Molecular barcoding of Australian ticks

by

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Author's Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Globally, ticks (Acari: Ixodida) are one of the most important vectors of disease due to their ability to transmit a wide variety of pathogenic bacteria, protozoa, and viruses during blood feeding. The microbes transmitted by ticks varies by species, and so it is essential that ticks are able to be identified correctly. However, the identification and discrimination of tick species still relies on traditional morphological techniques, which can at times be ambiguous, particularly in the case of subadult ticks. This study tested previously developed molecular barcoding assays to facilitate the identification of Australian ticks using DNA sequences in the case that morphology is inconclusive. Using reference ticks from eight native species of medical and veterinary importance (*Amblyomma triguttatum, Bothriocroton auruginans, Haemaphysalis bancrofti, Haemaphysalis humerosa, Ixodes cornuatus, Ixodes hirsti, Ixodes holocyclus and Ixodes tasmani*), four potential barcoding genes were trialled (Cytochrome c oxidase (COI), Internal transcribed spacer 2 (ITS2), 16S rRNA and 12S rRNA). Amplification was successful in 98.3% of samples (n=58) for COI, 89.6% (n=48) of samples for ITS2, and 100% of samples for both 16S rRNA and 12S rRNA (n=58 and n=48 respectively). Following Sanger sequencing, all four genes were found to be suitable for specimen identification using BLAST when genetic data was available. However, analysis of the sequences generated using Automatic Barcode Gap Discovery (ABGD) indicated that COI was the most efficient gene for species delimitation. This analysis revealed three new species group hypotheses and pairwise distances confirmed high levels of genetic divergence within these species. Further research is required to investigate the validity of these species groups, and to ensure that the assays tested in this study are suitable for all Australian ticks. Lastly, this study provided new genetic information for nine species based on 17 sequences, and these data will become publicly available on the genetic database, GenBank.

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List of abbreviations

Abbreviation/Symbol	Meaning
ABGD	Automatic barcode gap discovery
BLAST	Basic local alignment search tool
Base pairs	Base pairs
COI	Cytochrome c oxidase I
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
et al.	and others
gDNA	Genomic DNA
ITS2	Internal transcribed spacer 2
$MgCl_2$	Magnesium chloride
min	Minute
mL	Millilitre
mM	Millimolar
µ	Micro
µL	Microlitre
mt	Mitochondrial
n	Number of samples
NCBI	National Centre for Biotechnology Information
PCR	Polymerase chain reaction
RAxML	Randomized axelerated maximum likelihood
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
P_{AB}	Rosenberg's test statistic
s	Second
sp./spp.	Species
Taq	*Thermus aquaticus* DNA polymerase
TBD(s)	Tick-borne disease(s)
w/v	Weight of solute per volume of solvent
12S	12S rRNA gene
16S	16S rRNA gene
3'	3’ Hydroxyl-terminus of DNA molecule
5'	5’ Phosphate-terminus of DNA molecule
°C	°C Degrees Celsius
<	Less than
≤	Less than or equal to
%	Percentage

Chapter 1: Introduction

Globally, ticks are one of the most important vectors of disease due to their ability to transmit a wider variety of pathogenic bacteria, protozoa, and viruses than any other vector group (Jongejan and Uilenberg, 2004). Tick-borne diseases (TBD) are a serious health threat to humans and animals, and cause significant morbidity and economic loss worldwide (Esteve-Gassent et al., 2016). Australia is among the countries experiencing an increasing emergence of TBD, and there is a great amount of research conducted on the microorganisms carried by Australian ticks, their potential for transmission, and any pathological consequences (e.g. van Nunen, 2018; Burnard et al., 2017). It is an essential requirement of such studies that the ticks are first taxonomically identified.

Identification of most adult Australian ticks is possible using published morphological keys (e.g. Barker and Walker, 2014; Roberts, 1970). However, morphological similarities between species can make accurate identification problematic, and existing keys do not describe the morphology of every tick species in Australia. Furthermore, for many species only adult morphology is described, and nymph and larval morphology is unknown.

Molecular barcoding is a powerful tool that is frequently used to identify and distinguish biological species, even in the absence of morphological data. The technique uses short DNA sequences that are conserved within a species, but variable between species, providing more accuracy than can be achieved through morphological studies (Hebert et al., 2003a). The objective of this project is to develop molecular barcoding protocols that identify eight Australian ticks of medical and veterinary importance, with representatives within each of the Australian hard tick genera (Barker and Walker, 2014).

This introductory chapter will review the literature pertaining to ticks, the identification of ticks by species, and molecular barcoding. It will begin with taxonomy and evolution, and tick lifecycle before discussing Australian ticks specifically. The importance of ticks and TBD will then be covered briefly. Following this, tick morphology

and the limitations inherent to the morphological identification of tick species will be discussed, leading into molecular barcoding and how this tool has performed efficiently in the identification of many animal species, including ticks. The chapter will conclude with the thesis aims and hypotheses.

1.1 Ticks

Ticks are obligate hematophagous (blood-feeding) ectoparasites of vertebrate hosts. They parasitise mammals, birds, reptiles, and amphibians, and new host relationships are recorded frequently (D'Amico et al., 2017; Keskin et al., 2017; Krige et al., 2018; Kwak and Madden, 2017). Almost 900 species of tick have been described to date and ticks are distributed extensively worldwide (Jongejan and Uilenberg, 2004). While ticks are currently found in almost every region of the world, the changing climate and associated factors have led to the gradually shifting geographic range of several tick species and it is expected this will continue into the future (Sonenshine, 2018; Cumming and Van Vuuren, 2006). This section will discuss the taxonomy and evolution of ticks, the lifecycle of Ixodidae (hard) ticks, and provide an overview of Australian ticks.

1.1.1 Taxonomy and evolution

Ticks belong to the most vast and diverse animal phylum, Arthropoda, within the subphylum of Chelicerata (Nava et al., 2009). They are classified into the class Arachnida, and subclass Acari, which also encompasses taxa commonly referred to as mites. Within the superorder Parasitiformes, ticks belong to the order Ixodida (Cupp, 1991).

There are three families within Ixodida: Argasidae (soft ticks), Ixodidae (hard ticks), and Nuttalliellidae (Nava et al., 2009). Nuttalliellidae is a monotypic family, comprised only of *Nuttalliella namaqua*, a species restricted to regions of South Africa and Tanzania (Cupp, 1991). *Nuttalliella namaqua* will not be discussed further in this review, however there are a number of papers that provide further information on this species (e.g. Mans et al., 2014; Latif et al., 2012). The taxonomic classification of ticks is summarised in **table 1.1.**

Table 1.1. Taxonomic classification of ticks.

Phylum	Arthropoda
Subphylum	Chelicerata
Class	Arachnida
Subclass	Acari
Superorder	Parasitiformes
Order	Ixodida
Family	Argasidae Ixodidae Nuttalliellidae

The most important family of ticks both numerically and medically is the Ixodidae, or hard ticks, comprised of over 700 species in 14 genera, and characterised by a sclerotized dorsal shield (Guglielmone et al., 2010). This family is divided into the Prostriata and Metastriata (figure 1.1). The Prostriata is comprised of a single subfamily (Ixodinae) and the genus *Ixodes*. The Metastriata encompasses five subfamilies (Bothriocrotoninae, Amblyomminae, Haemaphysalinae, and Rhipicephalinae and Hyalomminae) which collectively contain 11 genera (Barker and Murrell, 2004; Black and Piesman, 1994).

The remaining family is the Argasidae, or soft ticks, which are characterised by their flexible, leathery exoskeleton and includes approximately 193 species within five genera (Guglielmone et al., 2010). The Argasidae are divided into two major subfamilies, Argasinae and Ornithodorinae (Barker and Murrell, 2004; Black and Piesman, 1994); however recent research into this family suggests reclassification is needed (Mans et al. 2018).

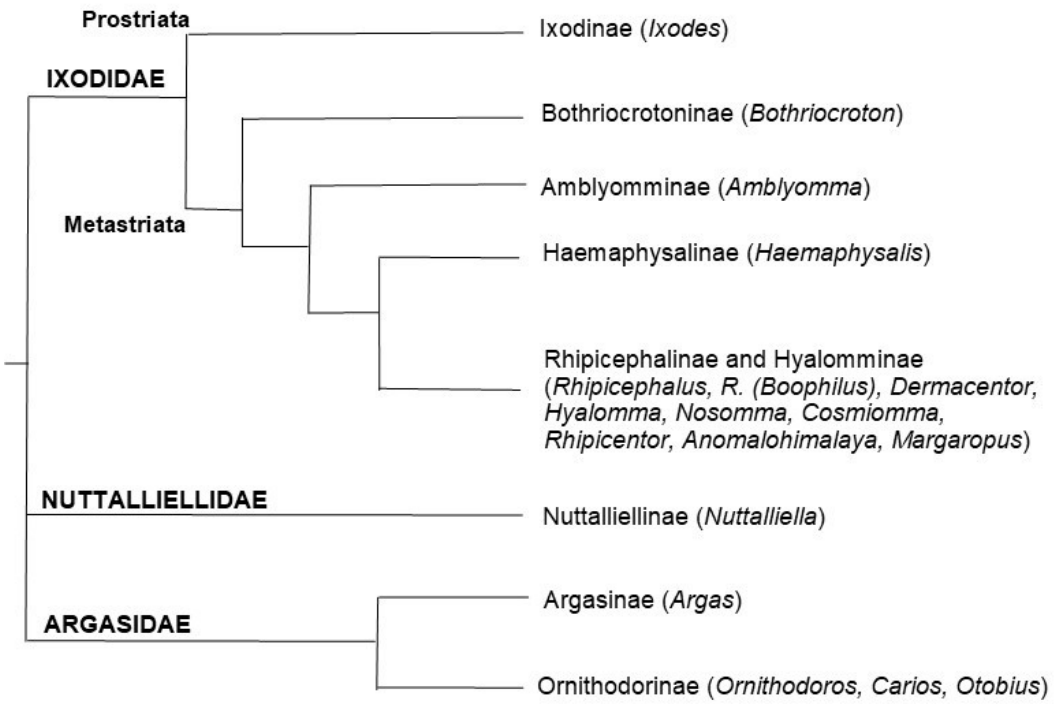


Figure 1.1. Phylogeny of families, subfamilies and genera of the order Ixodidae. Barker and Murrell (2004).

Regarding the evolution of this subphylum, records suggest that the Chelicerata arose more than 500 million years ago (mya), and that the Acari subclass diverged within Arachnida 397 ± 23 mya, at approximately the same time as the spider-scorpion clades diverged (Sonenshine and Roe, 2013). There are several hypotheses surrounding the origin of the Ixodida, mainly based on tick-host associations, phylogenetics, and morphological and biological evidence from present day tick species, due to a lack of information gained from fossil records (Nava et al., 2009). Hoogstraal's hypothesis suggested that the first ticks were associated with reptiles and arose during the late Paleozoic to early Mesozoic eras, 300-200 mya (Hoogstraal and Aeschlimann, 1982). Dobson and Barker asserted that the first hosts of ticks were in fact amphibians, and place the origin of ticks 408-362 mya, during the Denovian period of the Paleozoic era (Dobson and Barker, 1999). This hypothesis also suggests that all ticks originate from the region of Gondwana that became Australia following the breakup of Pangea. Evidence supporting this hypothesis includes that six of the eight tick subfamilies are endemic to Australia, and that the closest living relatives to ticks, the holothyrid mites, are also endemic to Australia (Barker et al., 2014; Murrell et al., 2005). Other hypotheses have

placed the origin in the Triassic and Cretaceous periods of the Mesozoic era, 250-202 mya and 145-66 mya respectively (Black and Piesman, 1994; de la Fuente, 2003; Klompen et al., 1996). While the timeline of tick origin is still unknown, the Ixodida certainly diverged prior to the mid-cretaceous period, as fossil records have shown that Ixodidae and Argasidae lineages were well differentiated by this time (Poinar and Brown, 2003; Klompen and Grimaldi, 2001).

1.1.2 The tick lifecycle

All ticks undergo four stages of development throughout their lifecycle: eggs, larva, nymph, and adult. However, other aspects of the lifecycles of hard and soft ticks differ substantially, including the number of nymphal instars, feeding patterns, and mating behaviour; therefore, this section will describe the lifecycle of hard ticks specifically.

The lifecycle of hard ticks is typically completed in 2-3 years, though it range from 6 months up to 6 years depending on tick species, host availability, and environmental conditions (Parola and Raoult, 2001). As obligate hematophagous parasites, hard ticks can be one-, two- or, three-host feeding, and the specificity of ticks for a host organism varies greatly by species (McCoy et al., 2013). One-host feeding ticks spend their entire life on the same host where they moult at the end of each feed, then reattach to the host at the next instar, only detaching as an engorged adult to lay eggs (Jongejan and Uilenberg, 1994). Similarly, two-host feeding ticks moult after feeding at the larva and nymphal stages but will then detach to seek a new host as an adult (Jongejan and Uilenberg, 1994). While most one- and two-host feeding ticks are obligate in this behaviour, certain species can be more opportunistic (Oliver, 1989). However, the majority of Ixodidae require three individual hosts throughout their lifecycle (figure 1.2).

The length of time required for each feeding varies by instar and by the host animal (Parola and Raoult, 2001). When feeding on warm-blooded animals, larval and nymphal ticks generally require 3-7 and 4-8 days respectively, while adult females typically require 7-12 days, and all instars require longer if feeding on reptiles (Oliver, 1989). Female adults feed once per life stage and require large blood meals over several

days to engorge with blood, while males do not engorge; rather males remain on the host for weeks to months and feed intermittently (Parola and Raoult, 2001; Oliver, 1989). After feeding, mating between adults usually occurs on the host, after which the female will detach to digest the blood meal, lay eggs (anywhere from 400-20,000, depending on species), and die (Parola and Raoult, 2001).

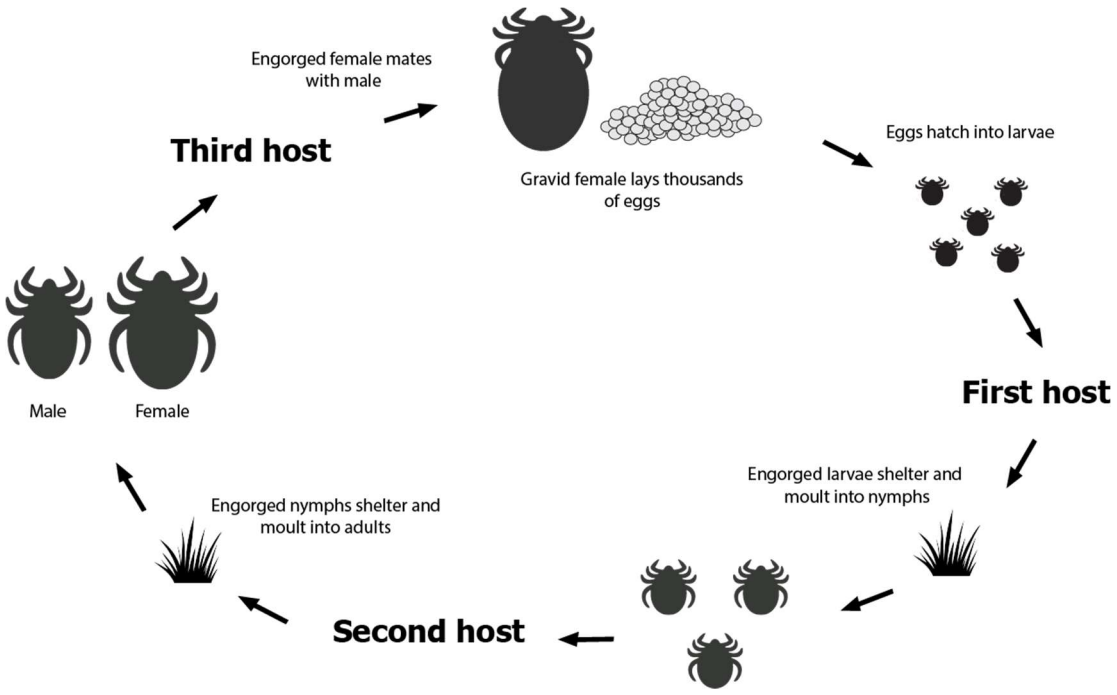


Figure 1.2. A typical three-host Ixodidae life cycle. Adapted from Parola and Raoult (2001).

1.1.3 Australian ticks

There are 72 tick species (58 hard; 14 soft) currently described in Australia (Kwak et al., 2018b; Ash et al., 2017; Barker et al., 2014), which include 67 native species (55 hard; 12 soft). When the recently described hard tick *Ixodes woyliei* was described in 2017, it marked the first discovery of a new Australian *Ixodes* tick species in over 50 years (Ash et al., 2017). This was followed by the discovery of *Ixodes heathi* in 2018 (Kwak et al., 2018b). The five species of tick not native to Australia (three hard; two soft) were

introduced during European settlement (Barker et al., 2014). All five of these introduced tick species infest domestic animals, and four have been recorded to bite humans (Barker and Walker, 2014). While most native Australian ticks feed exclusively on wildlife, 11 species commonly parasitise domestic animals, and humans are known incidental hosts of eight of these (Barker and Walker, 2014). These species are of medical and veterinary concern due to their proven ability to frequently transmit pathogenic microorganisms and toxins to the host; this will be discussed further in section 1.1.4. The importance of ticks. A complete list of Australian ticks, indicating which have been introduced, and those that have been recorded to bite humans and domestic animals is presented in table 1.2. It should be noted that it is likely that many more of these ticks will feed from humans and domestic animals opportunistically, however records are not yet extensive enough to confirm this (Kwak et al., 2018a; Kwak et al., 2017a).

Table 1.2. Australian ticks. A complete list of tick species found in Australia with indication of introduced species and those recorded to bite humans and domestic animals. A summary of information from Barker and Walker, 2014.

Subfamily	Genus	Species	Introduced species	Domestic animal biting (D) Human biting (H)
IXODIDAE				
Amblyomminae	*Amblyomma*	*albolimbatum , calabyi, breviscutatum , echidnae, glauerti, limbatum, macropi, moreliae, moyi, papuanum, postoculatum, triguttatum, vikirri, australiensis, loculosum, fimbriatum, flavomaculatum, trimaculatum*	Nil	*A. triguttatum (H)(D)*
Bothriocrotinae	*Bothriocroton*	*auruginans, concolor, hydrosauri, tachyglossi, undatum, glebopalma*	Nil	*B. auruginans (D)* *B. hydrosauri (H)(D)*
Haemaphysalinae	*Haemaphysalis*	*bancrofti, longicornis, novaeguinae, doenitzi, bremneri, humerosa, lagostrophi, petrogalis, ratti*	*H. longicornis*	*H. bancrofti (H)(D)* *H. longicornis (H)(D)*
Ixodinae	*Ixodes*	*confusus, cordifer, cornuatus, hirsti, holocyclus, myrmecobii, trichosuri, antechini, australiensis, fecialis, vestitus, victoriensis, hydromyidis, tasmani, auritulus, eudyptidis, kerguelenensis, kohlsi, kopsteini, ornithorhynchi, simplex, uriae, woyliei, heathi*	Nil	*I. cornuatus (H)(D)* *I. hirsti (D)* *I. holocyclus (H)(D)* *I. tasmani (H)(D)*
Rhipicephalinae	*Rhipicephalus*	*australis, sanguineus*	*R. australis* *R. sanguineus*	*R. australis (H)(D)* *R. sanguineus (H)(D)*
ARGASIDAE				
Argasinae	*Argas*	*lagenoplastis, falco, lowryae, australiensis, macrodermae, daviesi, deawe, nullarborensis, persicus, robertsi*	*A. persicus*	*A. persicus (D)* *A. robertsi (D)*
Ornithodorinae	*Ornithodoros*	*capensis, gurneyi, macmillani*	Nil	*O. capensis (H)(D)* *O. gurneyi (H)(D)*
	Otobius	*megnini*	*Ot. megnini*	*Ot. megnini (H)(D)*

1.1.4 The importance of ticks

Ticks are of significant medical, veterinary, and economic importance because of the deleterious effects of tick blood-feeding on the host, which can take a range of forms due to the injection of saliva (which may include potent neurotoxins) following the bite, the procurement of blood, and most seriously, the disease agents that ticks transmit (Cupp, 1991). Ticks are one of the world's most important vectors of pathogenic microorganisms. Approximately 10% of known tick species transmit microorganisms that cause disease to humans and domestic animals, many of which are zoonotic, and it is anticipated that the rate of identification of novel tick-borne pathogens (TBP) will continue to rise (Sonenshine and Roe, 2013; Jongejan and Uilenberg, 2004).

Endemic to the Northern Hemisphere, Lyme borreliosis is the most common infectious TBD and is transmitted by the *Ixodes ricinus* complex of ticks (Stanek et al., 2012). There are a vast number of other TBD of public health importance, including human babesiosis, human granulocytic anaplasmosis, Q-fever, and spotted fever (Piesman and Eisen, 2008).

While Australia is not considered to have many of the TBDs endemic overseas, particularly those in the Northern Hemisphere such as Lyme borreliosis, there is still relatively little known about the microorganisms carried by Australian ticks, their potential for transmission, and any pathological consequences (Gofton et al., 2015b). Of particular interest to researchers are Australian ticks known to bite humans, such as the paralysis tick *Ixodes holocyclus*, which can cause dermatological disease, neurological disease, and tick-induced allergies (Gofton et al., 2015b; van Nunen, 2015). There have also been reports of an illness that presents similarly to Lyme borreliosis, however the cause of these symptoms has not yet been identified (Gofton et al., 2015a; Gofton et al., 2015b).

Although important, a thorough discussion of TBD is outside the scope of this study. A number of thorough reviews on TBD worldwide are available (Dantas-Torres et al., 2012; Parola and Raoult, 2001), as well as information relating to the increasing incidence of TBD in Australia specifically (Graves and Stenos, 2017).

1.2 Tick morphology

While the Argasidae and Ixodidae vary morphologically (figure 1.3), there are characteristics common to all ticks. All adult and nymph ticks have eight legs, while all larvae have six, and all ticks possess a circulatory system, bathed in hemolymph (Cupp, 1991). The body of ticks is divided into the capitulum and the opisothoma (Jongejan and Uilenberg, 1994). The capitulum is the anterior part of the body and contains the mouthparts, which consist of sensory and cutting organs, and the hypostome, the structure that, barbed with recurved teeth, allows the tick to anchor to its host (Parola and Raoult, 2001; Jongejan and Uilenberg, 1994). Periphery sensory organs are located on the body and legs. The most complex of these is Haller's organ; a cluster of gustatory and olfactory receptors located on the tarsal of the front pair of legs, which are vital for communication and to locate hosts (Parola and Raoult, 2001).

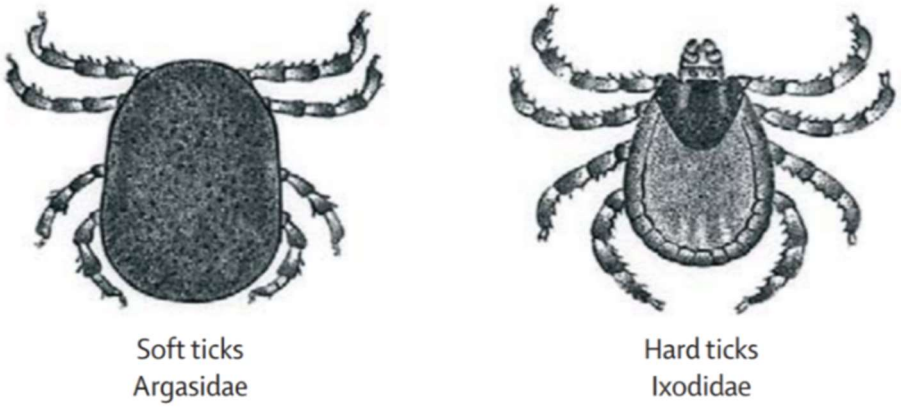


Figure 1.3: An illustration demonstrating the typical differences in dorsal view between soft ticks and hard ticks. Taken from Dante-Torres (2007) .

Hard ticks are characterised by the presence of a dorsal scutum, with a typically tear-shaped body (Cupp, 1991). In males, this scutum almost completely covers the dorsum and limits the quantity of blood that can be ingested at one time, yet in females it is smaller and allows them to become greatly distended during blood-feeding (Hoskins, 1991). The markedly anterior capitulum is easily seen from the dorsal surface and pulvilli are well developed (Cupp, 1991). Eyes may be present, although it is not considered that eyes enable detailed perception (Parola and Raoult, 2001).

Soft ticks lack the presence of a dorsal scutum and are instead characterised by a leathery integument, with a body shape that tends toward oval (Cupp, 1991). Due to this absence of a scutum, soft ticks do not exhibit sexual dimorphism (Hoskins, 1991). Contrary to that of hard ticks, much of the capitulum is situated anteriorly on the ventral surface and cannot be seen from the dorsal surface (Cupp, 1991). In soft ticks, pulvilli are absent or poorly developed, while eyes are usually absent (Hoskins, 1991).

1.2.1 Morphological determination of tick species

To study and control TBD transmission, the identification of ticks by species is essential and is currently achieved primarily by microscopic observation of external morphological characteristics (Diarra et al., 2017). Differences in morphology observed between tick species are too large to discuss within the context of this thesis, however within the Australian context they are detailed in morphological guides such as *Ticks of Australia. The species that infest domestic animals and humans* by Barker and Walker 2014, and *Australian Ticks* by Roberts 1970 (Barker and Walker, 2014; Roberts, 1970).

1.2.2 Limitations of determining species morphologically

While morphological differences between closely related species may be slight, their capacity as vectors for pathogenic microorganisms can differ dramatically, and therefore accurate identification of tick species is crucial (Abdullah et al., 2016; Jongejan and Uilenberg, 1994). However, there are several reasons that identification of ticks by morphology can be challenging.

One major limitation of morphological identification is that it relies heavily on entomological expertise and dichotomous key availability (Diarra et al., 2017). In the case of many species in Australia, identification keys have only been developed for adults, and a lack of available descriptions means that immature ticks are frequently identified only to genus level (e.g. Ernieenor et al., 2017). In Australia, *Australian Ticks* by F.H.S. Roberts is the pillar of native tick morphology, however this text lacks descriptions of all Australian species and life stages (Roberts, 1970). An updated book detailing the morphology of Australian ticks known to bite humans and domestic animals was released

in 2014, however this covers just 16 of the total 72 species (Barker and Walker, 2014). Some species of tick are so similar morphologically that even experienced taxonomists cannot distinguish between them, and if a tick specimen is damaged or engorged, this further complicates the process of identification (Lv et al., 2014b; Caporale et al., 1995).

1.3 Molecular barcoding

Molecular barcoding provides a highly efficient alternative for the identification of biological species, and has been used extensively in the study of arthropod taxonomy (Castalanelli et al., 2014; Taylor and Harris, 2012; Gariepy et al., 2007; Smith et al., 2005). The technique uses short DNA sequences that are conserved within a species, but variable between species, as molecular markers to identify organisms more easily and with more accuracy than can be achieved through morphological studies alone (Hebert et al., 2003a). The process involves amplification of a genetic marker gene by PCR, sequencing of the amplified DNA, and comparison of the DNA sequence to available genetic information of an online database (i.e. GenBank or Barcode of Life) in order to identify the species the DNA originated from (Hebert et al., 2003a). This section will first detail the selection of suitable genetic markers for DNA barcoding. It will then discuss the tick genome, and the use of molecular barcoding for identification of tick species, including recent advances in knowledge.

1.3.1 Genetic markers for molecular barcoding

Appropriate selection of genetic markers for use in molecular barcoding is essential for successful species identification. Ideally, for a gene region to be suitable as a marker, it should satisfy three criteria: 1) it must contain significant divergence and genetic variation at the species level; 2) it must have conserved flanking regions to allow for the development of universal primers; and 3) the sequence must be short enough to conform with current DNA extraction and amplification capabilities (Kress and Erickson, 2008).

Markers from the mitochondrial (mt) genome provide several advantages over nuclear DNA, namely: a high copy number, lack of introns, a high evolutionary rate, and limited exposure to recombination (Yang et al., 2014). Protein coding genes of the mtDNA are considered the best choice for many species; due to four-fold degeneracy, the third nucleotide of the codons of these genes are weakly constrained by selection, allowing for sufficient variation at species level to facilitate identification (Hebert et al., 2003a). A 658bp sequence from the 5' region of the cytochrome c oxidase subunit 1 (COI) protein coding gene has been selected as the standard marker for barcoding of animal species (Hebert et al., 2003a). Although originally selected by the Consortium for the Barcode of Life (CBOL) primarily for the sake of standardisation, COI does offer the advantage of existing universal primers that have repeatedly been shown to be efficient in the recovery of COI sequences from most animal phyla (Che et al., 2012; Ivanova et al., 2007; Hebert et al., 2003b; Folmer et al., 1994). However, limitations have been found in the ability of COI to distinguish at species level within certain groups of organisms, and in some taxa amplification of COI is unreliable (Chee, 2015; Deagle et al., 2014; Vences et al., 2005). Mitochondrial markers 12S rRNA (12S) and 16S rRNA (16S) have been used widely to identify many species with accuracy and provide a good alternative for identification of taxa in which COI is not successful (Yang et al., 2014; Vences et al., 2005).

Certain genes of the nuclear rRNA can also serve well as phylogenetic markers, due to the tandem organisation and high copy numbers of rRNA. The internal transcribed spacer (ITS1 and ITS2) genes of the nuclear rDNA spacer regions are an option for barcoding, as the spacers evolve much faster than the nDNA coding regions (Hwang and Kim, 1999). The 5.8S rRNA gene, imbedded between ITS1 and ITS2, has a similar evolution rate to the ITS, however the short length of the gene (ca. 150bp) means that while useful in phylogenetic studies, it is not an ideal candidate for barcoding (Hwang and Kim, 1999). Similarly, rRNA genes 18S and 28S have proven invaluable for phyla to family level phylogenetic studies but are not suitable candidates as barcoding genes due to inefficiency in discrimination at species level (; Pereira and Baldwin, 2016; Lv et al., 2014b; Taylor et al., 2007; Mallatt and Sullivan, 1998).

1.3.2 Tick genomes

In 2016, the 2.1 gigabase pair nuclear genome of the medically important *Ixodes scapularis* tick (from the northern hemisphere) was assembled, marking the first complete genome sequencing of any tick species (Gulia-Nuss et al., 2016). However, most tick genome studies have focussed on the mitochondrial DNA. As is typical for the majority of metazoa, the tick mitochondrial genome is 14-16kb long and contains 37 genes: two rRNA, 22 tRNA, and 13 protein coding (Boore, 1999). 12S, 16S, and COI have been a mainstay of tick molecular phylogenetics, and as such sequences for these genes are available for a limited number of Australian tick species (Barker and Murrell, 2004). Of the 72 total species, 39 have at least one of these molecular sequences available, and nine Australian tick species have had their entire mitochondrial genome sequenced (table 1.3).

Table 1.3. Molecular data of Australian ticks. Current availability of COI, ITS2, 12S rRNA and 16S rRNA genetic data (shaded green). * Introduced tick species.

Subfamily	Genus	Species	COI	ITS2	12S	16S	Complete mt genome
IXODIDAE							
Amblyomminae	*Amblyomma*	*albolimatum*					
	Amblyomma	*australiensis*					
	Amblyomma	*breviscutatum*					
	Amblyomma	*calabyi*					
	Amblyomma	*echidnae*					
	Amblyomma	*fimbriatum*	■		■	■	
	Amblyomma	*glauerti*			■	■	
	Amblyomma	*limbatum*	■				
	Amblyomma	*loculosum*					
	Amblyomma	*macropi*					
	Amblyomma	*moreliae*					
	Amblyomma	*moyi*					
	Amblyomma	*papuanum*					
	Amblyomma	*postoculatum*					
	Amblyomma	*triguttatum*	■	■	■	■	■
	Amblyomma	*trimaculatum*					
	Amblyomma	*vikirri*	■	■	■	■	
Bothriocrotinae	*Bothriocroton*	*auruginans*					
	Bothriocroton	*concolor*	■	■	■	■	■
	Bothriocroton	*glebopalma*			■		
	Bothriocroton	*hydrosauri*	■				
	Bothriocroton	*tachyglossi*					
	Bothriocroton	*undatum*	■	■	■	■	■
Haemaphysalinae	*Haemaphysalis*	*bancrofti*					
	Haemaphysalis	*bremneri*					
	Haemaphysalis	*doenitzi*	■	■	■		
	Haemaphysalis	*humerosa*	■	■	■		
	Haemaphysalis	*lagostrophi*					
	Haemaphysalis	*longicornis* *	■	■	■	■	■
	Haemaphysalis	*novaeguinae*					
	Haemaphysalis	*petrogalis*					
	Haemaphysalis	*ratti*					
Ixodinae	*Ixodes*	*antechini*	■			■	
	Ixodes	*auritulus*	■		■	■	
	Ixodes	*australiensis*	■				
	Ixodes	*confusus*					
	Ixodes	*cordifer*					
	Ixodes	*cornuatus*	■	■			
	Ixodes	*eudyptidis*					

	Ixodes	*fecialis*					
	Ixodes	*heathi*					
	Ixodes	*hirsti*					
	Ixodes	*holocyclus*					
	Ixodes	*hydromyidis*					
	Ixodes	*kerguelenensis*					
	Ixodes	*kohlsi*					
	Ixodes	*kopsteini*					
	Ixodes	*myrmecobii*					
	Ixodes	*ornithorhynchi*					
	Ixodes	*simplex*					
	Ixodes	*tasmani*					
	Ixodes	*trichosuri*					
	Ixodes	*uriae*					
	Ixodes	*vestitus*					
	Ixodes	*victoriensis*					
	Ixodes	*woylei*					
Rhipicephalinae	*Rhipicephalus*	*australis **					
	Rhipicephalus	*sanguineus **					
ARGASIDAE							
Argasinae	*Argas*	*australiensis*					
	Argas	*daviesi*					
	Argas	*dewae*					
	Argas	*falco*					
	Argas	*lagenoplastis*					
	Argas	*lowryae*					
	Argas	*macrodermae*					
	Argas	*nullarborensis*					
	Argas	*persicus **					
	Argas	*robertsi*					
Ornithodorinae	*Ornithodoros*	*capensis*					
	Ornithodoros	*gurneyi*					
	Ornithodoros	*macmillani*					
	Otobius	*megnini **					

1.3.3. Molecular barcoding for tick identification

Molecular barcoding protocols have been successfully developed for several tick species overseas. In one example of note, Lv et al. examined the best gene for use in Ixodida barcoding and determined that there was no significant difference between success rates of COI, 12S, 16S, and ITS2 in correctly identifying species but suggested that, as the standard barcoding gene, COI should be the first choice for tick species identification (Lv et al., 2014a). In a subsequent study, Lv et al. developed a barcoding system for five species of Ixodida based on COI and 16S (Lv et al., 2014b). This study primarily focussed on Asian tick species and species delimitation was inferred through Neighbour-Joining trees.

Ondrejicka et al. created the first reference DNA barcode library based on COI genes of morphologically identified medically important *Ixodes* spp. in Canada (Ondrejicka et al., 2017). During this, barcoding uncovered several morphological misidentifications of immature ticks, highlighting the difficulties involved in identification even for experts.

In an Australian study, Song et al. determined that COI and ITS2 sequences of the paralysis ticks *I. holocyclus* and *I. cornuatus* were efficient at differentiating these two morphologically equivocal species (Song et al., 2011). Using methodologies described in this study, Kwak et al. later used COI and ITS2 sequences to investigate phylogenetic relationships between Australian paralysis ticks and their relatives (Kwak et al., 2017b).

1.4 Species delimitation

Considering the generation of large numbers of single locus sequences by molecular barcoding, methods of delimitating species based on this data provide an excellent contribution to the taxonomic process (Kekkonen and Hebert, 2014). There are several methods available for species delimitation, which all differ in their analytical and theoretical approach (e.g. Fujisawa and Barraclough, 2013; Ratnasingham and Hebert, 2013; Zhang et al., 2013). For the purposes of this introduction, discussion will be limited to Automatic Barcode Gap Discovery (ABGD).

1.4.1 Automatic Barcode Gap Discovery (ABGD)

Automatic Barcode Gap Discovery is a method for primary species delimitation which sorts sequences into species group hypotheses, ready for further analysis (Puillandre et al., 2012). This method makes use of the barcode gap in the distribution of pairwise differences, observed when the intraspecific divergence is less than the interspecific divergence. ABGD uses prior intraspecific divergence to infer a confidence limit for intraspecific divergence, and identifies the barcode gap as the first significant gap beyond that limit (Puillandre et al., 2012). The data is then partitioned by use of this inference and gap detection over repeated steps, until no further partitioning occurs, and the data has been separated into hypothesised species groups (Puillandre et al., 2012). Many studies have made use of ABGD for species delimitation, reporting a reduced tendency to overestimate species groups when compared to alternative methods (Cao et al., 2016; Guillemin et al., 2016; Kekkonen and Hebert, 2014).

1.5 Conclusion and further research

Over the course of ongoing research driven by the increasing incidence of TBD in Australia, it has become apparent that the current methods of Australian tick identification are not sufficient. The traditional morphological methods that are still relied on are often difficult and time consuming, and in some cases identification by morphology alone is not possible. Molecular barcoding of Australian tick species will allow for a faster and more robust method for identifying Australian tick species at all life stages, and will be accessible for researchers that lack taxonomical experience. Furthermore, barcoding of Australian ticks will continue work towards the development of a more complete genetic database of Australian ticks and contribute to studies of Ixodida globally.

1.6 Thesis aims and hypothesis

The broad aim of this study is to test molecular barcoding assays that can distinguish eight native Australian hard tick species of veterinary and medical importance (table 1.4).

Table 1.4. Tick species at the focus of this study.

Species	Common name
Amblyomma triguttatum	Ornate kangaroo tick
Bothriocroton auruginans	Wombat tick
Haemaphysalis humerosa	Bandicoot tick
Haemaphysalis bancrofti	Wallaby tick
Ixodes cornuatus	Southern paralysis tick
Ixodes hirsti	Hirst's marsupial tick
Ixodes holocyclus	Paralysis tick
Ixodes tasmani	Common marsupial tick

The specific objectives of this study are to:

(i) Test published PCR assays targeting COI, ITS2, 16S rRNA and 12S rRNA that can be used to discriminate the specified Australian tick species,

(ii) Use these assays to produce multi-loci DNA barcode data for Australian ticks that have morphological descriptions but lack any genetic data and undertake a rigorous species delimitation assessment.

(iii) Test the robustness of assays to determine the identification of immature instars and specimens that are unable to be accurately identified to species based on current morphological keys.

(iv) Produce molecular phylogenies to examine the phylogenetic relationships of Australian ticks.

The hypotheses for this study are:

(i) Molecular barcoding assays examined in this study will be able to determine species identification of immature tick life stages.

(ii) All four loci tested in this study will be able to successfully determine species identification (where reference genetic data is available).

(iii) Species delimitation analysis will vary between the four barcoding genes tested in this study.

(iv) While species delimitation analysis will vary, all four genes will produce similar phylogenies showing genetic relatedness and similar tree topologies.

By amplifying and sequencing available barcoding genes, this project will begin to fill in extensive gaps in Australian tick genetic data. This study will focus on the molecular identification of ticks that commonly parasitise humans and companion animals in Australia. Whilst morphological aspects are beyond the scope of this project, it will allow molecular taxonomic assignment to morphologically undescribed nymph and larval specimens, providing reference material for future morphological descriptions of immature life stages.

Chapter 2: Methods and materials

2.1 Tick selection and species determination

The ticks selected for the PCR testing and optimisation stages of this study were chosen due to their medical and veterinary importance, in addition to representing Ixodidae genera. Ticks were provided by researchers who had previously collected and morphologically identified them as part of their own investigations, using identification keys from Australian Ticks (Barker and Walker 2014 and Roberts, 1970). Ticks included in the subset of reference ticks for this study were identified to instar and species. These reference ticks are summarised in table 2.1. Additional ticks were introduced at later stages of the study; where morphological identification was ambiguous, specimens were identified to instar and genus. All tick specimens used during this study had previously undergone genomic DNA extraction using a modified Qiagen Blood and Tissue kit – insect protocol (see Gofton et al., 2015a).

Table 2.1. Reference ticks selected for PCR testing and optimisation.

Sample Reference	Species	Collected & Identified By	Questing ○ From host ●
AT2F	*Amblyomma triguttatum*	A. Gofton	○
AT3M	*Amblyomma triguttatum*	A. Gofton	○
AT4M	*Amblyomma triguttatum*	A. Gofton	○
BA1F	*Bothriocroton auruginans*	S-M. Loh	●
BA2F	*Bothriocroton auruginans*	S-M. Loh	●
HB2F	*Haemaphysalis bancrofti*	S. Egan	●
HB3N	*Haemaphysalis bancrofti*	S. Egan	●
HH1M	*Haemaphysalis humerosa*	S. Egan	●
HH3M	*Haemaphysalis humerosa*	S. Egan	●
HH4M	*Haemaphysalis humerosa*	S. Egan	●
IC1F	*Ixodes cornuatus*	T. Greay	●
IHi1F	*Ixodes hirsti*	T. Greay	●
Q92	*Ixodes holocyclus*	A. Gofton	●
Q93	*Ixodes holocyclus*	A. Gofton	●
IH1F	*Ixodes holocyclus*	S. Egan	●
IT3	*Ixodes tasmani*	M. Ruiz Aravena (collected) S. Egan (identified)	●

2.2 PCR Amplification

Based on a thorough literature review, the following genes were selected for PCR analyses and optimisation, if required: COI, ITS2, 16S and 12S. Details of primers and their original references can be seen in table 2.2. No template controls were included in every assay.

Table 2.2. Primers used for each targeted barcoding gene.

Gene	Sequence (5'-3')	~ Amplicon size (bp)	Reference
COI	GGT GGG CTC ATA CAA TAA ATC C CCA CAA ATC ATA AAG ACA TTG G	850	Song et al., 2011
ITS2	ACA TTG CGG CCT TGG GTC TT TCG CCT GAT CTG AGG TCG AC	700-1500	Lv et al., 2013
16S rRNA	TTA AAT TGC TGT RGT ATT CCG GTC TGA ACT CAS AWC	450	Lv et al, 2013
12S rRNA	AAA CTA GGA TTA GAT ACC CT AAT GAG AGC GAC GGG CGA TGT	370	Beati and Kierans, 2001

2.2.1 Cytochrome c oxidase subunit I (COI)

A modified protocol using primer pair HCO2064/HCO1240 from Song et al., 2011 was used to amplify an approximately 850 bp fragment of COI (Song et al., 2011). Each PCR reaction was carried out in a final volume of 25µL and consisted of 19.85µL dH_2O, 1 X KAPA Taq buffer & dye with 1.5mM $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 0.5mM additional $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 2.0mM dNTPs (FisherBiotech, Australia), 0.12mM each of forward and reverse primers, 1.25 U KAPA Taq (KAPA Biosystems, Massachusetts, USA), and 1µL undiluted DNA. PCR conditions were as follows: An initial step of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 48°C for 30 s and 72°C for 50 s, followed by a final extension step of 72°C for 2 min before being held at 12°C.

2.2.2 Internal transcribed spacer 2 (ITS2)

A 700-1500 bp fragment of ITS2 was amplified using previously published primer pair ITS2-F/ITS2-R (Lv et al., 2014a). Each reaction was carried out in a final volume of 25μL and consisted of 19.05μL dH_2O, 1 X KAPA Taq buffer & dye with 1.5mM $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 2.5mM dNTPs (FisherBiotech, Australia), 0.4mM each of forwards and reverse primers, 0.5 U KAPA Taq (KAPA Biosystems, Massachusetts, USA), and 1μL undiluted DNA. PCR conditions were as follows: An initial step of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 2 min, followed by a final extension step of 68°C for 5 min before being held at 12°C.

2.2.3 16S rRNA

Primer pair 16S-F/16S-R1 from Lv et al., 2014 was used to amplify an approximately 450 bp fragment of 16S rRNA (Lv et al., 2014a). Each reaction was carried out in a final volume of 25μL and consisted of 18.05μL dH_2O, 1 X KAPA Taq buffer & dye with 1.5mM $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 1.0mM additional $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 2.5mM dNTPs (FisherBiotech, Australia), 0.4mM each of forward and reverse primers, 0.5 U KAPA Taq (KAPA Biosystems, Massachusetts, USA), and 1μL undiluted DNA. A touchdown PCR was used, with conditions as follows: An initial step of 94°C for 5 min, followed by 5 cycles of 94°C for 30 s, 49°C for 30 s and 68°C for 30 s, then 5 cycles of 94°C for 30 s, 47°C for 30 s and 68°C for 30 s, then 5 cycles of 94°C for 30 s, 45°C for 30 s and 68°C for 30 s, then 25 cycles of 94°C for 30 s, 43°C for 30 s and 68°C for 30 s followed by a final extension step of 58°C for 5 min before being held at 12°C.

2.2.4 12S rRNA

An approximately 370 bp fragment of 12S rRNA was amplified using previously published primer pair T1B/T2A (Beati and Keirans, 2001). Each reaction was carried out in a final volume of 25μL and consisted of 19.05μL dH_2O, 1 X KAPA Taq buffer & dye with 1.5mM $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 1.0mM additional $MgCl_2$ (KAPA Biosystems, Massachusetts, USA), 2.5mM dNTPs (FisherBiotech, Australia), 0.4mM each

of forward and reverse primers, 0.5 U KAPA Taq (KAPA Biosystems, Massachusetts, USA), and 1µL undiluted DNA. A touchdown PCR was used, with conditions as follows: An initial step of 94°C for 5 min, followed by 5 cycles of 94°C for 15 s, 51°C for 30 s and 68°C for 30 s, then 25 cycles of 94°C for 15 s, 53°C for 30 s and 70°C for 30 s, followed by a final extension step of 70°C for 5 min before being held at 12°C.

2.3 Gel electrophoresis

PCR products were run on a 2% (*w/v*) agarose gel. Agarose (Fisher Biotech, Australia) was dissolved in 1x TAE buffer and stained with SYBR® Safe (Invitrogen™, Australia). PCR products were run alongside a 100 bp molecular weight ladder (Axygen, FisherBiotech, Australia) to determine amplicon size. Ultra-Violet transillumination and AlphaDigiDoc transillumination system (BioRad, Hercules, CA, USA) was used to visualize DNA. Images were captured using a cannon camera and AlphaDigiDoc software.

2.4 DNA purification

Immediately following visualisation, amplified DNA bands of expected size were purified for Sanger sequencing using the filter tip method (Yang et al., 2013). P100 aerosol barrier pipette tips (Interpath Services, Australia) were cut approximately 5mm below the filter and placed inside 1.5mL tubes (Eppendorf™, Germany). DNA bands were excised from agarose gel using sterile scalpel blades and placed inside the filter tips within the tubes. Tubes were centrifuged at 14600 rpm for one minute. The filter tips now embedded with agarose gel were removed and discarded, and Eppendorf tubes containing the purified DNA was stored at -20°C until they were prepared for sequencing.

2.5 Sanger sequencing

Purified DNA was sequenced in both forward and reverse directions using primers specified in section 2.3, table 2.2 on an ABI 373096 Capillary Sequencer (Life Technologies, USA). Sequencing reactions were performed in a total volume of 10µL

containing 1µL of primer (3.2pM), 1µL of 5x reaction buffer (Applied Biosystems, Massachusetts, USA), 2µL of Big Dye version 3.1 (Applied Biosystems, Massachusetts, USA), 4-6µL of DNA, and 0-2µL of sterile dH_2O. PCR conditions were one cycle of 96°C for 2 min, followed by 25 cycles of 96°C for 10 s, the PCR annealing temperature for 5 s (COI: 48°C ; ITS2: 55°C ; 16S: 49°C ; 12S: 53°C) , and 60°C for 4 min, after which is was held at 12°C until removed and stored at -20°C until ethanol precipitation. For ethanol precipitation, 1µL of 125mM EDTA, 1µL of 3M sodium acetate and 35µL of 100% ethanol were added to each 10µL reaction, and each strip tube vortexed for 15 seconds to mix. Tubes were incubated at room temperature for 15-20 min, then placed in a centrifuge and spun at 4100 rpm for 30 minutes. Following this, the tubes were opened, inverted and spun up to 1000 rpm to expel the supernatant. DNA pellets were washed by adding 35µL of 70% ethanol to each tube, and vortexed for 15 seconds before being returned to the centrifuge and spun at 3300 rpm for 15 min. The tubes were again opened and inverted, and spun up to 1000 rpm 1-3 times, until all ethanol had been expelled. The samples were stored at -20°C until sequenced.

2.6 Sequence alignment and BLAST

Forward and reverse sequences were merged, quality filtered, and primers were trimmed using Geneious (version 11.1.3) (Kearse et al., 2012). A final consensus sequences was compared to the NCBI nr/nt database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Where genetic data was available, the top BLAST hit for the genes of each sample were used to confirm the species identity of the specimen.

2.7 Species delimitation

The suitability of COI, ITS2, 16S rRNA and 12S rRNA as genetic barcodes was investigated further through species delimitation using Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012).

Reference sequences for all available Australian hard ticks were downloaded from GenBank, along with a reference subset of non-Australian hard ticks to represent the

major clades. The genetically distant soft tick *Argas persicus* was used as an outgroup for COI, 16S and 12S analyses and *Argas walkerae* was used in analysis of ITS2. 16S, 12S and ITS2 were aligned using ClustalW with a ClustalW cost matrix, an open gap cost of 15 and a gap extend cost of 6.66 (Thompson et al., 1994). Sequences were trimmed to the same length and realigned using ClustalW with the same parameters as the initial alignment. COI sequences were first aligned using Geneious alignment (Kearse et al., 2012). Parameters for alignment were a global alignment with free end gaps, a 65% similarity cost matrix, gap open penalty 12, gap extension penalty 3, and 2 refinement iterations. Once trimmed, sequences were realigned using ClustalW with a ClustalW cost matrix, open gap cost of 15 and extend cost of 6.66 (Thompson et al., 1994). To allow for the inclusion of reference sequences, only relatively small fragments of each gene could be used. In the case of COI, both a short and longer alignment were analysed, with the longer including less reference sequences. Gaps in the consensus of each alignment were removed by eye. The final length of each alignment is summarised in table 2.3.

Table 2.3. Final lengths of each gene alignment used for species delimitation analysis.

Gene	Alignment length (bp)	Sequences
COI	332 (short) 571 (long)	79 91
ITS2	591	35
16S rRNA	358	46
12S rRNA	313	46

As ABGD parameters for Australian ticks have not been established, four Pmax values (Pmax= 0.05, 0.1, 0.15, 2.0) were trialled for each gene. The resulting number of species hypotheses generated was not altered by the change in Pmax, and so the default setting of Pmax= 0.1 was used. X (relative gap width) was kept at the default X= 1.5 for COI screening, but lowered to X= 1.0 in the case of ITS2, 16S rRNA and 12S rRNA following advice from the program. Pmin, steps and Nb bins were kept at default settings. COI, 16S rRNA and 12S rRNA were screened using the Kimura 2-P distance (Kimura, 1980).

However, this model failed to produce results in the case of ITS2, and so the Jukes-Cantor distance (Jukes and Cantor, 1969) was used for this gene. The output of ABGD was then imported into Geneious (version 11.1.3) and analysed using the species delimitation plugin (Masters et al., 2011). Trees were edited in FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/ (2016) by A. Rambaut). Species delimitation species group hypotheses were considered in conjunction with Rosenberg's test statistic (P_{AB}). The null hypothesis of random branching is rejected, and reciprocal monophyly observed, at a significance level of $P_{AB} \leq 0.1$.

2.8 Phylogenetic analysis

2.8.1 Analysis using RAxML (Randomised Axelerated Maximum Likelihood)

Phylogenetic analysis made use of reference sequences downloaded from Gen Bank in section 2.8. *Argas persicus* was used as an outgroup for COI, 16S and 12S analyses and *Argas walkerae* was used in analysis of ITS2. Sequence alignments for each gene were performed as described in section 2.8. The final length of the alignments analysed, and the total number of sequences included for each gene is summarised in table 2.4. Gaps in the consensus of each alignment were removed by eye.

Table 2.4. Final lengths of each gene alignment used for species delimitation analysis.

Gene	Alignment length (bp)	Sequences
COI	329 (short)	51
	585 (long)	56
ITS2	596	26
16S rRNA	350	46
12S rRNA	313	46

Phylogenetic analysis was performed using RAxML (Randomized Axelerated Maximum Likelihood). The best fit model for each gene was assessed using MEGA7 (Kumar et al., 2016), based on the lowest Bayesian Information Criterion (BIC) score. Bayesian phylogenetic reconstruction using sequence alignments were produced using the Geneious RAxML plugin (Stamatakis, 2014). GTR GAMMA substitution model was used for all genes, with rapid bootstrapping, search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. The resulting best ML tree with bootstrapping support values was then edited in Geneious (version 11.1.3).

2.8.2 Sequence concatenation

Sequences were concatenated using Geneious version 11.1.3. Twenty-three sequences were used, including *Argas walkerae* as an outgroup. The total alignment length was 1,441 bp. Bayesian phylogenetic reconstruction using sequence alignments were produced using the Geneious RAxML plugin (Stamatakis, 2014) using GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. The resulting tree was edited in Geneious (version 11.1.3).

Chapter 3: Results

3.1 Amplification of target barcoding genes

3.1.1 Cytochrome c oxidase subunit I (COI)

An approximately 850 bp fragment of COI was amplified consistently across three of the four genera tested (*Ixodes, Haemaphysalis*, and *Bothriocroton*). However, amplification of two of the three samples of *Amblyomma triguttatum* produced only trace amounts of amplified DNA, visualised as a weak band produced on an agarose gel which was not viable for Sanger sequencing and required re-amplification of the PCR product. Amplification of COI was achieved for all 16 reference tick samples tested. The use of undiluted DNA was optimal for amplification of most genera (figure 3.1a), although in the case of *Ixodes* samples strong amplification was also seen at 1/10 and 1/100 dilutions.

3.1.2 Internal transcribed spacer 2 (ITS2)

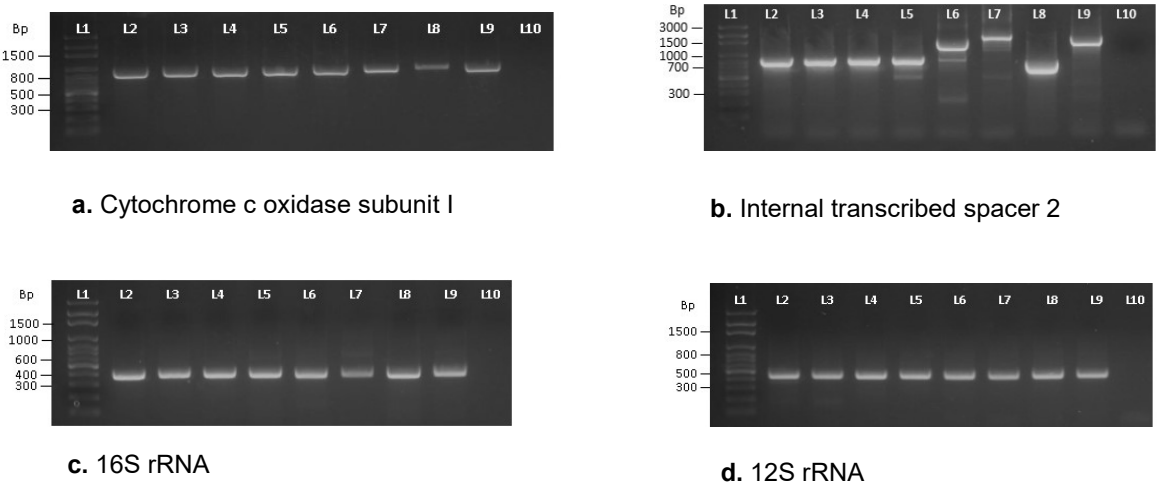
Amplification of ITS2 resulted in a fragment ranging from approximately 700 bp - 1500 bp depending on genus (figure 3.1b). *Bothriocroton* and *Haemaphysalis* samples produced the largest amplicons (1300-1350 bp and 1400-1500 bp respectively), while *Ixodes* and *Amblyomma* samples produced smaller fragments (both of approximately 600 – 700 bp). Amplification was successful in 14 out of 16 reference tick samples, with one *Haemaphysalis* and one *Bothriocroton* sample each failing to amplify despite multiple attempts. The use of undiluted DNA resulted in the strongest amplification across all genera, although in most cases a 1/10 dilution also amplified sufficiently.

3.1.3 16S rRNA

Amplicon size of 16S rRNA was approximately 450 bp across all genera tested (figure 3.1c). Amplification was successful in all 16 reference tick samples. Undiluted DNA achieved strong amplification across all genera consistently, and in 14 of the 16 samples 1/10 and 1/100 dilutions amplified equally well.

3.1.4 12S rRNA

Amplification of 12S rRNA resulted in an approximately 370 bp fragment across all genera (figure 3.1d). Amplification was successful in all 16 reference tick samples. The use of undiluted DNA or 1/10 dilutions resulted in the strongest amplification in all samples, while 1/100 dilutions usually did not amplify sufficiently.



a. Cytochrome c oxidase subunit I

b. Internal transcribed spacer 2

c. 16S rRNA

d. 12S rRNA

Figure 3.1 a–d. Amplification of target barcoding genes across Australian Ixodidae genera using undiluted DNA. L1: Ladder; L2: *Ixodes holocyclus*; L3: *Ixodes cornuatus*; L4: *Ixodes hirsti*; L5: *Ixodes tasmani*; L6: *Haemaphysalis humerosa*; L7: *Haemaphysalis bancrofti*; L8: *Amblyomma triguttatum*; L9: *Bothriocroton auruginans*; L10: No template control.

3.2 Confirmation of species identity using BLAST

The top NCBI BLAST result for each reference sequence generated are summarised in table 3.1 and were used to confirm the identity of specimens. Where relevant genetic data from target genes was available, the top BLAST hit matched the morphological identification.

Table 3.1. Top GenBank BLAST matched for each reference tick sample gene sequenced.

* No genetic data currently available on NCBI database for the species that was identified morphologically.

Sample	Morphological Identification	Gene	Closest GenBank Match	Accession	Similarity (%)	Query Coverage (%)	E value
AT2F	*Amblyomma triguttatum*	COI	*Amblyomma triguttatum*	AB113317	98	100	0
		ITS2*	*Amblyomma aureolatum*	AF469611	82	42	4e-58
		16S	*Amblyomma triguttatum*	AB113317	99	100	0
		12S	*Amblyomma triguttatum*	AB113317	99	100	1e-180
AT3M	*Amblyomma triguttatum*	COI	*Amblyomma triguttatum*	AB113317	98	100	0
		ITS2*	*NOT SEQUENCED*				
		16S	*Amblyomma triguttatum*	AB113317	99	100	0
		12S	*Amblyomma triguttatum*	AB113317	99	100	0
AT4M	*Amblyomma triguttatum*	COI	*Amblyomma triguttatum*	AB113317	98	100	0
		ITS2*	*Amblyomma aureolatum*	AF469611	82	43	4e-58
		16S	*Amblyomma triguttatum*	AB113317	100	100	0
		12S	*Amblyomma triguttatum*	AB113317	99	100	0
BA1F	*Bothriocroton auruginans*	COI*	*Bothriocroton sp.*	KM821512	100	85	0
		ITS2*	*Aponomma concolor*	AF119116	87	89	0
		16S*	*Bothriocroton concolor*	JN863727	92	99	2e-169
		12S*	*Aponomma hydrosauri*	U95860	88	99	6e-119
BA2F	*Bothriocroton auruginans*	COI*	*Bothriocroton sp.*	KM821512	99	82	0
		ITS2*	*FAILED TO AMPLIFY*				
		16S*	*Bothriocroton concolor*	JN863727	92	100	1e-160
		12S*	*Aponomma hydrosauri*	U95860	89	95	7e-113
HB2F	*Haemaphysalis bancrofti*	COI*	*Haemaphysalis sp.*	KM821505	99	84	0
		ITS2*	*FAILED TO AMPLIFY*				
		16S*	*Haemaphysalis doenitzi*	JF979402	96	100	0
		12S*	*Haemaphysalis doenitzi*	JQ346679	97	100	2e-168
HB3F	*Haemaphysalis bancrofti*	COI*	*Haemaphysalis sp.*	KM821505	99	85	0

		ITS2*	*Haemaphysalis humerosa*	AF199115	86	90	2e-96
		16S*	*Haemaphysalis doenitzi*	JF979402	96	100	0
		12S*	*Haemaphysalis doenitzi*	JQ346679	97	99	2e-178
HH1M	*Haemaphysalis humerosa*	COI	*Haemaphysalis humerosa*	JX573138	99	100	0
		ITS2	*NOT SEQUENCED*				
		16S	*Haemaphysalis humerosa*	JX573138	100	100	0
		12S	*Haemaphysalis humerosa*	AF031852	98	87	4e-150
HH3M	*Haemaphysalis humerosa*	COI	*Haemaphysalis humerosa*	JX573138	95	100	0
		ITS2	*Haemaphysalis humerosa*	AF199115	99	100	0
		16S	*Haemaphysalis humerosa*	JX573138	97	99	0
		12S	*Haemaphysalis humerosa*	AF031852	95	87	4e-137
HH4M	*Haemaphysalis humerosa*	COI	*Haemaphysalis humerosa*	JX573138	99	100	0
		ITS2	*Haemaphysalis humerosa*	AF199115	99	100	0
		16S	*Haemaphysalis humerosa*	JX573138	96	99	0
		12S	*Haemaphysalis humerosa*	AF031852	95	83	9e-139
IC1F	*Ixodes cornuatus*	COI	*Ixodes cornuatus*	KY213793	99	85	0
		ITS2	*Ixodes cornuatus*	KY213761	99	82	0
		16S*	*Ixodes holocyclus*	AF113930	98	100	0
		12S*	*Ixodes holocyclus*	AB075955	93	100	2e-153
IHi1F	*Ixodes hirsti*	COI	*Ixodes hirsti*	KY213775	99	84	0
		ITS2	*Ixodes hirsti*	KY213764	99	81	0
		16S*	*Ixodes holocyclus*	AB075955	93	99	0
		12S*	*Ixodes holocyclus*	AB075955	91	100	3e-136
IH1F	*Ixodes holocyclus*	COI	*Ixodes holocyclus*	AB075955	99	100	0
		ITS2	*Ixodes holocyclus*	AB025592	100	99	0
		16S	*Ixodes holocyclus*	AB051844	99	99	0
		12S	*Ixodes holocyclus*	AB075955	99	100	0
Q92	*Ixodes holocyclus*	COI	*Ixodes holocyclus*	AB075955	99	100	0
		ITS2	*Ixodes holocyclus*	AB025592	100	98	0
		16S	*Ixodes holocyclus*	AB075955	99	100	0
		12S	*Ixodes holocyclus*	AB075955	99	98	8e-172

Q93	*Ixodes holocyclus*	COI	*Ixodes holocyclus*	AB075955	99	100	0
		ITS2	*Ixodes holocyclus*	AF208344	99	99	0
		16S	*Ixodes holocyclus*	AB075955	99	100	0
		12S	*Ixodes holocyclus*	AB075955	98	100	0
IT3	*Ixodes tasmani*	COI	*Ixodes tasmani*	KY213771	97	86	0
		ITS2*	*Ixodes uriae*	D88307	78	28	6e-38
		16S	*Ixodes tasmani*	U95906	99	100	0
		12S	*Ixodes tasmani*	U95905	97	100	2e-178

3.3 Species determination of *Ixodes* nymphs and larvae

Identification to species level was required for ten subadult *Ixodes* ticks (nymph n=7, larvae n=3) to facilitate use of these specimens in another study. COI (figure 3.2a) and 16S (figure 3.2b) genes were successfully amplified and sequenced in 8/10 and 10/10 samples respectively. The top NCBI BLAST match of the COI sequences (table 3.2) showed 7/8 sequences generated were able to obtain a sufficient similarity (>99%) and E value to confirm species identity of subadults.

a. Cytochrome c oxidase subunit I **b.** 16S rRNA

Figure 3.2 a & b. Amplification of target genes of subadult *Ixodes* ticks.
L1: Ladder; L2: UIN1; L3: UIN2; L4: UIN3; L5: UIN4; L6: UIN5; L7: UIN6; L8; UIN7; L9: UIL1; L10: UIL2; L11: UIL3; L12: No template control.

Table 3.2 *Ixodes* subadult specimen COI sequence BLAST search results.

Sample	Closest GenBank Species	Accession	Simularity (%)	Query Coverage (%)	E value
UIN1	*Ixodes holocyclus*	KY213782	100.0	94.1	0.0
UIN2	*Ixodes holocyclus*	KY213782	100.0	95.4	0.0
UIN3	*Ixodes trichosuri*	KY213778	99.9	85.9	0.0
UIN4	*Ixodes trichosuri*	KY213778	99.9	84.7	0.0
UIN5	*Ixodes trichosuri*	KY213778	99.9	85.1	0.0
UIN6	*Ixodes sp.*	KM488530	99.5	82.2	0.0
UIN7					
UIL1	*Ixodes trichosuri*	KY213778	99.8	91.0	0.0
UIL2					
UIL3	*Ixodes trichosuri*	KY213777	100.0	87	0.0

The COI amplicon of UIN7 was not successfully sequenced, and UIL2 COI failed to amplify. However, a BLAST search comparing the 16S sequences of these samples to a custom database containing the 16S sequences generated in this study confirmed that both UIN7 and UIL2 were identical to the species identified for UIN3, UIN4, UIN5, UIL1, and UIL3. The top match for UIN6 was a sequence of an unspecified species. Furthermore, it did not match any of the other sequences obtained during this study and therefore was not identified at this stage.

3.4 Species delimitation using Automatic Barcode Gap Discovery

3.4.1 Species delimitation of COI sequences

Species delimitation was performed using a short (342 bp) alignment of COI to facilitate the inclusion of a larger number of reference sequences (figure 3.3). Forty-two species were represented across 91 sequences, including 15 sequences generated from ticks in this study and 76 reference sequences obtained from GenBank. The soft tick *Argas persicus* was included as an outgroup. Species delimitation using Automatic Barcode Gap Discovery (ABGD) revealed 45 monophyletic species group hypotheses, of which 22 had $P_{AB} \leq 0.1$. Two *Ixodes tasmani* sequences grouped together, separately from the other *I. tasmani* samples. The Rosenburg test statistic (P_{AB}) for the two groups were both $P_{AB}=0.01$. The *H. humerosa* sequence also grouped away from the others of that species ($P_{AB}= 0.1$ for both groups), and the two *Ixodes simplex* sequences obtained from GenBank each formed an individual group ($P_{AB}=1.0$ for both).

To investigate the impact of a longer sequence length on this outcome, several reference sequences were discarded, and the same analysis was performed on a longer COI sequence (572bp) (figure A1.1). This saw the *H. humerosa* groups differentiate further, with one group containing three sequences ($P_{AB}=1.7$) and the other two groups containing one sequence each ($P_{AB}=0.17$ and $P_{AB}=0.1$). The other species groupings remained the same as for the shorter alignment.

3.4.2 Species delimitation of ITS2 sequences

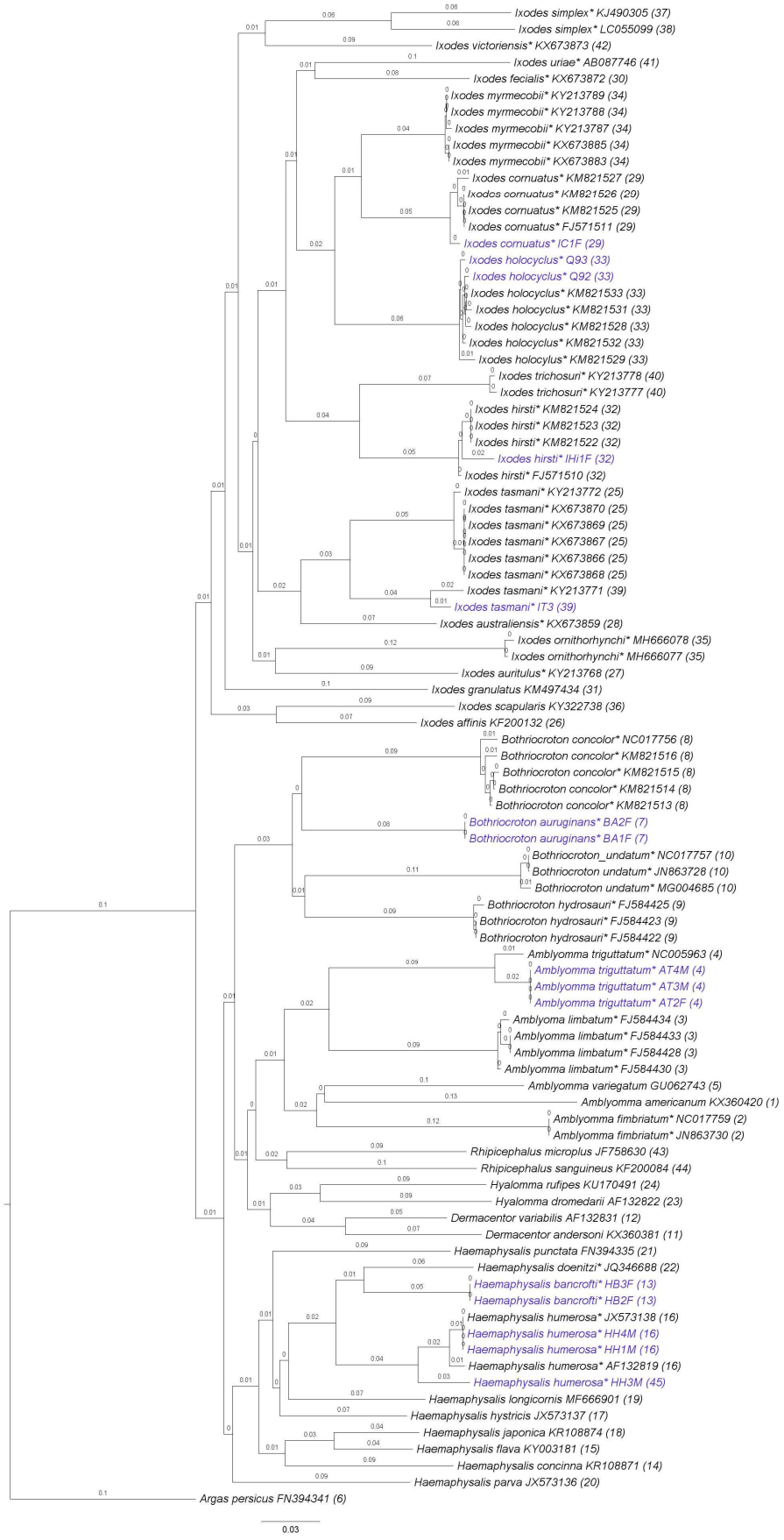
Species delimitation was performed on a 453 bp alignment of ITS2 due to a lack of available longer reference sequences and high numbers of gaps in alignment (figure A1.2). While 30 species were included within 35 sequences (11 generated from this study and 24 reference sequences from GenBank including one *Argas walkerae* outgroup sequence), ABGD screening resulted in only 17 species groups, all with a significant P_{AB} value. Changes to Pmax were trialled but further species groups were not generated.

3.4.3 Species delimitation of 16S sequences

Species delimitation was performed using a 350 bp alignment of 16S (figure 3.4). Thirty-five species were represented across 46 sequences (16 sequences generated from ticks in this study and 30 reference sequences obtained from GenBank, including one *Argas persicus* outgroup sequence). Screening with ABGD revealed 34 monophyletic species group hypotheses, with failure to discriminate between *H. bancrofti* and *Haemaphysalis doenitzi* (P_{AB}= 0.01). Sixteen of the species groups had a $P_{AB} \leq 0.1$.

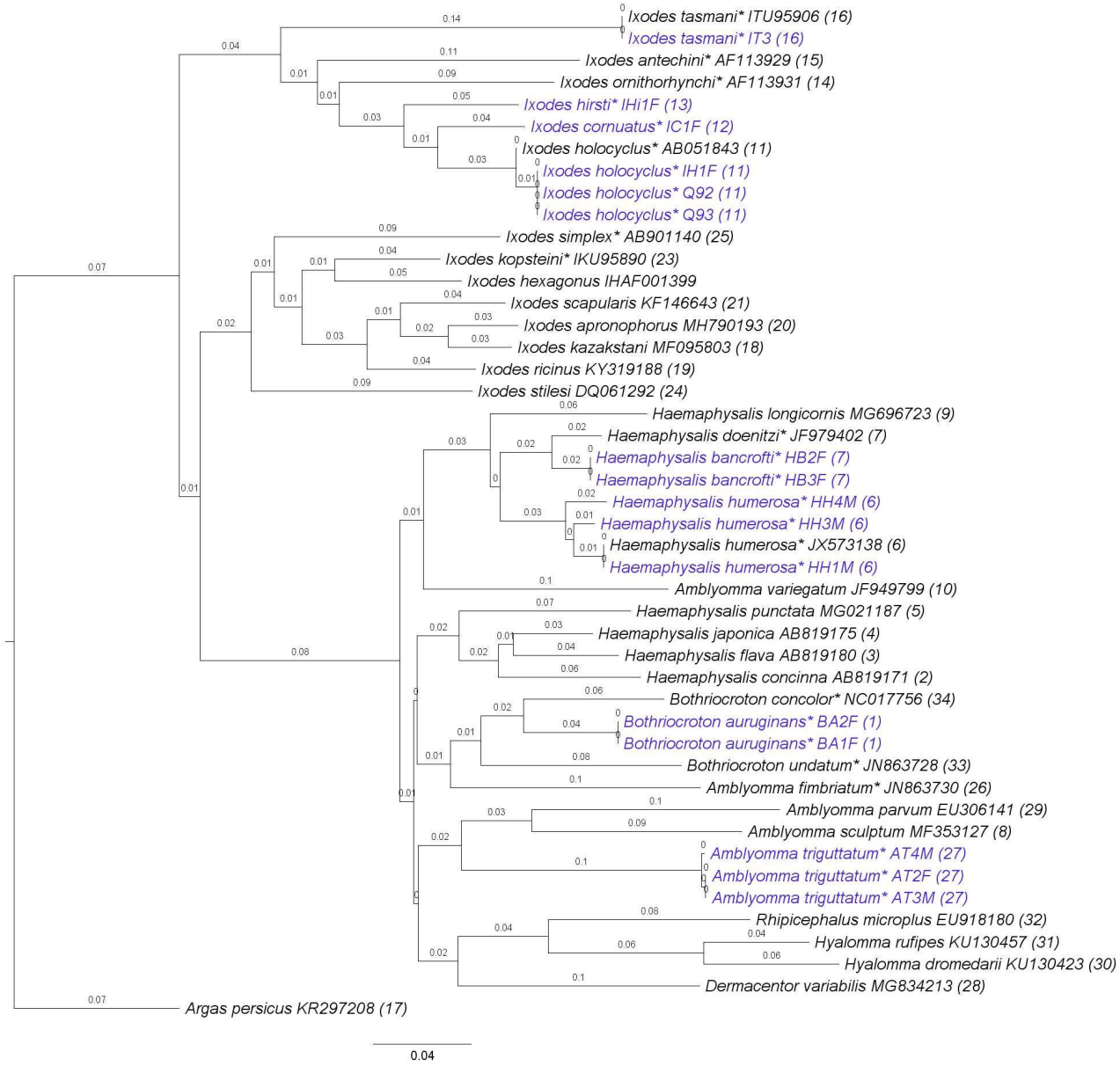
3.4.4 Species delimitation of 12S sequences

Species delimitation was performed using a 313 bp alignment of 12S (figure 3.5). Thirty-six species were represented across 46 sequences (17 sequences generated from ticks in this study and 29 reference sequences obtained from GenBank, including one *Argas persicus* outgroup sequence). ABGD screening resulted in 34 monophyletic species group hypotheses, with *H. bancrofti* and *H. doenitzi* grouped as one species (P_{AB}= 0.04). Fifteen of the 34 groups had a $P_{AB} \leq 0.1$.



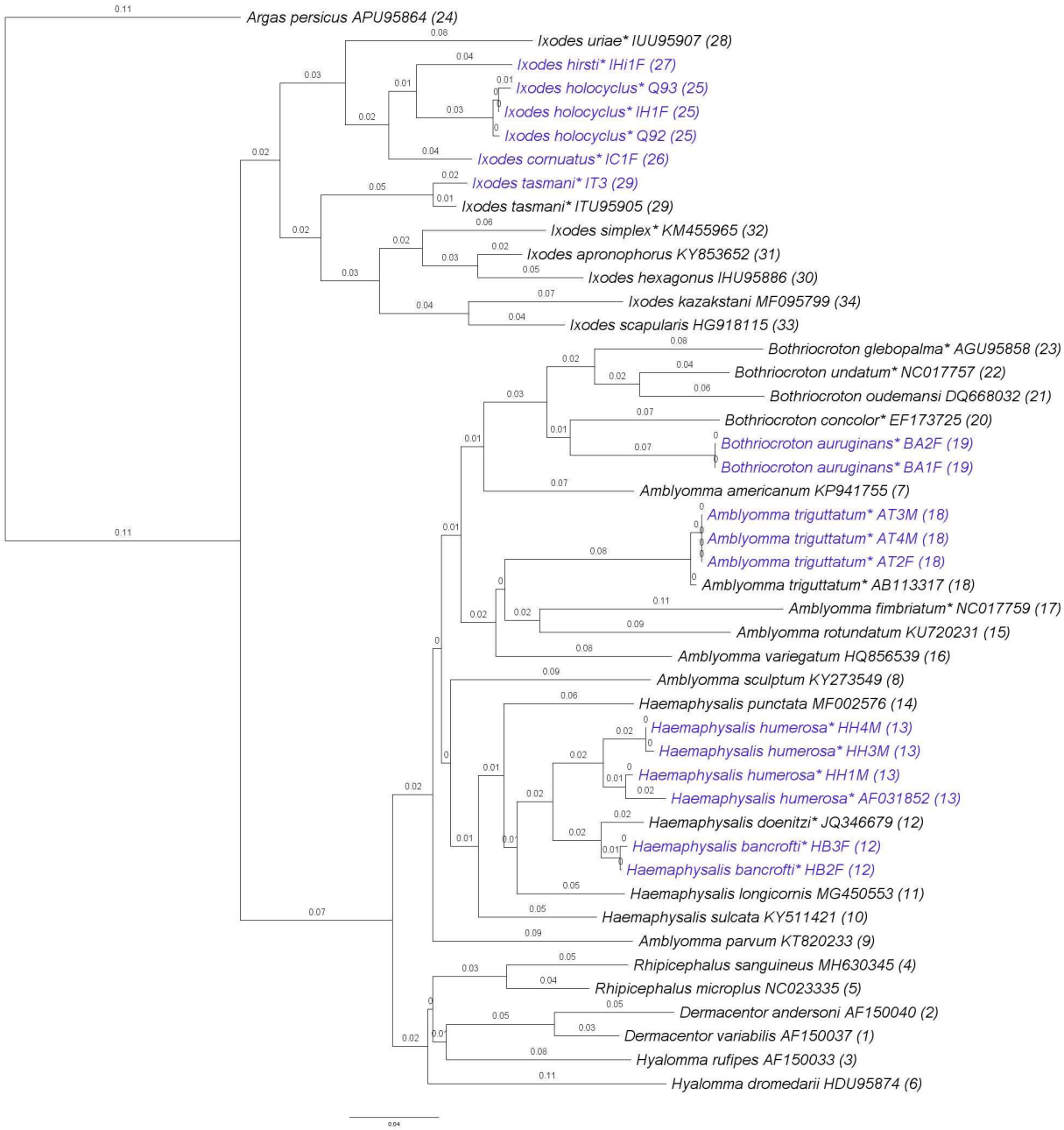
Group	P_{AB}
1	1
2	0.11
3	4.08E-03
4	4.08E-03
5	1
6	2.40E-04
7	0.02
8	0.02
9	0.02
10	0.02
11	1
12	1
13	0.33
14	0.33
15	1
16	0.1
17	0.02
18	1
19	0.03
20	0.01
21	0.02
22	0.33
23	1
24	1
25	0.01
26	1
27	0.33
28	0.03
29	8.80E-04
30	1
31	1.16E-03
32	0.02
33	6.40E-06
34	8.80E-04
35	0.33
36	1
37	1
38	1
39	0.01
40	0.02
41	1
42	0.33
43	1
44	1
45	0.1

Figure 3.3. Species delimitation of reference sample COI sequences (342 bp) using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.15) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent the Rosenberg test statistic. Sequences in blue indicate those sequenced in this study, and asterisks indicate native Australian Ixodidae.



Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
P_{AB}	0.33	0.33	1	1	0.17	0.01	0.01	1	4.00E-02	0.03	0.1	0.07	0.05	0.05	0.04	4.94E-03	9.60E-04
Group	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**
P_{AB}	1	0.17	1	0.33	1	0.05	0.04	0.05	0.1	0.05	0.17	1	1	1	0.33	0.17	0.33

Figure 3.4. Species delimitation of reference samples 16S rRNA sequences using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.1) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent the Rosenberg test statistic. Sequences in blue indicate those sequenced in this study, and asterisks indicate native Australian Ixodidae.



Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
P_{AB}	1	1	0.33	1	1	0.07	0.05	0.02	3.08E-03	0.02	0.04	0.01	0.01	0.03	1	0.05	1
Group	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**
P_{AB}	0.03	0.33	0.33	1	1	0.33	9.60E-04	0.17	0.1	0.17	0.07	0.02	0.33	0.33	0.33	1	1

Figure 3.5. Species delimitation of reference samples 12S rRNA sequences using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.1) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent Rosenberg's test statistic. Sequences in blue indicate those sequenced in this study, and asterisks indicate native Australian Ixodidae.

3.5 Phylogenetic analysis and sequence concatenation

3.5.1 Phylogenetic analysis of COI using RAxML

The topography produced by COI was investigated using RAxML to analyse a 585 bp alignment of 52 sequences including the soft tick *Argas persicus* used as an outgroup (figure 3.6). The RAxML output resulted in a monophyletic *Ixodes* clade encompassing both Australian and Non-Australian *Ixodes*. *Amblyomma* and *Bothriocroton* both form monophyletic clades. The *Dermacentor, Hyalomma,* and *Rhipicephalus* genera form a group, in which each genus forms a monophyletic clade. The genus *Haemaphysalis* is paraphyletic due to the branching of *H. parva*.

To investigate the effect of sequence length on tree topology, the same analysis was performed on a 329 bp alignment of the same 52 sequences as above. The resulting tree showed a very similar topology to that of the longer alignment, with the exception of *I. uriea* and I. *auritulus* (figure A1.3).

3.5.2 Phylogenetic analysis of ITS2 using RAxML

Following the unsuccessful use of ITS2 for species delimitation, analysis was conducted to investigate whether the gene was useful in constructing phylogenies. RAxML analysis was performed on a 332 bp alignment of 37 sequences, using soft tick *Argas walkerae* as an outgroup. This produced no useable results, and nor did subsequent analyses of 395 bp and 453 bp. A longer 596 bp alignment of 26 sequences resulted in topology resembling that of the COI analysis (figure A1.4). *Amblyomma Bothriocroton*, *Haemaphysalis,* and *Ixodes* each formed monophyletic clades. *Dermacentor* and *Rhipicephalus* grouped together, within which *Rhipicephalus* formed a monophyletic clade.

3.5.3 Phylogenetic analysis of 16S using RAxML

Phylogenetic analysis of 16S and 12S was conducted to investigate whether the phylogenies constructed by these individual genes are similar to that generated by COI.

A 350 bp alignment of 46 16S sequences was analysed using RAxML (figure A1.5). The *Ixodes* were paraphyletic, with the Australian and Non-Australian species grouping separately, except for *I. simplex*. *Bothriocroton*, *Dermacentor, Hyalomma*, and *Rhipicephalus* each formed a monophyletic clade, while *Amblyomma* and *Haemaphysalis* were paraphyletic.

3.5.4 Phylogenetic analysis of 16S using RAxML

For 12S, a 313 bp alignment of 46 sequences was analysed using RAxML (figure A1.6). Again the *Ixodes* were paraphyletic, and the grouping of the Australian and Non-Australian species less apparent. *Amblyomma* was also paraphyletic. *Bothriocroton, Dermacentor, Haemaphysalis, Hyalomma*, and *Rhipicephalus* each formed monophyletic clades.

3.5.5 Sequence concatenation of COI, ITS2 and 16S and 12S sequences

RAxML was further used for analysis of the concatenated COI, ITS2, 16S and 12S sequences (figure 3.7). Twenty-three specimens were included, and the full concatenated sequence length was 1,441 bp. The concatenated tree presented a similar topography to the individual COI tree (figure 3.8). Each genus formed a monophyletic clade, with the Australian and Non-Australian *Ixodes* grouping separately.

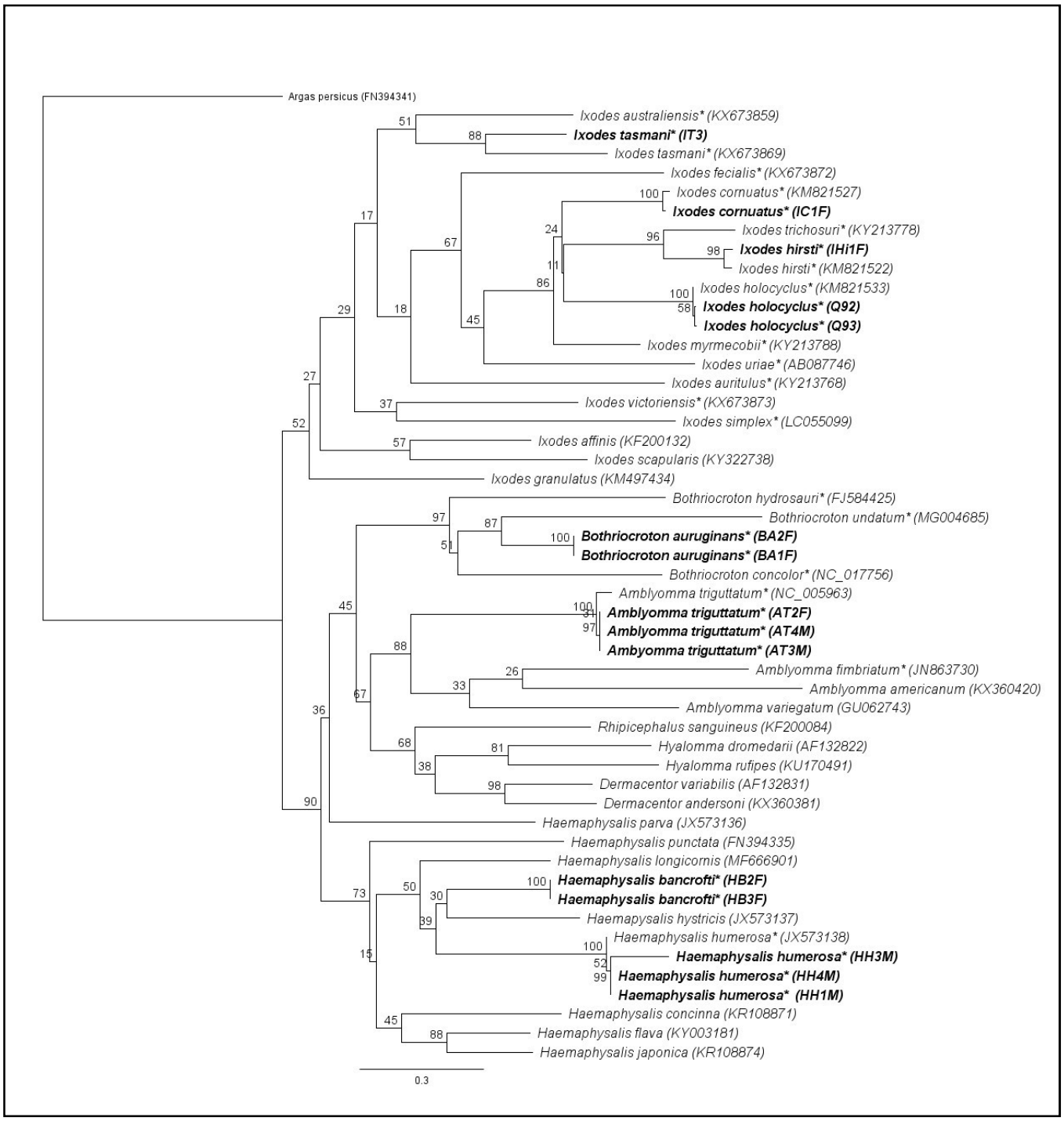


Figure 3.6. RAxML analysis of a 585 bp alignment of tick COI sequences. Analysis was conducted using a GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. Bolded sequences indicate those generated from this study, and asterisks indicate native Australian Ixodidae.

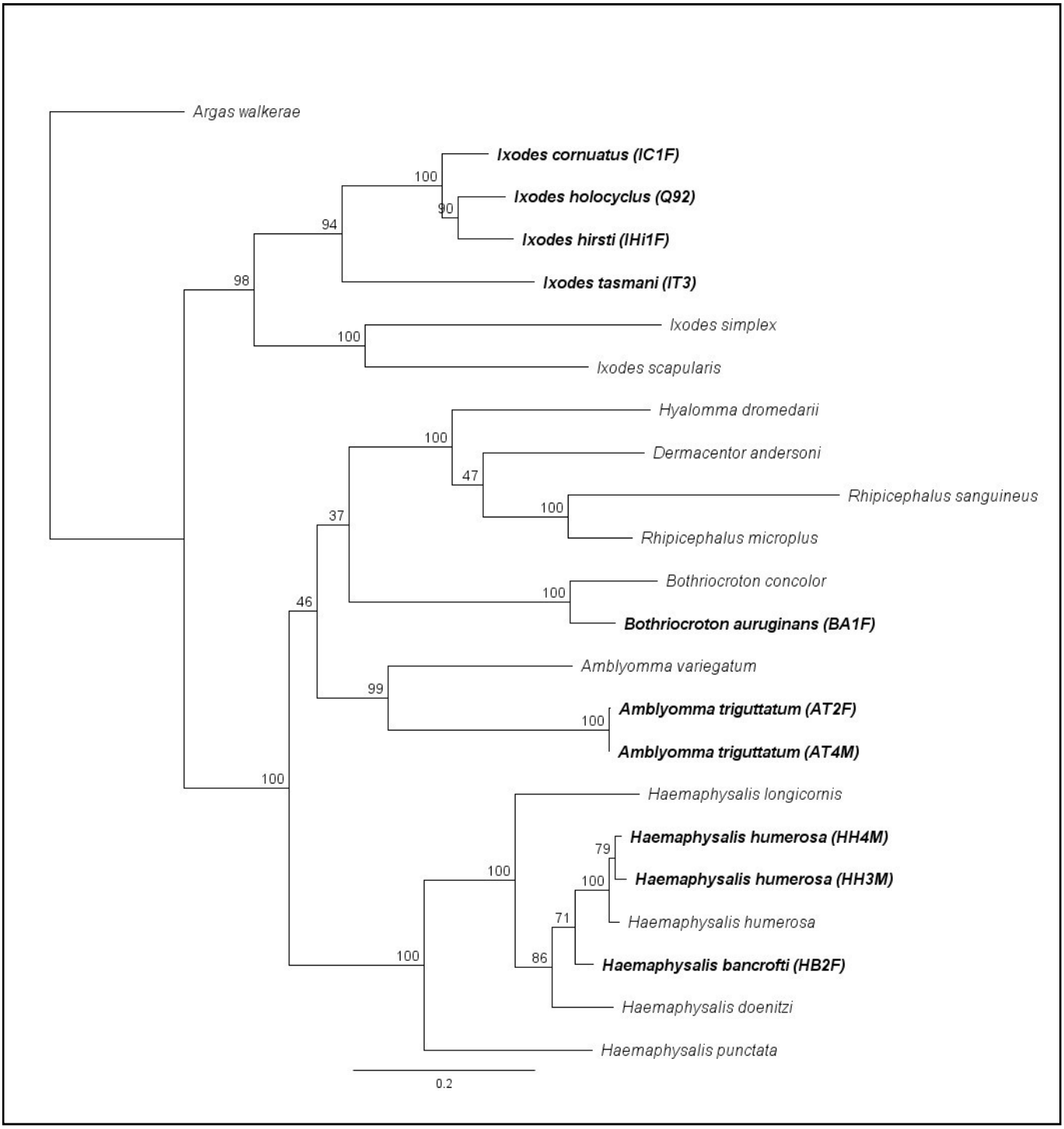


Figure 3.7. RAxML analysis of concatenated reference tick COI, ITS2, 16S and 12S sequences. Analysis was conducted using a GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. Bolded sequences indicate those generated from this study.

3.6 Comparison of COI genetic distances and ABGD group hypotheses

3.6.1 Genetic variation within COI sequences

The highest genetic variation of *Amblyomma* ticks was seen in *A. triguttatum* (number of sequences= 4; pairwise differences= 0 – 3.22%), followed by *A. limbatum* (n= 4; 0 – 1.32%) and *A. fimbriatum* (n= 2; 0%) showed no variation. Of *Bothriocroton*, *B. concolor* (n=5; 0.29 – 2.05%) showed highest variation, followed by *B. undatum* (n=3; 0 – 0.88), *B. hydrosauri* (*n*= 3; 0 – 0.59%*)*, while *B. auruginans* showed no genetic variation (n= 2; 0%). The highest variation of the two *Haemaphysalis* species was observed in *H. humerosa* (n=5; 0-5.56%) while *H. bancrofti* showed no variation (*n*=2; 0%). Among *Ixodes*, *I. simplex* variation was the highest (n= 2; 11.44%), followed by *I. tasmani* (n= 8; 0 – 10.82%), *I. hirsti* (n=5; 0 – 2.05%), *I. holocyclus* (n= 7; 0.29 – 1.90%); *I. cornuatus* (n= 5; 0 – 1.02%); and *I. myrmecobii* (n=5; 0 – 0.59%), *I. orinthorhynchi* (n= 2; 0 – 0.59%) and *I. trichosuri* (*n*= 2; 0 – 0.59%) all displayed the same amount of variation. These distances are summarised in table 3.3.

3.6.2 Comparison of COI distances to ABGD group hypotheses

The largest variation observed within any individual ADBG species group was within *A. triguttatum* with a 3.22% pairwise distance. Three groups thought to each contain one species split into two groups each (*H. humerosa*, *I. simplex* and *I. tasmani*); the lowest amount of variation between two of these groups was *H. humerosa* (group 16 and 45) with a 5.56% difference (table 3.3). According to this, the threshold for species grouping is in the range of a 3.23 – 5.55% genetic pairwise difference.

Table 3.3. Variation within and between species groups that split during species delimitation analysis (pairwise distance %).

Species	ABGD Species Group	Number of sequences	Variation within group (%)	Variation between groups (%)
Haemaphysalis humerosa	16	4	0–1.46	5.56
	45	1	-	
Ixodes simplex	37	1	-	11.4
	38	1	-	
Ixodes tasmani	25	6	0–0.88	9.934–10.82
	39	2	2.63	

Table 3.4. Pairwise differences between COI sequences within Species. ***** Includes sequences generated by this study

Species	Number of Sequences	Distance (%)
Amblyomma fimbriatum	2	0
Amblyomma limbatum	4	0–1.32
*Amblyomma triguttatum**	4	0–3.22
*Bothriocroton auruginans**	2	0
Bothriocroton concolor	5	0.29–2.05
Bothriocroton hydrosauri	3	0–0.59
Bothriocroton undatum	3	0–0.88
*Haemaphysalis bancrofti**	2	0
*Haemaphysalis humerosa**	5	0–5.56
*Ixodes cornuatus**	5	0–1.02
*Ixodes hirsti**	5	0–2.05
*Ixodes holocyclus**	7	0.29–1.90
Ixodes myrmecobii	5	0 –0.59
Ixodes orinthorhynchi	2	0.59
Ixodes simplex	2	11.44
*Ixodes tasmani**	8	0–10.82
Ixodes trichosuri	2	0.59

3.7 Application of barcoding to wildlife ticks of undetermined species

A selection of 32 adult and nymphal ticks removed from Australian wildlife were used to further assess the efficiency of COI, 16S and 12S for barcoding across a wider variety of more poorly described genera. Specimen identification based on morphology and the top COI BLAST result for each sample is summarised in table 3.4. All but two specimens (UWN52 and UWN59) matched most closely with the genus they had been morphologically identified as belonging to. UWN52 was identified as the genus *Bothriocroton* but matched most closely with *Amblyomma*. In the case of UWN59, only host COI DNA was amplified and as such this sample was excluded from further analyses.

Species delimitation was used to investigate the species of the UWN samples using COI, 16S and 12S sequences. The UWN samples were aligned with reference sequences from the ticks known to be present in Australia. For COI, 27 species were represented in addition to the unknown species of the UWN samples. Analysis using ABGD resulted in 30 monophyletic species groups, of which 20 had a $P_{AB} \leq 0.1$. The grouping of the reference sequences was identical to that of the previous COI species delimitation analysis with the exception that *H. humerosa* was no longer split into two species groups ($P_{AB}= 0.1$). The 16S alignment contained 18 species in addition to the UWN samples. ABGD revealed 20 monophyletic species group hypotheses, with $P_{AB} \leq 0.1$ in 14 of them. The 12S alignment also contained 12 species in addition to the UWN samples. ABGD hypothesised 21 monophyletic species groups, of which 12 had a $P_{AB} \leq 0.1$.

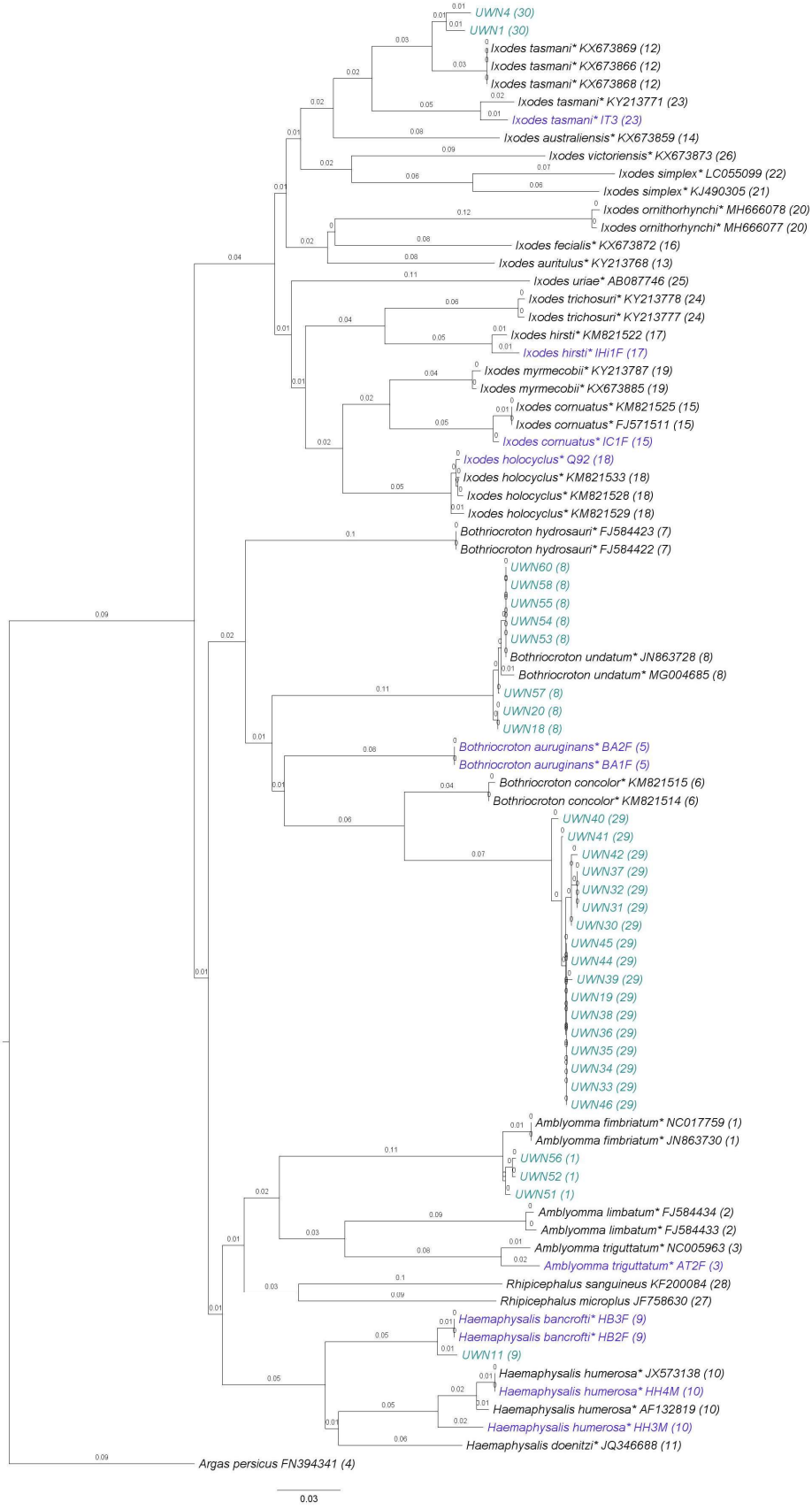
Genetic variance was calculated for the samples that displayed both a high BLAST identity to a reference sequence (table 3.5), and that had grouped accordingly during species delimitation (table 3.6). This resulted in 12 of 31 samples being assigned to a species (table 3.7). The remaining 19 samples could only be resolved to the genus level.

Table 3.5. Top GenBank BLAST match for COI sequences of wildlife ticks of undertermined species

* Host DNA was amplified; sample excluded from further analyses.

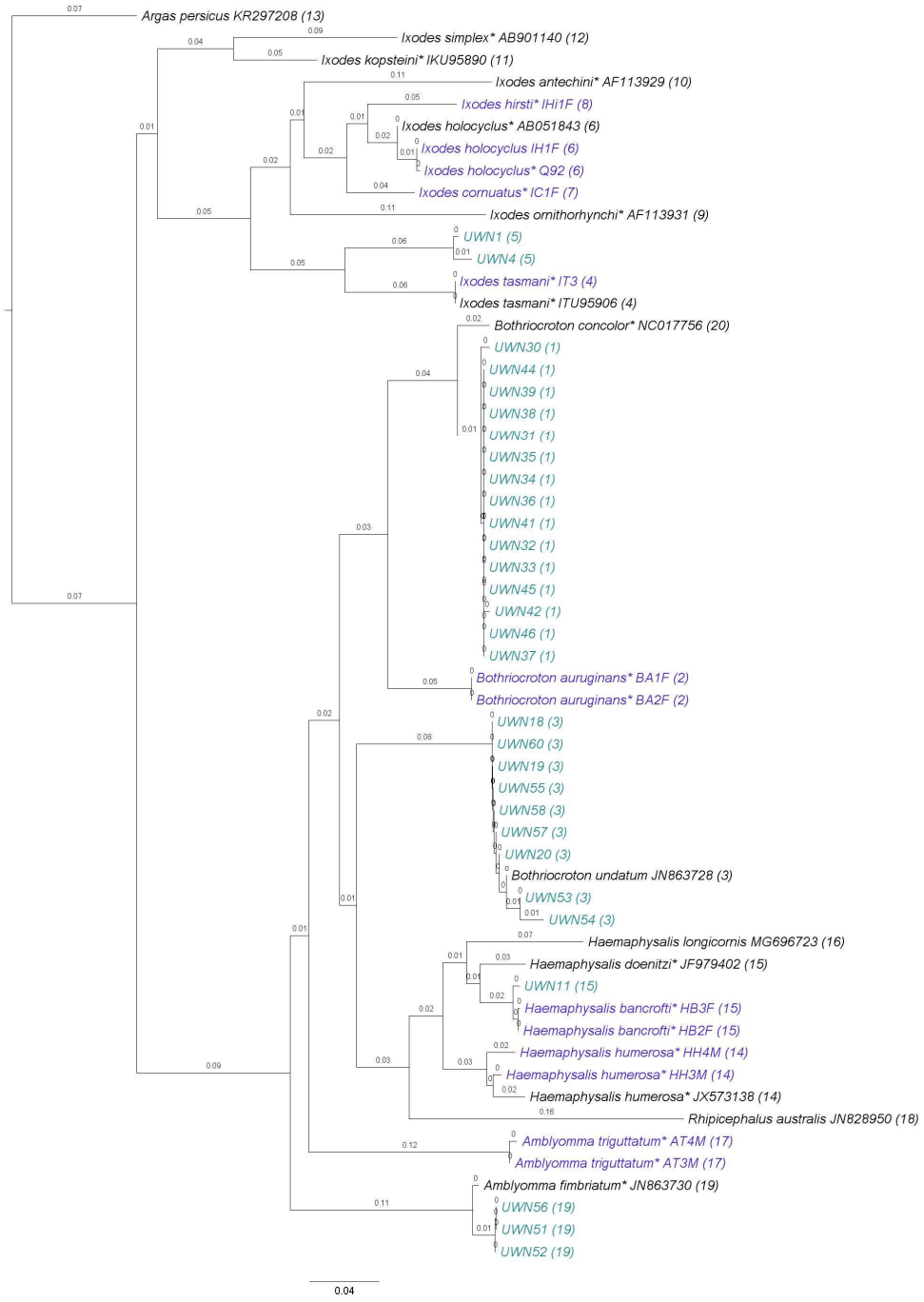
Sample	Morphological Identification	Closest GenBank Match	Accession	Similarity (%)	Query Coverage (%)	E value
UWN1	*Ixodes* sp.	*Ixodes tasmani*	KX673867	96	98	0.0
UWN4	*Ixodes* sp.	*Ixodes tasmani*	KX673867	96	99	0.0
UWN11	*Haemaphysalis* sp.	*Haemaphysalis* sp.	KM821505	98	84	0.0
UWN18	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN19	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN20	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN30	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN31	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN32	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN33	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN34	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN35	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	99	0.0
UWN36	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN37	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN38	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	99	0.0
UWN39	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN40	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	93	100	0.0
UWN41	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN42	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN44	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN45	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	99	0.0
UWN46	*Bothriocroton* sp.	*Bothriocroton concolor*	JN863727	92	100	0.0
UWN51	*Amblyomma* sp.	*Amblyomma fimbriatum*	JN863730	97	100	0.0

UWN52	*Bothriocroton* sp.	*Amblyomma fimbriatum*	JN863730	98	100	0.0
UWN53	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN54	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN55	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN56	*Amblyomma* sp.	*Amblyomma fimbriatum*	JN863730	98	100	0.0
UWN57	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN58	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0
UWN59*	*Bothriocroton* sp.	*Varanus komodoensis*	AB080275	89	100	0.0
UWN60	*Bothriocroton* sp.	*Bothriocroton undatum*	JN863728	99	100	0.0



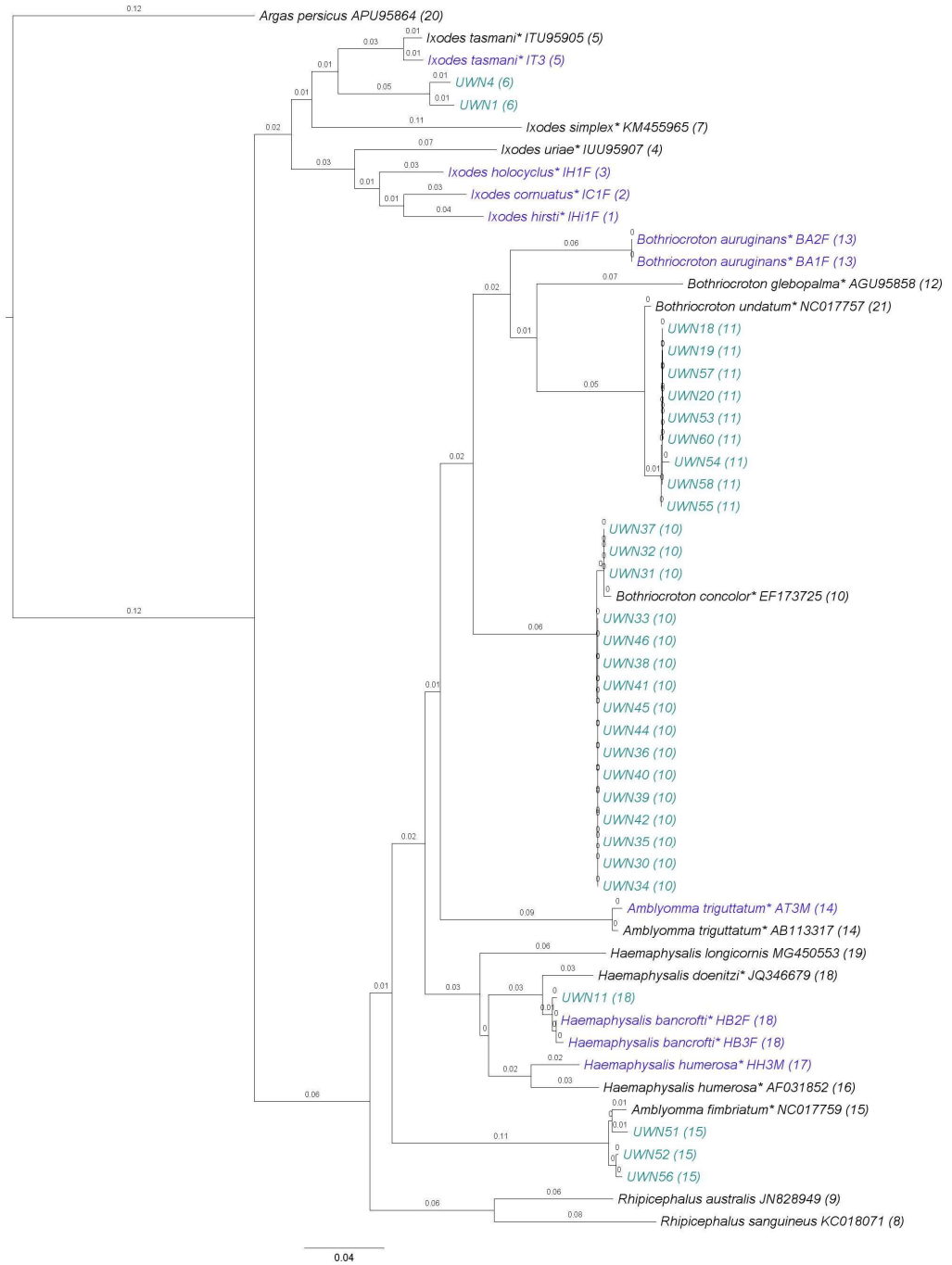
Group	P_{AB}
1	1.98E-03
2	0.11
3	0.11
4	3.00E-04
5	4.70E-04
6	6.40E-04
7	1.10E-04
8	1.50E-09
9	0.01
10	0.1
11	0.1
12	0.05
13	0.17
14	0.04
15	0.05
16	0.33
17	0.11
18	1.98E-03
19	0.05
20	0.33
21	0.1
22	0.1
23	0.02
24	0.11
25	0.01
26	0.33
27	1
28	1
29	6.40E-04
30	0.05

Figure 3.8. Species delimitation of UWN samples COI sequences using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.15) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent Rosenberg's test statistic. Sequences in blue indicate reference ticks sequenced in this study and those in green are the unidentified wildlife tick samples. Asterisks indicate Australian Ixodidae.



Group	1	2	3	4	5	6	7	8	9	10
P_{AB}	0.01	7.60E-04	1.20E-06	0.11	0.11	0.17	0.1	0.17	0.05	0.07
Group	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
P_{AB}	1	1	6.20E-04	0.01	0.1	0.1	7.10E-05	0.03	3.80E-07	0.01

Figure 3.9. Species delimitation of UWN samples 16S rRNA sequences using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.1) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} represent Rosenberg's test statistic. Sequences in blue indicate reference ticks sequenced in this study and those in green are the unidentified wildlife tick samples. Asterisks indicate native Australian Ixodidae.



Group	1	2	3	4	5	6	7	8	9	10	11
P_{AB}	1	1	0.33	0.17	0.11	0.11	0.1	1	1	5.70E-10	0.02
Group	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	
P_{AB}	0.02	2.14E-03	1.30E-04	3.80E-07	1	1	0.03	0.05	6.70E-04	0.02	

Figure 3.10. Species delimitation of UWN samples 12S rRNA sequences using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.1) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent the Rosenberg test statistic. Sequences in blue indicate reference ticks sequenced in this study and those in green are the unidentified wildlife tick samples. Asterisks indicate native Australian Ixodidae.

Table 3.6. Pairwise differences (%) between UWN samples and reference sequences.

UWN Sample	BLAST & Species Delimitation Match	Difference (%)
UWN11	*Haemaphysalis bancrofti*	1.73
UWN18	*Bothriocroton undatum*	0.29–1.16
UWN20	*Bothriocroton undatum*	0.29–1.16
UWN51	*Amblyomma fimbriatum*	1.73
UWN52	*Amblyomma fimbriatum*	1.73
UWN53	*Bothriocroton undatum*	0–0.87
UWN54	*Bothriocroton undatum*	0–0.87
UWN55	*Bothriocroton undatum*	0–0.87
UWN56	*Amblyomma fimbriatum*	2.02
UWN57	*Bothriocroton undatum*	0.29–1.16
UWN58	*Bothriocroton undatum*	0–0.87
UWN60	*Bothriocroton undatum*	0–0.87

Table 3.7. Species of wildlife ticks determined according to BLAST results, species delimitation and aligment distances.

Those shaded green were successfully identified to species level.

Sample	Determined species	Sample	Determined species
UWN1	*Ixodes* sp.	UWN40	*Bothriocroton* sp.
UWN4	*Ixodes* sp.	UWN41	*Bothriocroton* sp.
UWN11	*Haemaphysalis bancrofti*	UWN42	*Bothriocroton* sp.
UWN18	*Bothriocroton undatum*	UWN44	*Bothriocroto* n sp.
UWN19	*Bothriocroton* sp.	UWN45	*Bothriocroton* sp.
UWN20	*Bothriocroton undatum*	UWN46	*Bothriocroton* sp.
UWN30	*Bothriocroton* sp.	UWN51	*Amblyomma fimbriatum*
UWN31	*Bothriocroton* sp.	UWN52	*Amblyomma fimbriatum*
UWN32	*Bothriocroton* sp.	UWN53	*Bothriocroton undatum*
UWN33	*Bothriocroton* sp.	UWN54	*Bothriocroton undatum*
UWN34	*Bothriocroton* sp.	UWN55	*Bothriocroton undatum*
UWN35	*Bothriocroton* sp.	UWN56	*Amblyomma fimbriatum*
UWN36	*Bothriocroton* sp.	UWN57	*Bothriocroton undatum*
UWN37	*Bothriocroton* sp.	UWN58	*Bothriocroton undatum*
UWN38	*Bothriocroton* sp.	UWN59	
UWN39	*Bothriocroton* sp.	UWN60	*Bothriocroton undatum*

Chapter 4: Discussion

4.1 PCR testing and optimisation

While tick barcoding studies have been undertaken overseas, relatively little genetic work has been done on Australian tick species. Consequently, many of the available methods pertaining to barcoding gene amplification have not been tested extensively on Australian tick fauna. Each of the final assays used to amplify the four target barcoding genes were based on studies conducted elsewhere (Lv et al., 2014a; Song et al., 2011; Beati and Keirans, 2001). In the case of 12S and 16S, the methods described in previous studies performed exceptionally well across all samples with a 100% successful amplification rate, and so testing with alternative primers was not required. On the other hand, the primer pairs and assays initially used for COI and ITS2 were insufficient and further investigation was required to identify an assay that performed well across Australian Ixodidae.

4.1.1 Amplification of cytochrome c oxidase subunit I (COI)

The assay used to amplify COI was modified from the method described by Song et al., 2011 (Song et al., 2011). In their paper, Song et al. recommend the use of a hemi-nested PCR in the case that the primary PCR product failed to be visualised on an agarose gel. Kwak et al. later also used this method, achieving amplification in just 27 out of 64 samples (Kwak et al., 2017b). Trials during this study found that the primary primer pair (HCO2064/HCO1215) was inefficient at COI amplification, with no samples visualising on gel. When the primary PCR was bypassed, and a single step PCR was carried out using the secondary primers only (HCO2064/HCO1240), amplification was achieved across all samples. As the original method recommends using just 1µL of a 1/10 dilution of the primary PCR product, it is likely that the DNA concentration for the samples that failed to amplify was too low, hence the difficulty experienced in amplification.

It must be noted that the assay did not perform equally well across all genera tested. Only one of the three *Amblyomma triguttatum* samples amplified sufficiently

without requiring reamplification of the PCR product. Changes to the annealing temperature and concentrations of DNA, $MgCl_2$, primers, dNTPs and KAPA Taq were investigated, however these changes did not improve amplification efficiency in the *Amblyomma* samples, and in many cases impaired amplification of samples of other genera. Interestingly, the same difficulty was not experienced with amplifying COI from the *Amblyomma fimbriatum* samples UWN51, 52 and 56, indicating that this assay may be more efficient for *Amblyomma* amplification than this performance on the initial *A. triguttatum* samples suggested. A subsequent attempt was made to amplify the *A. triguttatum* samples in duplicate reactions and including a positive control. In this instance, all three amplified and were successfully sequenced, although two of the duplicate reactions failed (figure A1.5). To ensure reproducibility, several subsequent amplification attempts were made with varying and inconsistent results. However, in all instances that DNA was not visible or only weakly visible on an agarose gel, reamplification of the primary PCR product successfully resulted in strong amplification (figure A1.6). Therefore, while this assay is sufficient, it appears perhaps not as robust for amplification of all *Amblyomma* as it has so far been for other genera.

During this study, unsuccessful attempts were also made to amplify COI using primer pair cox1F/cox1R (Chitimia et al., 2010), which have previously been used to amplify COI from the Australian *Ixodes woyliei* tick (Ash et al., 2017). Dilutions of DNA were trialled to ensure the failure was not due to inhibition. A gradient PCR annealing temperature and a touchdown PCR protocol was also trialled. Bands were occasionally observed amidst non-specific binding for some *Ixodes* sp. samples, however results were not consistent when repeated and a reliable assay using these primers was not achieved.

4.1.2 Amplification of internal transcribed spacer 2 (ITS2)

The method described by Beati and Keirans (2001) using the primer pair T1B/T2A successfully amplified ITS2 across all genera (Beati and Keirans, 2001). However, despite multiple attempts, during testing of the initial 16 reference tick samples, amplification of one *Haemaphysalis bancrofti* and one *Bothriocroton auruginans* sample was not achieved. While ITS2 from the UWN samples (*see* section 3.5) was not used for species delimitation analysis, the gene was amplified in these samples to

investigate whether the assay was suitable for use across a wider range of Australian Ixodidae. Amplification was successful in 29 out of 32 samples, and so it can be considered that this assay is efficient in Australian Ixodidae ITS2 amplification despite the failure to amplify two of the reference tick samples.

Primers ITS862 and ITS130 were also trialled for amplification of ITS2 using the same assay concentrations that were successful for COI (Song et al., 2011). Amplification was consistently successful only in the case of *Ixodes.* This primer pair has previously been used to amplify ITS2 from Australian *Ixodes* with poor success rates (15 out of 64 samples amplified) (Kwak et al., 2017b). Although varying concentrations of DNA and a gradient annealing temperature was tested, amplification was not achieved for *Amblyomma* or *Bothriocroton*, and was occasionally successful but unreliable in the case of *Haemaphysalis*.

4.1.3. Amplification of genes from immature tick samples

Both the microbial diversity and microbial burden have been found to be greatest in nymphal ticks, so efficient methods of identifying ticks of this instar are essential (Egan, 2017; Zolnik et al., 2016). To ensure the assays tested would be suitable for use on subadult tick specimens, amplification of both the COI and 16S genes from nymph (n=7) and larval (n=3) ticks was attempted; of the 20 total reactions, just one larval COI gene failed to amplify (*see* section 3.3). At a later stage of the study, COI, 16S and 12S from nine nymphal ticks were amplified, with success across all 27 reactions (*see* section 3.7). These results indicate that the COI, 16S and 12S assays used in this study are efficient for amplification of nymphal tick DNA.

4.1.4. Amplification of genes from engorged tick samples

Previous studies have reported difficulty when to amplifying the DNA of engorged ticks, due to inhibitory substances present in mammalian blood (Abdullah et al., 2016; Wilson, 1997). With the exception of five ticks collected while questing, all of the

reference ticks in this study were taken from mammalian hosts, in addition to all of the UIN/UIL ticks, and 19 of the UWN specimens (table A1.1). All of these ticks exhibited some level of engorgement at the time of DNA extraction. Considering the success rates of gene amplification, it can be concluded that the presence of mammalian host DNA did not cause issues with inhibition in this study. The only interference by host DNA noted was in the case of UWN59, when PCR amplified the COI sequence of the lace monitor from which the tick was taken.

4.1.5. Amplification of diluted DNA

All of the PCR reactions carried out over the course of this study used gDNA extracted from whole ticks. Serial dilutions were tested to investigate the viability of these assays in situations where the DNA input is significantly lower, such as when whole tick extraction is not feasible (e.g. when the gDNA needs to be conserved for pathogen detection, or when extracting from museum specimens)(Krige et al., 2018). However, dilutions were only used in one trial of each assay, using one sample of each reference tick species. Therefore, while the results indicate that some level of DNA dilution <1/10 will be adequate, more extensive testing at varying dilutions across a greater number of samples would be necessary to conclude the dilutions that generate consistent results across all genera.

4.2 The use of BLAST for confirmation of species identity

This study made use of BLAST to support the morphological identification of ticks (see section 3.2). In the case of six out of the eight reference tick species used, there was at least one of the four genes sequenced available on GenBank, and so the top BLAST hit reinforced the species identification that had been assigned. However, for the *H. bancrofti* and *B. auruginans* specimens, there was no genetic data available, and so BLAST results could only confirm a genus match. The use of BLAST to determine the species of *Ixodes* nymphs and larvae in section 3.3 is a demonstration of how efficient the tool can be for species with genetic data available; seven of eight *Ixodes* samples were immediately matched to a species with >99% similarity using COI.

4.2.1 Limitations of using BLAST to determine species

With the currently limited understanding of intra- and interspecific genetic divergence within Australian Ixodidae, and the lack of available genetic data, reliance cannot be placed solely on BLAST results to unequivocally confirm whether a specimen belongs to a given species. Until variance thresholds have been determined and thoroughly tested, the results from tools such as BLAST must be considered in conjunction with other factors, particularly tick morphology, geographical location and tick host, to be sure that ticks are accurately assigned to species (Estrada-Peña et al., 2013). It is crucial to acknowledge that tools such as BLAST are only as useful as the quality of the information submitted to the GenBank database. The continually improving efficiency of molecular research techniques has led to an enormous increase in submissions to online genetic databases, and consequently a great accumulation of errors (Shen et al., 2013). It is recognised that errors frequently arise in this data due to the misidentification of species, contamination, and sample mix-up (Shen et al., 2013). Such errors are further enhanced by the lack of curation of sequences when they are submitted. For this reason, future contributions to Australian tick molecular barcoding research should ensure *a priori* identification of the specimens is described and documented as part of the investigation, to ensure the correct morphological identification has been assigned.

4.3 Species delimitation using ABGD

Species delimitation analysis performed in this study indicates that COI is more capable of discriminating at species level than ITS2, 16S, or 12S. This is not unexpected in the case of COI, as it has been shown to be more divergent at species level within clades than 16S in Australian ticks (Moon et al., 2015). Furthermore, as the standard barcoding gene, the use of COI for species delimitation has been comparatively well established (Kekkonen and Hebert, 2014).

16S and 12S both performed relatively well, forming groups that mirrored the species assignments of all but one species; both 16S and 12S grouped *H. doenitzi* with *H. bancrofti* samples. A conclusive determination of the suitability of use of these genes in species delimitation of Australian Ixodidae cannot be formed on the basis of this study

due to the underrepresentation of most species. Future research should endeavour to include multiple reference sequences from each species so that both intraspecific and interspecific distances can be investigated.

Significantly, very recent research has now been released demonstrating the suitability of COI for species delimitation of overseas tick species using ABGD (Mans et al., 2018). While Mans et al. focussed primarily on soft ticks, some Ixodidae were included. The paper reports the possibility of a large number of cryptic soft tick species following species delimitation and comparison of genetic distances. With so much to still be learned about Australian ticks, the possibility must be considered that future research could uncover a similar scenario amongst Australian Ixodidae.

While this study focused only on the use of ABGD for species delimitation, this is just one of several approaches available. Future research into the delimitation of tick species should also make use of other available methods of analysis (e.g. Zhang et al., 2013) as congruent results from two or more analytical approaches will provide supporting evidence for the robustness of the species grouping (Kekkonen and Hebert, 2014).

4.3.1 Factors impacting the use of ITS2 for species delimitation

This study did not find ITS2 suitable for species delimitation, despite a previous study indicating that low intraspecific and relatively high interspecific divergence occurs within the ITS2 of several Australian *Ixodes* (Song et al., 2011). As shown in figure A1.2, analysis the use of ITS2 revealed considerable under-grouping of species. It is possible that this was due to the short sequence lengths used for analysis. Reference sequences available for ITS2 are scarce, and many of those available are relatively short fragments. The amplicon size of ITS2 obtained in this study was widely variable between genera, and individual sequence gaps were abundant following alignment. Given the need to trim the sequences for analysis, it is possible that much of the genetic data vital to discriminating of species was lost. While discarding some sequences would have allowed for a longer alignment, an alignment of so few sequences is detrimental for the purpose of species

delimitation analysis. This leads to a second likely contributing cause for the under-splitting of sequences; that there were too few sequences per species included in the analysis (*see* section 4.2.5).

4.3.2 Limitations of ABGD

Simulation studies have shown that ABGD performs poorly when there are less than 3–5 species representatives per group, as so few specimens often mean an absence in a barcoding gap (Puillandre et al., 2012). This is problematic for the study of Australian ticks, whereby so few reference sequences are available, and for species with molecular data available, only one or two specimens are represented. Underestimation of species groups in this study occurred at some level for three of the four genes tested (ITS2, 16S, and 12S), and all three of these genes were extremely limited in the reference sequences available for use.

Although considered a much more conservative estimator of species when compared to some other methods of species delimitation, some studies have reported the overestimation of species groups during analysis using ABGD (Ortiz and Francke, 2016; Fernández and Giribet, 2014; Paz and Crawford, 2012). The few species split during analysis in this study (*H. Humerosa*, *I. simplex* and I. *tasmani*) each showed significant genetic variance in comparison to the intraspecific differences observed within the other species. Nevertheless, this known tendency to overestimate species groups will need to be considered when investigating the possibility of new lineages in future.

4.4 Phylogenetic analysis

Phylogenetic analysis of COI using RAxML Baysian reconstruction revealed a topology that supported the species delimitation grouping. Additionally, the results were mostly in line with what is currently known regarding Ixodidae phylogeny (Barker and Murrell, 2004). However, the *Dermacentor*, *Hyalomma,* and *Rhipicephalus* genera would normally form a clade branching from *Haemaphysalis*, yet in this study these groups are seen to have most recently diverged from *Amblyomma*. The 16S and 12S genes were not individually useful for phylogenetic analysis due to high incidences of paraphyly

accompanied by low bootstrapping values. However, when all four sequences were concatenated, the results resembled that of the COI tree and what is considered to be the correct topology; with the one exception of the *Dermacentor/Hyalomma/ Rhipicephalus* clade, which does not include any native Australian tick species.

RAxML analysis of ITS2 sequences indicated that the gene may be useful for inferring phylogenetic relationships only when using longer (> approximately 500 bp) sequences (figure A1.3). RAxML analysis on alignments of 332 bp, 395 bp and 438 bp all resulted in nonsensical phylogenies, with no real partitioning of genera. When the same analysis was performed on a 594 bp alignment, the results were in line with what is currently understood regarding tick phylogeny (Barker and Murrell, 2004), with the exception that, as with the COI and 16S analysis, the Rhipicephalinae and Hyalomminae clade diverges at the same point as Amblyomminae rather than Haemaphysalinae. However, very low bootstrapping values at many points of divergence indicate a lack of support for these results (figure A1.3). Studies on Australian tick phylogeny within the *Ixodes* genus have previously made use of this gene (Kwak et al., 2017b; Song et al., 2011). However, these Australian studies have not yet made use of ITS2 across multiple genera in analysis that would require more numerous reference sequences, and until more sequences are available, doing so will remain problematic.

4.5 Intraspecific COI variation within hypothesised species groups

Intraspecific pairwise distances in alignment of COI sequences of the reference ticks mostly occurred in the 0–2% range, with some noticeable exceptions (*see* table 3.4). While *H. humerosa* displayed the smallest intraspecific distance (0–5.56%) of the three split species groups, it is still considerably higher than the divergence observed within other genera in this study. However, because the intraspecific divergence of only one other Australian *Haemaphysalis* species was able to be observed, the level of variation that should be expected within these species is uncertain. Future research is required to obtain and analyse reference sequences from other *Haemaphysalis* species, as well as more *H. humerosa* specimens, to investigate this genetic divergence further.

It was hypothesized by Roberts in 1970, and corroborated by Kwak et al. in 2017, that *I. tasmani* may contain cryptic species (Kwak et al., 2017b; Roberts, 1970) and the findings of this study support this. A distance of 0–10.82% was observed between *I. tasmani* samples, with the *I. tasmani* sequenced during this study differing drastically from all but one of the reference sequence. Furthermore, while not a species match for any genetic sequences available, samples UWN1 and UWN4 were determined during species delimitation to be most closely related to the *I. tasmani* reference sequences. Research encompassing a large sample of *I. tasmani* collected from varying geographical locations is necessary to further investigate this possibility.

Two *I. simplex* sequences were included in the analyses of this study for reference purposes, and no new data for this species was generated. However, the species group was split during species delimitation and the alignment distance revealed a pairwise distance of 11.44% between the sequences. To investigate, a longer alignment containing all of the available *I. simplex* sequences (n=5) was generated. This found the interspecific distance to range from 0.3–10.48%. While not a species at the focus of this study, future research should investigate this further.

Amblyomma triguttatum sequences were not split into different species groups during species delimitation. Nevertheless, the divergence seen is still relatively high (0-3.22%). Currently *A. triguttatum* is split into four subspecies (Barker and Walker, 2014; Roberts, 1962) which are differentiated based on morphological traits; however the true status of these distinct subspecies remains to be substantiated and more research is necessary.

4.5.1 Geographical separation and genetic divergence

Investigation into the possibility of new lineages must take into account the intraspecies differentiation that can arise due to geographical distance (De Queiroz, 2007). The interspecific genetic differences between two recently separated lineages may be comparatively smaller than the intraspecific differences seen within a single, older, geographically widespread species (De Queiroz and Good, 1997). Based on the small number of sequences analysed in this study and others, the variation of COI seen within

Ixodes species is generally less than approximately 2% (Kwak et al., 2017b; Song et al., 2011), yet this can increase substantially between geographically separated clades. For example, the differentiation between Australian and New Zealand clades of *I. eudyptidis* is in the range of 4.5–5% due to long term separation and allopatric divergence (Moon et al., 2015). Pertinently, the three tick species that split within their groups during species delimitation in this study, as well as *A. triguttatum*, are all geographically widespread (Roberts, 1970).

4.6 Integration of methods for species determination

Ideally the most efficient method for species determination would be one that can match a barcoding sequence to that of a known species in a database. However, as outlined during this study, this is not always possible, nor practical (*see* section 4.2.1 Limitations of using BLAST to determine species). Combining the use of morphological and genetic methods will provide the most accurate species determination in cases of doubt.

In this study, species delimitation was also employed to ensure species were grouping in accordance with BLAST results. In section 3.7, species delimitation was used as a complementary tool when morphology was ambiguous and BLAST inconclusive for a large number of *Bothriocroton* UWN specimens. Species delimitation determined these specimens were one species, and that they were most closely related to the *Bothriocroton concolor* group. Based on these observations, the alignment distances, and current knowledge of Australian tick phylogeny, it could be inferred that these specimens are most likely *B. tachyglossi* (Andrews et al., 2006; Roberts, 1970).

4.7 Molecular barcoding of Australian Ixodidae

As the standard molecular barcoding gene for eukaryotes and having demonstrated effectiveness in discriminating the ticks used in this study, COI is the ideal candidate for molecular barcoding of Australian Ixodidae. Having the most available reference sequences by far, also makes COI the optimal gene to work with for the

purposes of species delimitation and phylogenetic analysis. However, it should be noted that ITS2, 16S, and 12S are all also suitable for species determination using BLAST.

4.7.1 Limitations of molecular barcoding

While molecular barcoding is an excellent tool for the efficient determination of species, it is important to acknowledge the limitations of the process. Molecular barcoding is not a replacement for current taxonomical practices, but rather a complementary approach (Stepanovic et al., 2016; Stoeckle, 2003). The analysis of single genes is not sufficient for resolving deep phylogenies (DeSalle et al., 2005). Furthermore, it cannot be considered a tool sufficient for the discovery of new species, as species should not be discovered on the basis of a single gene (Hickerson et al., 2006). Barcoding does have a role to play in the discovery of new species; obtaining barcoding sequences can provide a starting point or exploratory tool for unexplored groups or can provide evidence to support existing hypotheses (Puillandre et al., 2012; DeSalle et al., 2005). However, barcoding is only one component of a wide range of relevant data that must be considered, including ecology, morphology, behaviour, and genetics (Cao et al., 2016; Stoeckle, 2003).

4.8 Conclusion

This study tested assays for the amplification of four potential Australian Ixodidae barcoding genes (COI, ITS2, 16S, and 12S), and determined an assay efficient for the amplification of each gene in eight Australian ticks of medical and veterinary importance. All genera of Australian hard ticks were represented in this study and while further testing is needed, it is expected that the assays will perform well across all Ixodidae species. The amplification and sequencing of barcoding genes will contribute valuable genetic data on Australian ticks. Furthermore, this study made use of ABGD on Australian Ixodidae for the first time, and thereby identified Australian tick species with large intraspecific divergence which require further investigation. The results of this study indicate that the standard barcoding gene, COI, is efficient for barcoding Australian Ixodidae.

References

Abdullah, H.H., El-Molla, A., Salib, F.A., Allam, N.A., Ghazy, A.A., Abdel-Shafy, S., 2016. Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (Acari: Ixodidae) vectors of Rickettsioses in Egypt. Vet World 9, 1087-1101.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J Mol Biol 215, 403-410.

Andrews, R.H., Beveridge, I., Bull, C.M., Chilton, N.B., Dixon, B., Petney, T., 2006. Systematic status of *Aponnoma tachyglossi* Roberts (Acari: Ixodidae) from echidnas, *Tachyglossus aculeatus,* from Queensland, Australia. Syst Appl Acarol 11, 23-39.

Ash, A., Elliot, A., Godfrey, S., Burmej, H., Abdad, M.Y., Northover, A., Wayne, A., Morris, K., Clode, P., Lymbery, A., Thompson, R.C., 2017. Morphological and molecular description of *Ixodes woyliei* n. sp. (Ixodidae) with consideration for co-extinction with its critically endangered marsupial host. Parasit Vectors 10, 70.

Barker, S.C., Murrell, A., 2004. Systematics and evolution of ticks with a list of valid genus and species names. Parasitology 129, S15-S36.

Barker, S.C., Walker, A.R., 2014. Ticks of Australia. The species that infest domestic animals and humans. Zootaxa, 1-144.

Barker, S.C., Walker, A.R., Campelo, D., 2014. A list of the 70 species of Australian ticks; diagnostic guides to and species accounts of *Ixodes holocyclus* (paralysis tick), *Ixodes cornuatus* (southern paralysis tick) and *Rhipicephalus australis* (Australian cattle tick); and consideration of the place of Australia in the evolution of ticks with comments on four controversial ideas. Int J Parasitol 44, 941-953.

Beati, L., Keirans, J.E., 2001. Analysis of the systematic relationships among ticks of the genera *Rhipicephalus* and *Boophilus* (Acari : Ixodidae) based on mitochondrial 12S ribosomal DNA gene sequences and morphological characters. Journal of Parasitology 87, 32-48.

Black, W.C., Piesman, J., 1994. Phylogeny of hard- and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proc Nati Acad Sci 91, 10034-10038.

Boore, J.L., 1999. Animal mitochondrial genomes. Nucleic Acids Res 27, 1767-1780.

Burnard, D., Weaver, H., Gillett, A., Loader, J., Flanagan, C., Polkinghorne, A., 2017. Novel Chlamydiales genotypes identified in ticks from Australian wildlife. Parasit Vectors 10, 46.

Cao, X., Liu, J., Chen, J., Zheng, G., Kuntner, M., Agnarsson, I., 2016. Rapid dissemination of taxonomic discoveries based on DNA barcoding and morphology. Sci Rep 6, 37066.

Caporale, D.A., Rich, S.M., Spielman, A., Telford, S.A., Kocher, T.D., 1995. Discrimination between *Ixodes* Ticks by Means of Mitochondrial DNA sequences. Mol Phylogenet Evol 4, 361-365.

Castalanelli, M.A., Teale, R., Rix, M.G., Kennington, W.J., Harvey, M.S., 2014. Barcoding of mygalomorph spiders (Araneae : Mygalomorphae) in the Pilbara bioregion of Western Australia reveals a highly diverse biota. Invertebr Syst 28, 375-385.

Che, J., Chen, H.M., Yang, J.X., Jin, J.Q., Jiang, K., Yuan, Z.Y., Murphy, R.W., Zhang, Y.P., 2012. Universal COI primers for DNA barcoding amphibians. Mol Ecol Resour 12, 247-258.

Chee, S.Y., 2015. Limitations of cytochrome oxidase I for the barcoding of Neritidae (Mollusca: Gastropoda) as revealed by Bayesian analysis. Genet Mol Res 14, 5677-5684.

Chitimia, L., Lin, R.Q., Cosoroaba, I., Wu, X.Y., Song, H.Q., Yuan, Z.G., Zhu, X.Q., 2010. Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial cox1 and nad5 genes. Exp Appl Acarol 52, 305-311.

Cumming, G.S., Van Vuuren, D.P., 2006. Will climate change affect ectoparasite species ranges? Glob Ecol Biogeogr 15, 486-497.

Cupp, E.W., 1991. Biology of Ticks. Vet Clin North Am Small Anim Pract 21, 1-26.

D'Amico, G., Dumitrache, M.O., Matei, I.A., Ionica, A.M., Gherman, C.M., Sandor, A.D., Modry, D., Mihalca, A.D., 2017. Ixodid ticks parasitizing wild carnivores in Romania. Exp Appl Acarol 71, 139-149.

Dantas-Torres, F., 2007. Rocky Mountain spotted fever. Lancet Infect Dis 7, 724-732.

Dantas-Torres, F., Chomel, B.B., Otranto, D., 2012. Ticks and tick-borne diseases: a One Health perspective. Trends Parasitol 28, 437-446.

de la Fuente, J., 2003. The fossil record and the origin of ticks (Acari: Parasitiformes: Ixodida). Exp Appl Acarol 29, 331-344.

De Queiroz, K., 2007. Species concepts and species delimitation. Syst Biol 56, 879-886.

De Queiroz, K., Good, D.A., 1997. Phenetic clustering in biology: a critique. Q Rev Biol 72, 4-27.

Deagle, B.E., Jarman, S.N., Coissac, E., Pompanon, F., Taberlet, P., 2014. DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biol Lett 10.

DeSalle, R., Egan, M.G., Siddall, M., 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Philos Trans R Soc Lond B Biol Sci 360, 1905-1916.

Diarra, A.Z., Almeras, L., Laroche, M., Berenger, J.M., Kone, A.K., Bocoum, Z., Dabo, A., Doumbo, O., Raoult, D., Parola, P., 2017. Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. PLoS Negl Trop Dis 11, e0005762.

Dobson, S.J., Barker, S.C., 1999. Phylogeny of the Hard Ticks (Ixodidae) Inferred from 18S rRNA Indicates That the Genus *Aponomma* Is Paraphyletic. Molecular Phylogenetics and Evolution 11, 288-295.

Egan, S., 2017. Profiling the bacterial microbiome of ticks that parasitise bandicoots in Australia. Murdoch University, Murdoch.

Ernieenor, F.C.L., Ernna, G., Mariana, A., 2017. Phenotypic and genotypic identification of hard ticks of the genus *Haemaphysalis* (Acari: Ixodidae) in Peninsular Malaysia. Exp Appl Acarol 71, 387-400.

Esteve-Gassent, M.D., Castro-Arellano, I., Feria-Arroyo, T.P., Patino, R., Li, A.Y., Medina, R.F., de Leon, A.A., Rodriguez-Vivas, R.I., 2016. Translating Ecology, Physiology, Biochemistry, and Population Genetics Research to Meet the Challenge of Tick and Tick-Borne Diseases in North America. Arch Insect Biochem Physiol 92, 38-64.

Estrada-Peña, A., Gray, J., Khal, O., Lane, R.S., Nijhof, A.M., 2013. Research on the ecology of ticks and tick-borne pathogens—methodological principles and caveats. Front Cell Infect Microbiol 3.

Fernández, R., Giribet, G., 2014. Phylogeography and species delimitation in the New Zealand endemic, genetically hypervariable harvestman species, Aoraki denticulata (Arachnida, Opiliones, Cyphophthalmi). Invertebr Syst 28, 401-414.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3, 294-299.

Fujisawa, T., Barraclough, T.G., 2013. Delimiting Species Using Single-Locus Data and the Generalized Mixed Yule Coalescent Approach: A Revised Method and Evaluation on Simulated Data Sets. Syst Biol 62, 707-724.

Gariepy, T.D., Kuhlmann, U., Gillott, C., Erlandson, M., 2007. Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of Arthropods. J Appl Entomol 131, 225-240.

Gofton, A.W., Doggett, S., Ratchford, A., Oskam, C.L., Paparini, A., Ryan, U., Irwin, P., 2015a. Bacterial Profiling Reveals Novel "Ca. *Neoehrlichia*", *Ehrlichia*, and *Anaplasma* Species in Australian Human-Biting Ticks. PLoS One 10, e0145449.

Gofton, A.W., Oskam, C.L., Lo, N., Beninati, T., Wei, H., McCarl, V., Murray, D.C., Paparini, A., Greay, T.L., Holmes, A.J., Bunce, M., Ryan, U., Irwin, P., 2015b. Inhibition of the endosymbiont "Candidatus *Midichloria* mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in *Ixodes* ticks from Australia. Parasit Vectors 8, 345.

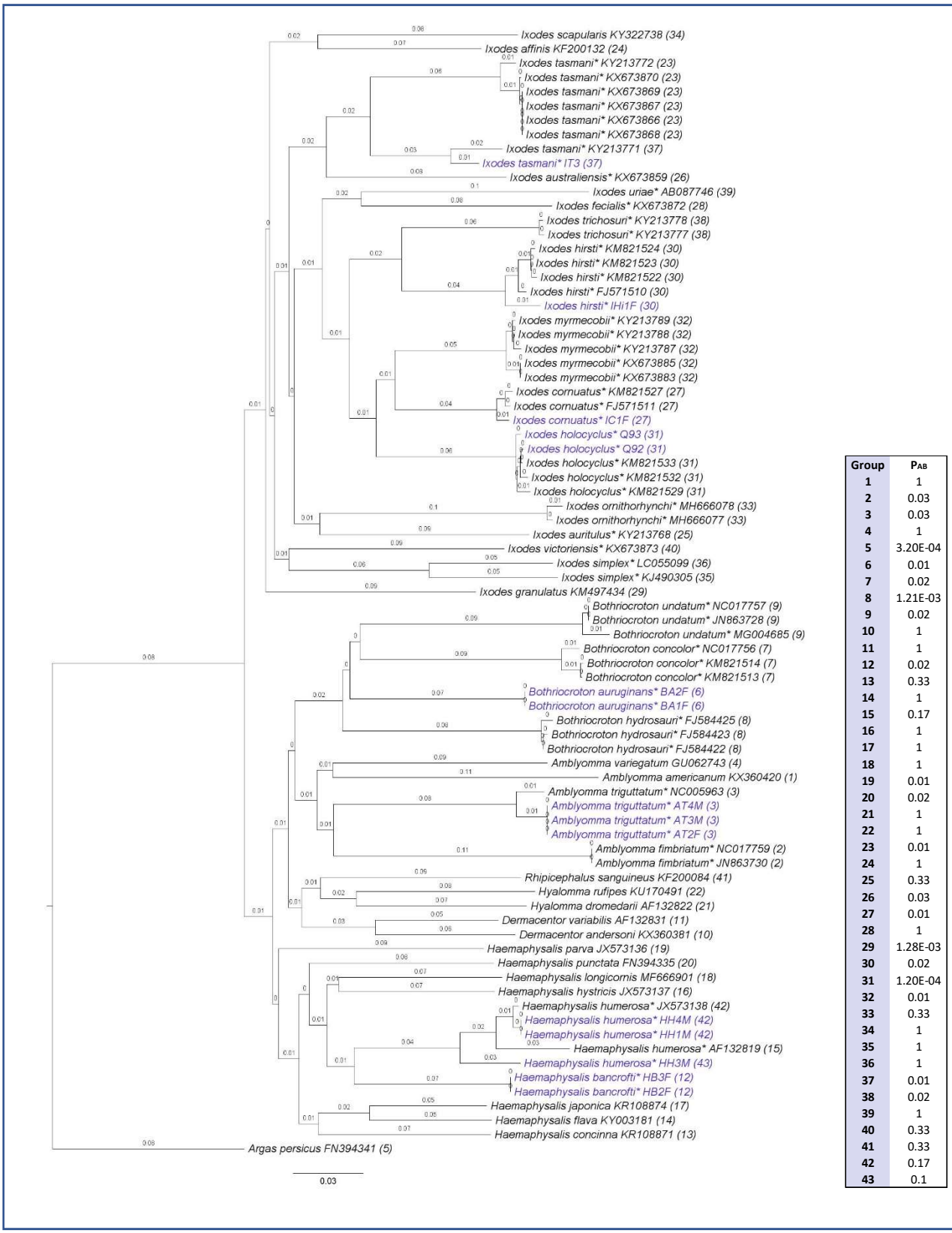
Graves, S.R., Stenos, J., 2017. Tick-borne infectious diseases in Australia. Med J Aust 206, 320-324.
Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Pena, A., Horak, I.G., Shao, R., Barker, S., 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. Zootaxa 2528, 1-28.
Guillemin, M.L., Contreras-Porcia, L., Ramirez, M.E., Macaya, E.C., Contador, C.B., Woods, H., Wyatt, C., Brodie, J., 2016. The bladed Bangiales (Rhodophyta) of the South Eastern Pacific: Molecular species delimitation reveals extensive diversity. Mol Phylogenet Evol 94, 814-826.
Gulia-Nuss, M., Nuss, A.B., Meyer, J.M., Sonenshine, D.E., Roe, R.M., Waterhouse, R.M., Sattelle, D.B., de la Fuente, J., Ribeiro, J.M., Megy, K., Thimmapuram, J., Miller, J.R., Walenz, B.P., Koren, S., Hostetler, J.B., Thiagarajan, M., Joardar, V.S., Hannick, L.I., Bidwell, S., Hammond, M.P., Young, S., Zeng, Q., Abrudan, J.L., Almeida, F.C., Ayllon, N., Bhide, K., Bissinger, B.W., Bonzon-Kulichenko, E., Buckingham, S.D., Caffrey, D.R., Caimano, M.J., Croset, V., Driscoll, T., Gilbert, D., Gillespie, J.J., Giraldo-Calderon, G.I., Grabowski, J.M., Jiang, D., Khalil, S.M., Kim, D., Kocan, K.M., Koci, J., Kuhn, R.J., Kurtti, T.J., Lees, K., Lang, E.G., Kennedy, R.C., Kwon, H., Perera, R., Qi, Y., Radolf, J.D., Sakamoto, J.M., Sanchez-Gracia, A., Severo, M.S., Silverman, N., Simo, L., Tojo, M., Tornador, C., Van Zee, J.P., Vazquez, J., Vieira, F.G., Villar, M., Wespiser, A.R., Yang, Y., Zhu, J., Arensburger, P., Pietrantonio, P.V., Barker, S.C., Shao, R., Zdobnov, E.M., Hauser, F., Grimmelikhuijzen, C.J., Park, Y., Rozas, J., Benton, R., Pedra, J.H., Nelson, D.R., Unger, M.F., Tubio, J.M., Tu, Z., Robertson, H.M., Shumway, M., Sutton, G., Wortman, J.R., Lawson, D., Wikel, S.K., Nene, V.M., Fraser, C.M., Collins, F.H., Birren, B., Nelson, K.E., Caler, E., Hill, C.A., 2016. Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease. Nat Commun 7, 10507.
Hebert, P.D., Cywinska, A., Ball, S.L., deWaard, J.R., 2003a. Biological identifications through DNA barcodes. Proc Biol Sci 270, 313-321.
Hebert, P.D., Ratnasingham, S., deWaard, J.R., 2003b. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc Biol Sci 270 Suppl 1, S96-99.
Hickerson, M.J., Meyer, C.P., Moritz, C., 2006. DNA barcoding will often fail to discover new animal species over broad parameter space. Syst Biol 55, 729-739.
Hoogstraal, H., Aeschlimann, A., 1982. Tick-Host Specificity. In: Second Symposium on Host Specificity among Parasites of Vertebrates, Paris.
Hoskins, J.D., 1991. Ixodid and Argasid Ticks. Vet Clin North Am Small Anim Pract 21, 185-197.
Hwang, U.-W., Kim, W., 1999. General properties and phylogenetic utilities of nuclear ribosomal DNA and mitochondrial DNA commonly used in molecular systematics. Korean J Parasitol 37, 215-228.
Ivanova, N.V., Zemlak, T.S., Hanner, R.H., Hebert, P.D.N., 2007. Universal primer cocktails for fish DNA barcoding. Mol Ecol Notes 7, 544-548.
Jongejan, F., Uilenberg, G., 1994. Ticks and control methods. Rev Sci Tech 13, 1201-1226.
Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology 129.
Jukes, T.H., Cantor, C.R., 1969. Evolution of Protein Molecules. Academic Press, New York.
Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647-1649.
Kekkonen, M., Hebert, P.D., 2014. DNA barcode-based delineation of putative species: efficient start for taxonomic workflows. Mol Ecol Resour 14, 706-715.
Keskin, A., Selcuk, A.Y., Kefelioglu, H., 2017. Ticks (Acari: Ixodidae) infesting some small mammals from Northern Turkey with new tick-host associations and locality records. Exp Appl Acarol 73, 521-526.
Kimura, M., *1980*. A Simple Method for Estimating Evolutionary Rates of Base Substitutions Through Comparative Studies of Nucleotide Sequences. J Mol Evol 16, 111-120.

Klompen, H., Grimaldi, D., 2001. First Mesozoic Record of a Parasitiform Mite: a Larval Argasid Tick in Cretaceous Amber (Acari: Ixodida: Argasidae). Annals of the Entomological Society of America 94, 10-15.
Klompen, J.S., Black, W.C.t., Keirans, J.E., Oliver, J.H., Jr., 1996. Evolution of ticks. Annu Rev Entomol 41, 141-161.
Kress, W.J., Erickson, D.L., 2008. DNA barcodes: genes, genomics, and bioinformatics. Proc Natl Acad Sci U S A 105, 2761-2762.
Krige, A.-S., Loh, S.-M., Oskam, C., 2018. New host records for ticks (Acari : Ixodidae) from the echidna (*Tachyglossus aculeatus*) revealed in Australian museum survey. Aust J Zool 65, 379-382.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33, 1870-1874.
Kwak, M., Somerville, C., van Nunen, S., 2018a. A novel Australian tick *Ixodes* (Endopalpiger) *australiensis* inducing mammalian meat allergy after tick bite. Asia Pac Allergy 8, e31.
Kwak, M.L., Ash, A., Elliot, A., 2017a. Redescription of the numbat tick *Ixodes* (*Sternalixodes*) *myrmecobii* Roberts, 1962 (Acari: Ixodidae) with descriptions of the male and nymph, and new host records. Zootaxa 4272, 40-56.
Kwak, M.L., Beveridge, I., Koehler, A.V., Malipatil, M., Gasser, R.B., Jabbar, A., 2017b. Phylogenetic analysis of the Australasian paralysis ticks and their relatives (Ixodidae: *Ixodes*: *Sternalixodes*). Parasit Vectors 10, 122.
Kwak, M.L., Madden, C., 2017. The first record of infestation by a native tick (Acari: Ixodidae) on the Australian emu (*Dromaius novaehollandiae*) and a review of tick paralysis in Australian birds. Exp Appl Acarol 73, 103-107.
Kwak, M.L., Madden, C., Wicker, L., 2018b. *Ixodes heathi* n. sp. (Acari: Ixodidae), a co-endangered tick from the critically endangered mountain pygmy possum (*Burramys parvus*), with notes on its biology and conservation. Exp Appl Acarol.
Latif, A.A., Putterill, J.F., de Klerk, D.G., Pienaar, R., Mans, B.J., 2012. *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae): first description of the male, immature stages and re-description of the female. PLoS One 7, e41651.
Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C., Wang, Q., Mei, L., Lin, X., 2014a. Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasites & Vectors 7, 1-11.
Lv, J., Wu, S., Zhang, Y., Zhang, T., Feng, C., Jia, G., Lin, X., 2014b. Development of a DNA barcoding system for the Ixodida (Acari: Ixodida). Mitochondrial DNA 25, 142-149.
Mallatt, J., Sullivan, J., 1998. 28S and 18S rDNA sequences support the monophyly of lampreys and hagfishes. Mol Biol Evol 15, 1706-1718.
Mans, B.J., de Klerk, D.G., Pienaar, R., Latif, A.A., 2014. The host preferences of *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae): a generalist approach to surviving multiple host-switches. Exp Appl Acarol 62, 233-240.
Mans, B.J., Featherston, J., Kvas, M., Pillay, K.A., de Klerk, D.G., Pienaar, R., de Castro, M.H., Schwan, T.G., Lopez, J.E., Teel, P., Perez de Leon, A.A., Sonenshine, D.E., Egekwu, N.I., Bakkes, D.K., Heyne, H., Kanduma, E.G., Nyangiwe, N., Bouattour, A., Latif, A.A., 2018. Argasid and ixodid systematics: Implications for soft tick evolution and systematics, with a new argasid species list. Ticks Tick Borne Dis.
Masters, B.C., Fan, V., Ross, H.A., 2011. Species delimitation – a geneious plugin for the exploration of species boundaries. Mol Ecol Resour 11, 154-157.
McCoy, K.D., Leger, E., Dietrich, M., 2013. Host specialization in ticks and transmission of tick-borne diseases: a review. Front Cell Infect Microbiol 3, 57.
Moon, K.L., Banks, S.C., Fraser, C.I., 2015. Phylogeographic Structure in Penguin Ticks across an Ocean Basin Indicates Allopatric Divergence and Rare Trans-Oceanic Dispersal. PLoS One 10, e0128514.

Murrell, A., Dobson, S.J., Walter, D.E., Campbell, N.J.H., Shao, R., Barker, S.C., 2005. Relationships among the three major lineages of the Acari (Arthropoda:Arachnida) inferred from small subunit rRNA: paraphyly of the Parasitiformes with respect to the Opilioacariformes and relative rates of nucleotide substitution. Invertebr Syst 19.
Nava, S., Guglielmone, A.A., Mangold, A.J., 2009. An overview of systematics and evolution of ticks. Front Biosci 14, 2857-2877.
Oliver, J.H., 1989. Biology and Systematics of Ticks (Acari, Ixodida). Annu Rev Ecol Evol Syst 20, 397-430.
Ondrejicka, D.A., Morey, K.C., Hanner, R.H., 2017. DNA barcodes identify medically important ticks in Canada. Genome 60, 74-84.
Ortiz, D., Francke, O.F., 2016. Two DNA barcodes and morphology for multi-method species delimitation in Bonnetina tarantulas (Araneae: Theraphosidae). Mol Phylogenet Evol 101, 176-193.
Parola, P., Raoult, D., 2001. Ticks and Tickbome Bacterial Diseases in Humans: An Emerging Infectious Threat. Ticks Tick Borne Dis 32, 897-928.
Paz, A., Crawford, A.J., 2012. Molecular-based rapid inventories of sympatric diversity: a comparison of DNA barcode clustering methods applied to geography-based vs clade-based sampling of amphibians. J Biosci 37, 887-896.
Pereira, T.J., Baldwin, J.G., 2016. Contrasting evolutionary patterns of 28S and ITS rRNA genes reveal high intragenomic variation in *Cephalenchus* (Nematoda): Implications for species delimitation. Mol Phylogenet Evol 98, 244-260.
Piesman, J., Eisen, L., 2008. Prevention of tick-borne diseases. Annu Rev Entomol 53, 323-343.
Poinar, G., Jr., Brown, A.E., 2003. A new genus of hard ticks in Cretaceous Burmese amber (Acari: Ixodida: Ixodidae). Syst Parasitol 54, 199-205.
Puillandre, N., Lambert, A., Brouillet, S., Achaz, G., 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol Ecol 21, 1864-1877.
Ratnasingham, S., Hebert, P.D., 2013. A DNA-Based Registry for All Animal Species: The Barcode Index Number (BIN) System. PLoS One 8.
Roberts, F.H.S., 1962. On the status of morphologically divergent populations of *Amblyomma triguttatum* Koch (Acarina: Ixodidae). Aust J Zool 10, 367-381.
Roberts, F.H.S., 1970. Australian Ticks. Commonwealth Scientific and Industrial Research Organization, Melbourne.
Shen, Y.Y., Chen, X., Murphy, R.W., 2013. Assessing DNA barcoding as a tool for species identification and data quality control. PLoS One 8, e57125.
Smith, M.A., Fisher, B.L., Hebert, P.D., 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. Philos Trans R Soc Lond B Biol Sci 360, 1825-1834.
Sonenshine, D.E., 2018. Range Expansion of Tick Disease Vectors in North America: Implications for Spread of Tick-Borne Disease. Int J Environ Res Public Health 15.
Sonenshine, D.E., Roe, R.M., 2013. Biology of Ticks Volume 1, 2 Edition. Oxford University Press, USA.
Song, S., Shao, R., Atwell, R., Barker, S., Vankan, D., 2011. Phylogenetic and phylogeographic relationships in *Ixodes holocyclus* and *Ixodes cornuatus* (Acari: Ixodidae) inferred from COX1 and ITS2 sequences. Int J Parasitol 41, 871-880.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312-1313.
Stanek, G., Wormser, G.P., Gray, J., Strle, F., 2012. Lyme borreliosis. The Lancet 379, 461-473.
Stepanovic, S., Kosovac, A., Krstic, O., Jovic, J., Tosevski, I., 2016. Morphology versus DNA barcoding: two sides of the same coin. A case study of *Ceutorhynchus erysimi* and *C. contractus* identification. Insect Sci 23, 638-648.
Stoeckle, M., 2003. Taxonomy, DNA, and the Bar Code of Life. BioScience 53.

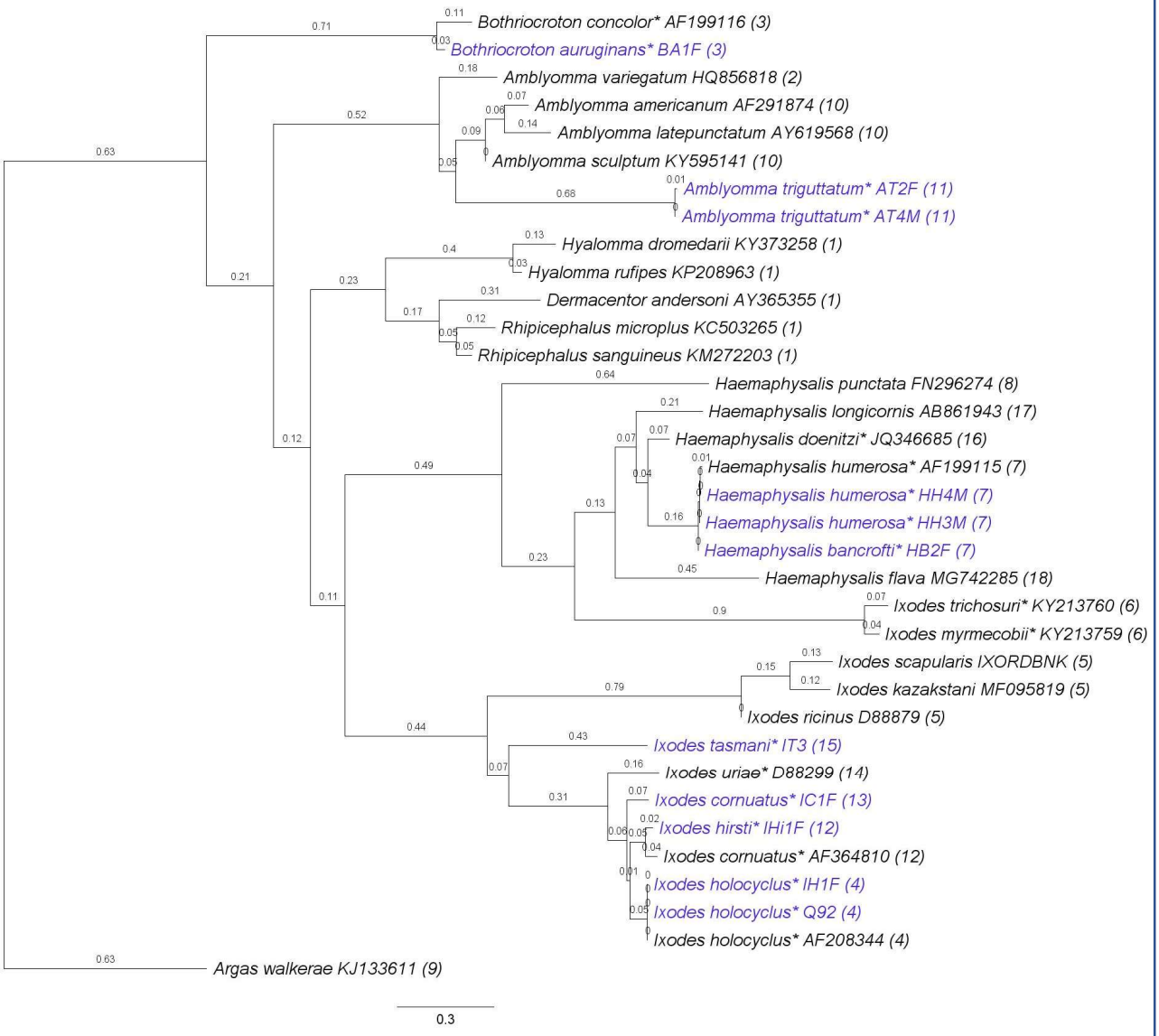
Taylor, H.R., Harris, W.E., 2012. An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding. Mol Ecol Resour 12, 377-388.
Taylor, J.D., Williams, S.T., Glover, E.A., Dyal, P., 2007. A molecular phylogeny of heterodont bivalves (Mollusca: Bivalvia: Heterodonta): new analyses of 18S and 28S rRNA genes. Zoologica Scripta 36, 587-606.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal-W - Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. Nucleic Acids Res 22, 4673-4680.
van Nunen, S., 2015. Tick-induced allergies: mammalian meat allergy, tick anaphylaxis and their significance. Asia Pac Allergy 5, 3-16.
van Nunen, S.A., 2018. Tick-induced allergies: mammalian meat allergy and tick anaphylaxis. Med J Aust 208, 316-321.
Vences, M., Thomas, M., van der Meijden, A., Chiari, Y., Vieites, D.R., 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Front Zool 2, 5.
Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63, 3741-3751.
Yang, L., Tan, Z., Wang, D., Xue, L., Guan, M.X., Huang, T., Li, R., 2014. Species identification through mitochondrial rRNA genetic analysis. Sci Rep 4, 4089.
Yang, R., Murphy, C., Song, Y., Ng-Hublin, J., Estcourt, A., Hijjawi, N., Chalmers, R., Hadfield, S., Bath, A., Gordon, C., Ryan, U., 2013. Specific and quantitative detection and identification of *Cryptosporidium hominis* and *C. parvum* in clinical and environmental samples. Exp Parasitol 135, 142-147.
Zhang, J., Kapli, P., Pavlidis, P., Stamatakis, A., 2013. A general species delimitation method with applications to phylogenetic placements. Bioinformatics 29, 2869-2876.
Zolnik, C., Prill, R.J., Falco, R.C., Daniels, T.J., Kolokotronis, S.-O., 2016. Microbiome changes through ontogeny of a tick pathogen vector. Mol Ecol 25, 4963-4977.

Appendix



Group	P_{AB}
1	1
2	0.03
3	0.03
4	1
5	3.20E-04
6	0.01
7	0.02
8	1.21E-03
9	0.02
10	1
11	1
12	0.02
13	0.33
14	1
15	0.17
16	1
17	1
18	1
19	0.01
20	0.02
21	1
22	1
23	0.01
24	1
25	0.33
26	0.03
27	0.01
28	1
29	1.28E-03
30	0.02
31	1.20E-04
32	0.01
33	0.33
34	1
35	1
36	1
37	0.01
38	0.02
39	1
40	0.33
41	0.33
42	0.17
43	0.1

Figure A1.1. Species delimitation of reference sample COI sequences (572 bp) using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.15) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Kimura two parameter model (KP2). Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent the Rosenberg test statistic. Sequences in blue indicate those sequenced in this study, and asterisks indicate native Australian Ixodidae.



Group	1	2	3	4	5	6	7	8	9
P_{AB}	1.20E-06	0.07	1.00E-04	0.05	1.21E-03	0.01	0.1	0.02	1.68E-03
Group	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
P_{AB}	0.05	0.05	0.05	0.07	0.05	0.04	0.1	0.07	0.05

Figure A1.2. Species delimitation of ITS2 reference sample sequences using Automatic Barcode Gap Discovery (ABGD). Pmin (0.001) to Pmax (0.1) were scanned over 10 steps. Relative gap width was 1.0 and distance distribution 20 Nb bins. Pairwise-distances were calculated using the Jukes-Cantor model. Resulting species grouping hypotheses are indicated in parentheses. P_{AB} values represent the Rosenberg test statistic. Sequences in blue indicate those sequenced in this study, and asterisks indicate native Australian Ixodidae.

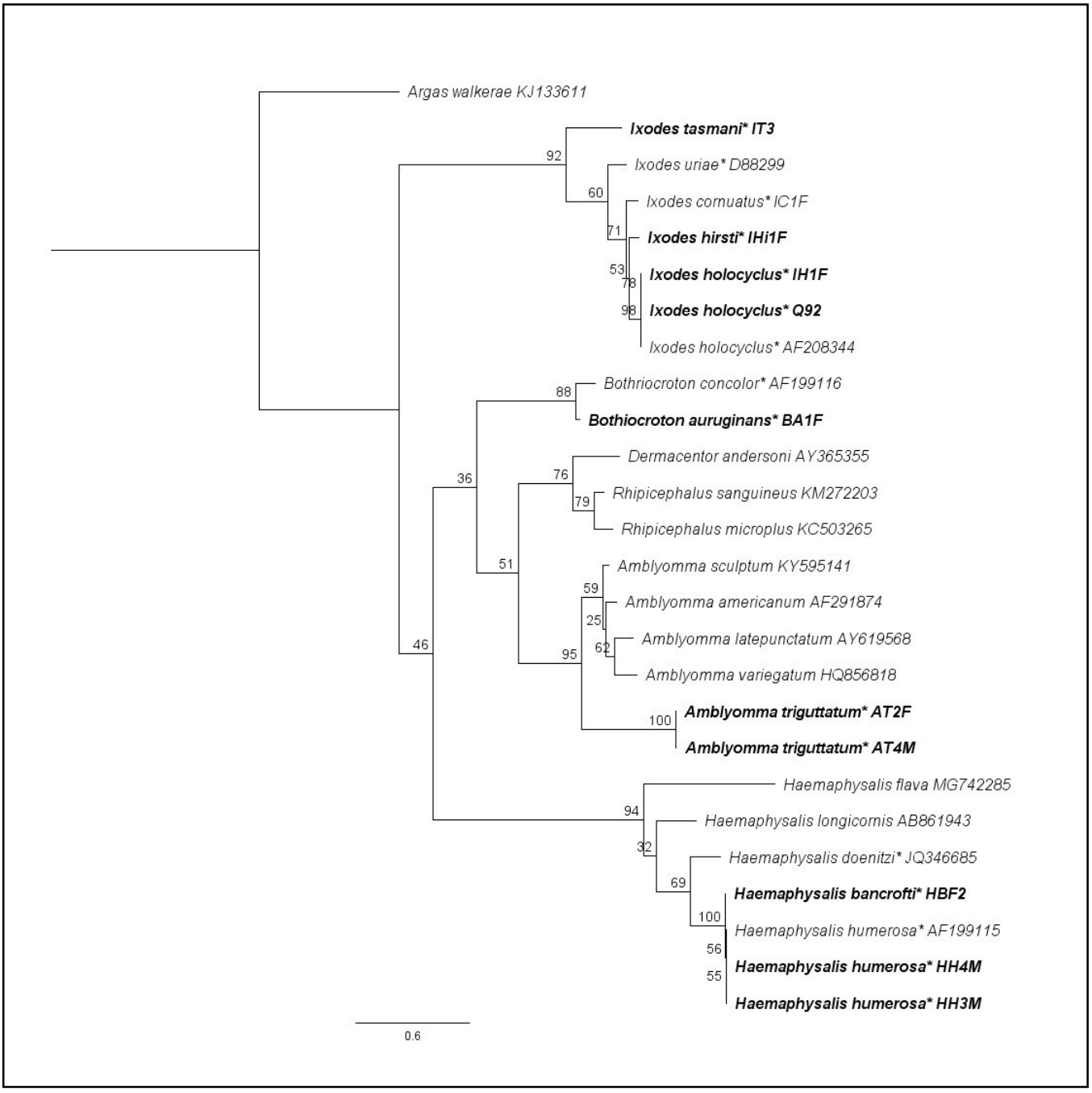


Figure A1.3. RAxML analysis of 594 bp alignment of tick ITS2 sequences. Analysis was conducted using a GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. Bolded sequences indicate those generated from this study, and asterisks indicate native Australian species.

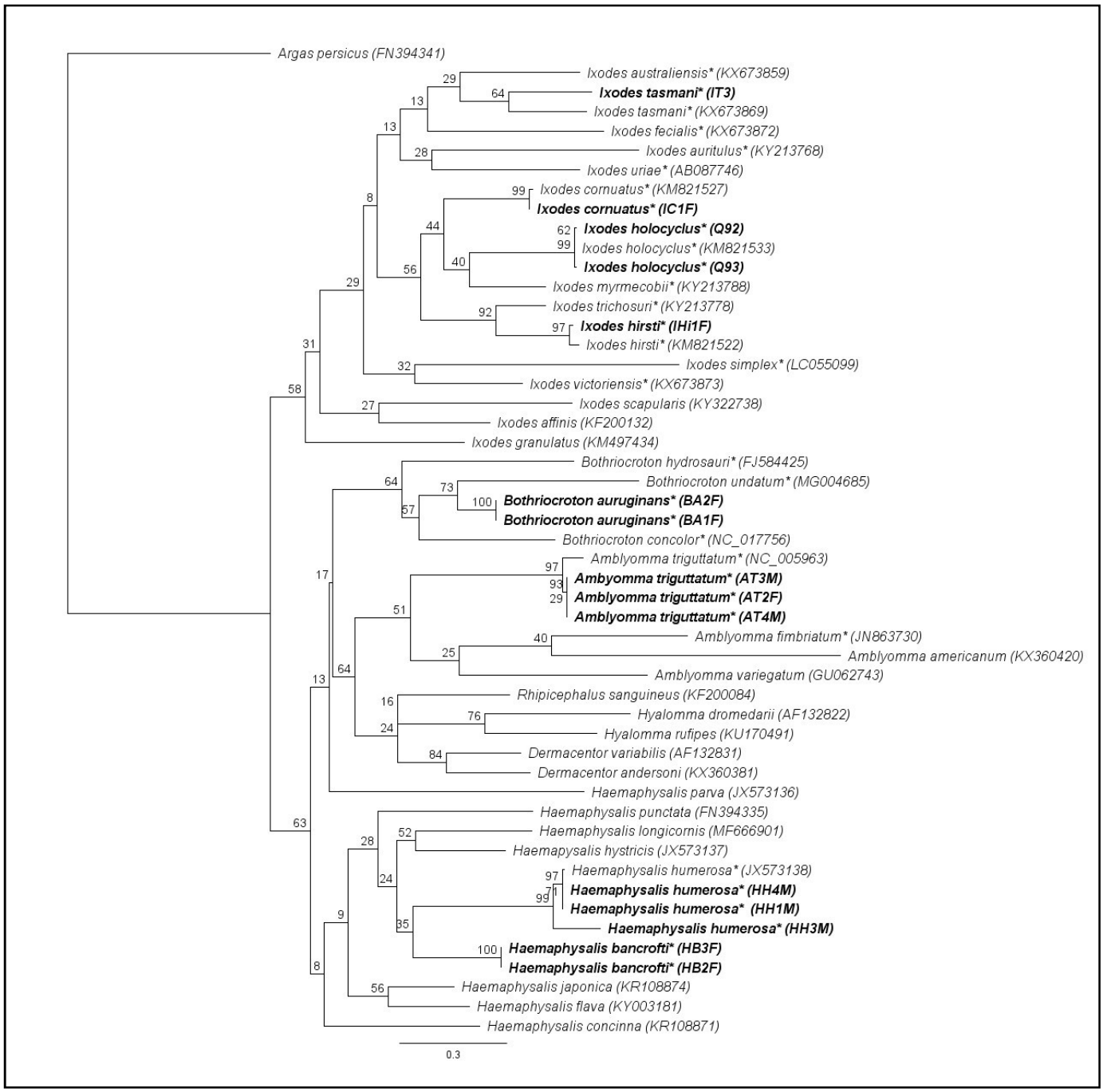


Figure A1.4. RAxML analysis of 329 bp alignment of tick COI sequences. Analysis was conducted using a GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. Bolded sequences indicate those generated from this study, and asterisks indicate native Australian species.

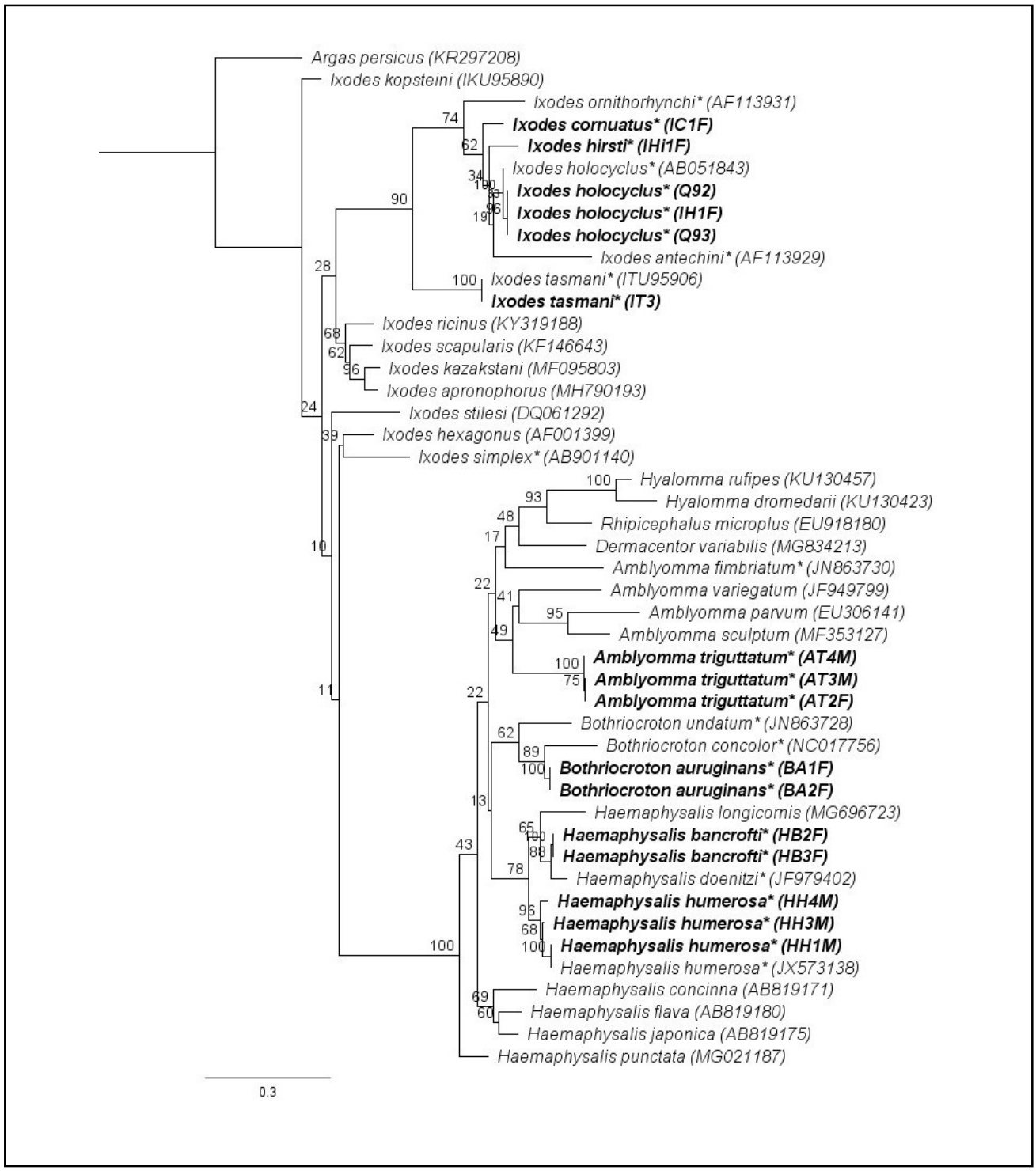


Figure A1.5. RAxML analysis of tick 16S rRNA sequences. Analysis was conducted using a GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. Bolded sequences indicate those generated from this study, and asterisks indicate native Australian Ixodidae.

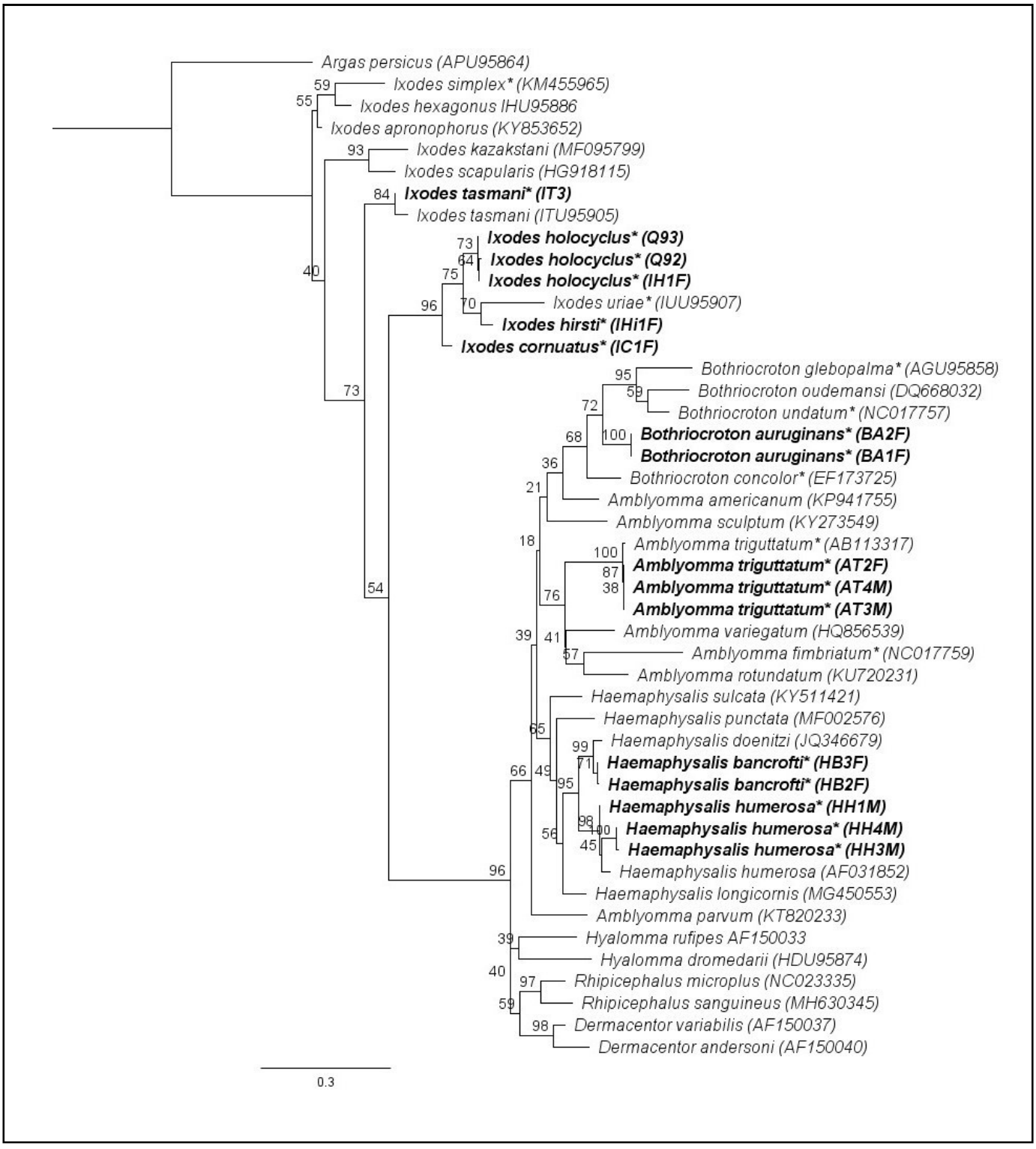


Figure A1.6. RAxML analysis of tick 12S rRNA sequences. Analysis was conducted using a GTR GAMMA model, rapid bootstrapping and search for best-scoring ML tree algorithm, 1000 bootstrapping replicates and a random parsimony seed of 1. Bolded sequences indicate those generated from this study, and asterisks indicate native Australian Ixodidae.

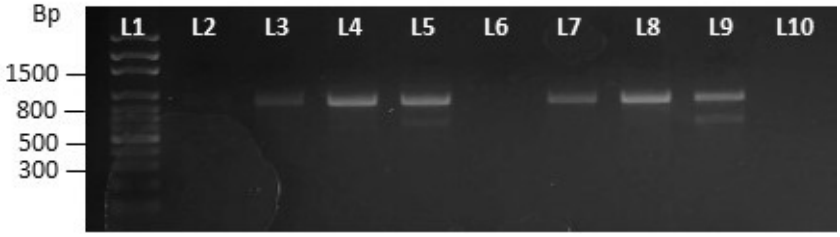


Figure A1.7. Amplification of *Amblyomma triguttatum* COI gene. L1: 100bp ladder; L2-3: Positive control; L4-5: AT2F; L6-7:AT3M; L8-9: AT4M; L10: No template control.

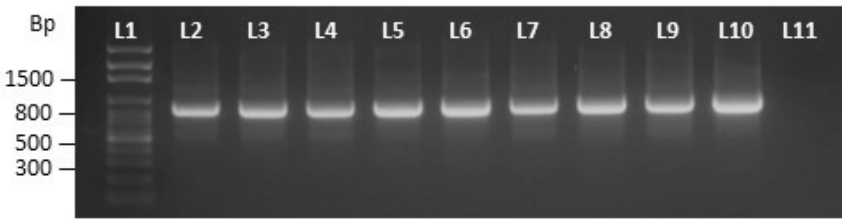


Figure A1.8. Secondary amplification of *Amblyomma triguttatum* COI gene following weak primary amplification. L1: 100bp ladder; L2-4: AT2F; L5-7:AT3M; L8-10: AT4M; L10: No template control.

Table A1.1. The host animals from which ticks used in this study were collected.

REFERENCE TICKS			
Sample	**Host**	**Sample**	**Host**
AT2F	None (questing)	HH3M	Northern brown bandicoot
AT3M	None (questing)	HH4M	Northern brown bandicoot
AT4M	None (questing)	IC1F	Domestic dog
BA1F	Wombat	IHi1F	Domestic cat
BA2F	Wombat	Q92	None (questing)
HB2F	Long-nosed bandicoot	Q93	None (questing)
HB3N	Long-nosed bandicoot	IH1F	Long-nosed bandicoot
HH1M	Northern brown bandicoot	IT3	Tasmanian devil
UIN & UIL SAMPLES (Section 3.3)			
Sample	**Host**	**Sample**	**Host**
UIN1	Domestic dog	UIN6	Domestic dog
UIN2	Domestic dog	UIN7	Domestic dog
UIN3	Domestic dog	UIL1	Domestic dog
UIN4	Domestic dog	UIL2	Domestic dog
UIN5	Domestic dog	UIL3	Domestic dog
UWN SAMPLES (Section 3.7)			
Sample	**Host**	**Sample**	**Host**
UWN1	Koala	UWN40	Echidna
UWN4	Platypus	UWN41	Echidna
UWN11	Kangaroo	UWN42	Echidna
UWN18	Lace monitor	UWN44	Echidna
UWN19	Lace monitor	UWN45	Echidna
UWN20	Lace monitor	UWN46	Echidna
UWN30	Echidna	UWN51	Lace monitor
UWN31	Echidna	UWN52	Lace monitor
UWN32	Echidna	UWN53	Lace monitor
UWN33	Echidna	UWN54	Lace monitor
UWN34	Echidna	UWN55	Lace monitor
UWN35	Echidna	UWN56	Lace monitor
UWN36	Echidna	UWN57	Lace monitor
UWN37	Echidna	UWN58	Lace monitor
UWN38	Echidna	UWN59	Lace monitor
UWN39	Echidna	UWN60	Lace monitor