLD) suggest geographical location may be a factor influencing variation in the mitochondrial genome. Perhaps this is expected as the distance between the two locations is substantial (> 1000 km), potentially resulting in subtle intraspecies population variation. Geographical based variation has been identified previously using partial mitochondrial cytochrome oxidase III (*cox3*) sequences in American *I. pacificus* ticks (Kain et al., 1999) where variation of approximately 2% was described. Further analyses on a larger number of geographically assorted mitochondrial genomes would obviously be required to confirm whether geography influences intraspecies variation for this tick species.

Our analysis of the newly acquired *I. holocyclus* mitochondrial genome sequences to the one previously described sequence revealed that all five sequences were also unique, having < 1% non-identity. Five unique haplotypes were determined with DnaSP, also with a strong level of variation (Hd: 1.0). Intraspecies variation observed in *I. holocyclus* mitochondrial genomes does not appear to be influenced heavily or at all by host species diversity. Sequence I7, a female tick pool removed from koalas is almost identical to the previously sequenced *I. holocyclus* mitochondrial genome sequence (NC_005293) derived from a male tick in Sydney, New South Wales. The mitochondrial genome sequences were nearly identical with only 60 SNPs across all genes in the genome, with the exception of *nad3* and *nad4* which remained conserved. Sequences B9b2, derived from a tick removed from a brushtail possum and H4, and a tick pool removed from koalas were also similar in nucleotide sequence. A total of 87 SNPs distinguish these two mitochondrial genomes. The only genes of the mitochondrial genomes not containing any nucleotide variation in either sequence are *cox2* and *atp8*, which is unexpected as *atp8* has been described as the least conserved protein coding gene in Ixodidae ticks (Liu et al., 2013; Guo et al., 2016). Sequence E5b2, a tick removed from a spotted tail quoll has 70–117 SNPs present in half of the protein coding genes including *nad1,2,4,5, cox1, atp6* and *cob*. It is unexpected to see variation present in *cox1* as it has been previously described as the most conserved protein coding gene in Ixodidae ticks (Liu et al., 2013; Guo et al., 2016). As all the mitochondrial genomes assembled from this study were from relatively close proximity in Queensland, expanded sampling of other regions will be required to assess the broader population genetics of this species across the species' home range. Although no obvious differences were observed between the small collection of *I. holocyclus* removed from different marsupial hosts, similar studies would be of interest to investigate whether host species is a factor influencing variation. The mitochondrial protein coding gene sequence variation observed was otherwise similar to the variation observed between individuals within populations of *I. holocyclus*, *I. pacificus* and *R. sanguineus* ticks examining *cox1/ITS, cox3 and cox1/cytb* genes respectively (Kain et al., 1999; Shaw et al., 2002; Song et al., 2011; Liu et al., 2013), yet significantly more than that seen in *ITS* of *I. holocyclus* (Song et al., 2011), although the sample sizes are too small to confirm this. Nevertheless, this genetic resource will serve as the basis for expanded studies to establish a population genetic framework for *I. holocyclus*.

4. Conclusion

The generation of this data provides an additional eight mitochondrial genomes for future analyses, including two species which had not previously been sequenced, *H. bancrofti* and *I. tasmani*, as well as multiple mitochondrial genomes for both *I. holocyclus* and *I. tasmani*. Phylogenetic analyses of the 13 protein coding genes supported

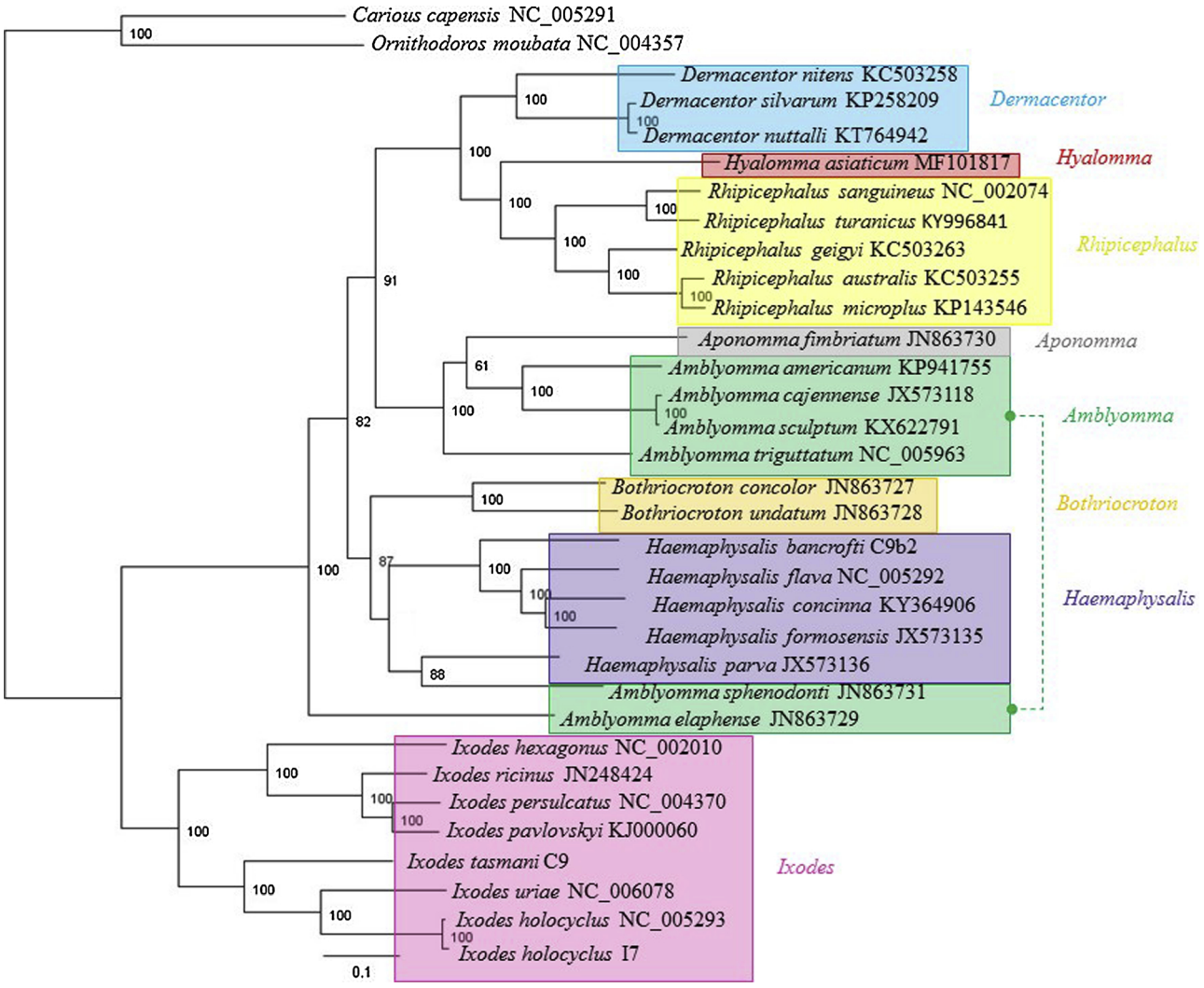

Fig. 1. Maximum likelihood phylogeny of the 13 protein-coding mitochondrial genes of hard tick species.
Maximum likelihood tree incorporating representative whole mitochondrial genome sequences of each genus within the Ixodidae from GenBank, as well as the eight mitochondrial genomes generated in this study. Tree was built using a 11,429 bp under the TN93 + G evolutionary model, bootstrap support exceeding 75 is shown at internal nodes. GenBank accession numbers are shown next to species name.

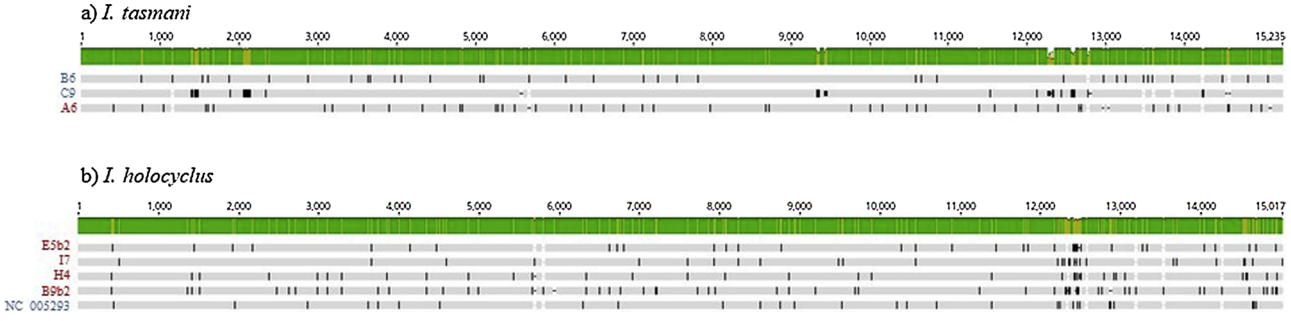

Fig. 2. Intraspecies variation within the whole mitochondrial genome of *Ixodes tasmani* and *holocyclus* ticks.
a) Whole mitochondrial genome alignment of *I. tasmani* tick pools. Pools B6 and C9 are coloured blue to represent their collection from the state of New South Wales. A6 is coloured maroon to represent its collection from Queensland. Nucleotide agreements in the alignment are shaded grey while disagreements and gaps are represented in black.
b) Whole mitochondrial genome alignment of *I. holocyclus* tick pools. The four tick pools used in this study are coloured maroon to represent their collection from Queensland, while the GenBank reference genome is coloured blue to represent its collection from New South Wales. Nucleotide agreements in the alignment are shaded grey while disagreements and gaps are represented in black.

placement of *H. bancrofti* in a separate lineage within the *Haemaphysalis* genus as well as *I. uriae* and *I. holocyclus* as sister species within the *Ixodes*. *I. tasmani* was confirmed to branch as a separate lineage within the Australasian *Ixodes* clade. Intraspecies variation was supported within *I. tasmani* genomes with evidence that genetic differences may exist amongst geographically dispersed individuals. Individual genetic variation for *I. holocyclus* ticks was also consistent with what was previously reported for other hard tick species. As such a limited number of mitochondrial genomes are available, additional data would be required to resolve outstanding sister species relationships within genera and to fully assess the intraspecies variation observed.

Ethics approval

Tick collection was carried out opportunistically on animals presenting for care or collected as a part of other animal health investigations. The approval to complete tick sampling was sought and approved by the University of the Sunshine Coast Animal Ethics Committee (ANS1539).

Availability of data and material

Nucleotide sequences obtained from this study were deposited in GenBank under accessions MH043264 to MH043271.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2019.02.013.

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