IJP: Parasites and Wildlife 10 (2019) 125–131

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

# Cryptic species diversity in ticks that transmit disease in Australia

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ARTICLE INFO

*Keywords:*
Ixodidae
Ticks
Cryptic species
Phylogenetics
Species diversity

ABSTRACT

Ticks are important vectors of a broad range of pathogens in Australia. Many tick species are morphologically similar and are therefore difficult to identify using morphology alone, particularly when collected in the larval and nymphal life stages. We report here the application of molecular methods to examine the species diversity of ixodid ticks at two sites in southern New South Wales, Australia. Our taxon sampling included six morphologically characterised adult stage voucher specimens of *Ixodes trichosuri, Ixodes tasmani, Ixodes fecialis* and *Ixodes holocyclus* (the paralysis tick) and ~250 field collected specimens that were in the larva or nymph stage and thus not morphologically identifiable. One nuclear and two mitochondrial amplicons were sequenced using a combination of Sanger and Illumina MiSeq sequencing. Phylogenetic relationships were estimated using both maximum likelihood and Bayesian methods. Two clades with strong bootstrap and Bayesian support were observed across trees estimated from each of three markers and from an analysis of the concatenated sequences. One voucher specimen of *I. trichosuri* was located in one of these clades, while the other *I. trichosuri* voucher specimen was in a second clade with the remaining three identified species, suggesting these morphologically similar ticks may represent different cryptic species. Unidentified specimens were found across both clades, and molecular divergence of many of these is equal to or greater than that observed between identified species, suggesting additional unidentified species may exist. Further studies are required to understand the taxonomic status of ticks in Australia, and how this species diversity impacts disease risk for livestock, domestic animals, wildlife and humans.

## 1. Introduction

Ticks are obligate hematophagous ecto-parasites responsible for transmitting a diverse range of micro- and macro- parasites to animals and humans (Klompen et al., 1996; Bonnet and Liu, 2012; McCoy et al., 2013; Lv et al., 2014b; Zhang and Zhang, 2014). Hard ticks are of particular medical and veterinary importance, as their broad host range makes them excellent vectors for disease transmission (Barker and Walker, 2014). For example, in Australia, *Ixodes* ticks attach to a very diverse range of vertebrate hosts that include native species, livestock and companion animals, and humans (Spratt and Haycock, 1988; Atwell et al., 2001; Jongejan and Uilenberg, 2004; Murdoch and Spratt, 2006; Barker and Walker, 2014) and have been shown to harbour an equally diverse range of known and potential pathogens identified through metagenomics (Barker and Murrell, 2004; Carpi et al., 2011; Gofton et al., 2015; Greay et al., 2018). Given that there is a range of potential pathogens that ticks can transmit, a thorough understanding of their species diversity and phylogenetic relationships may be beneficial in assessing disease and transmission risks.

The typical *Ixodes* life cycle has four stages: egg, larva, nymph and adult (Anderson et al., 2004). They feed on blood as larvae, moult to nymphs, feed, and then moult to adults. Adult females must feed a third time after mating before they can lay eggs (Black et al., 1997; Bonnet and Liu, 2012). Thus, ixodid ticks are three-host ticks, with moulting occurring off the host, so that all three stages (larva, nymph and adult) are found on vegetation as they quest for hosts.

This multi-host life cycle facilitates pathogen transmission and zoonotic pathogen detection. During feeding, ticks may ingest a broad range of blood- and tissue-dwelling pathogens from their hosts, which they can then transmit to subsequent hosts during feeding (Caporale et al., 1995; Commins and Platts-Mills, 2013). For example, ixodid ticks are known to carry rickettsial pathogens, including *Borrelia burgdorferi*, the causative organism of Lyme disease (Kilpatrick et al., 2017; Walter et al., 2017), protozoan parasites such as those causing babesiosis in cattle and humans (Spielman et al., 1985; de la Fuente and Kocan, 2006; Graves and Stenos, 2009; Izzard et al., 2009), and many viruses, such as those that cause yellow fever (Telford et al., 1997; de la Fuente et al., 2008; Gould and Solomon, 2008; Vilcins et al., 2008, 2009).

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https://doi.org/10.1016/j.ijppaw.2019.08.002
Received 1 May 2019; Received in revised form 5 August 2019; Accepted 5 August 2019
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In Australia, a "Lyme-like" disease of uncertain etiology has arisen (Mayne et al., 2014; Chalada et al., 2016). Ticks in the *I. ricinus* complex responsible for transmitting Lyme disease are not found in Australia (Schmid, 1985), but several tick-borne rickettsioses (Graves and Stenos, 2009; Izzard et al., 2009; Vilcins et al., 2009), including Q fever (Spelman, 1982), scrub typhus (Wang et al., 2009), and Flinders Island spotted fever (Stenos et al., 1998; Parola and Raoult, 2001) are all transmitted by native ixodid ticks. Although vector competence varies among tick species and is correlated with disease risk, there have been few studies examining tick species diversity and competence in Australian wild spaces. A first step in such a study is to develop a robust, preferably molecular, phylogeny of Australian ixodid ticks that could be used to identify species simultaneously with molecular screening for pathogen presence.

Previous research has focused on ticks collected from companion animals at veterinary clinics (Atwell et al., 2001; Day, 2011; Greay et al., 2016), bacterial studies (Murrell et al., 2003; Vilcins et al., 2009; Andreotti et al., 2011), and paralysis ticks (Stone et al., 1983; Grattan-Smith et al., 1997; Jackson et al., 2002, 2007; Eppleston et al., 2013). However, to appropriately quantify transmission potential and disease risk, we must first understand biogeographic variation in tick species diversity in environments where animals and humans might be exposed to ticks.

The strategy we have employed was to sample at two locations: one at a pasture/bushland margin where large numbers of Eastern grey kangaroos (*Macropus giganteus*, an Australian ecological equivalent of white-tailed deer in the north-eastern USA) are at high density and human contact with ticks is likely, and at a nearby regenerating native bush site where Eastern grey kangaroos are markedly less numerous, there is generally greater wildlife diversity, and human-tick encounters are less likely. At the bush/pasture margin site ticks were collected from vegetation, and at the regenerating bushland site we collected (mostly larval) ticks from Australian bush rats (*Rattus fuscipes*). Tick molecular diversity was analysed using DNA sequences for three markers: mitochondrial ribosomal RNA 12S and 16S genes and the nuclear internal transcribed spacer 2 (Cruickshank, 2002; Song et al., 2011; de Mandal et al., 2014; Lv et al., 2014a, 2014b; Lv et al., 2014a).

## 2. Materials and methods

### 2.1. Sample collection

Un-engorged ticks were collected by flagging on pasture and in clearings at or close to the bush/pasture margins at two locations on the Australian National University Coastal Campus, Kioloa, NSW (Fig. 1) in February, April, and June in 2015, and in October and November in 2016. To flag, an approximately 1 × 1.5 m piece of white fabric was dragged along the ground: ticks caught up on the fabric were picked off with tweezers and placed into tubes that contained a mix of plaster of Paris and charcoal (Konnai et al., 2008). In 2015, we collected a total of 633 larvae, 34 nymphs, and 3 adults: ~120 larvae, 13 nymphs and 1 adult at the dunes and 513 larvae, 21 nymphs and 1 adult on the ANU campus. In 2016, we collected ~300 larvae, ~250 nymphs, and ~80 adults from the ANU campus.

Engorged ticks were collected from Australian bush rats, *R. fuscipes*, that had been captured at the site reported by Spratt and Haycock (1988) in the Mogo State Forest, approximately 40 km south of Kioloa. This is an area of regenerating dry eucalypt forest that had been subjected to periodic commercial forestry of native vegetation and fire. Rats were captured in small Elliott traps and held in an approved animal facility at the ANU Coastal Campus for up to 3 days in wire-bottomed cages. Engorged ticks were collected each morning from collection containers beneath the cages, and stored live in glass vials containing dry, clean, fine sand. Trapping of bush rats, and the subsequent holding of captured rats for 3 days post-trapping to allow collection of engorged ticks, were performed with the appropriate regulatory permits (La Trobe University approval AEC 13–23, New South Wales – Scientific Licence 5 L 101280, Victorian – Scientific Permit 10007169). We collected ~343 engorged larvae, ~68 engorged nymphs, and no adults from native bush rats at Mogo State Forest.

Six (6) specimens preserved in 70% ethanol were identified morphologically and used as vouchers for this study: *I. tasmani* ex *Vombatus ursinus* (from Lucyvale, Victoria), *I. fecialis* ex *Dasyurus maculatus* (from Spirabo Nat Pk., NSW), *I. tasmani* ex *V. ursinus* (from Jerangle, NSW), *I. trichosuri* ex *R. norvegicus* (Lab-reared, experimental), and *I. trichosuri* ex *Perameles nasuta* (Lab reared, experimental). An adult *I. holocyclus* was identified after collection by flagging from Kioloa. The *R. norvegicus* host was laboratory bred and used as an experimental animal in parasite life cycle studies (Spratt and Haycock, 1988). The *P. nasuta* host had been removed from the Mogo study site, maintained in the laboratory, and used as an experimental animal in the same parasite life cycle studies.

### 2.2. Purifying tick DNA

DNA was extracted using a Bioline ISOLATE II Genomic DNA kit following the manufacturer's instructions (Bioline (Aust) Pty Ltd, Alexandria NSW, Australia, 2015). Tweezers or a scalpel blade were used to remove a back leg from live ticks and placed immediately into extraction buffer. Alternatively, whole bodies of dead, small ticks or whole carapaces from moulted ticks were used. In total, DNA was extracted from 128 larvae and 1 nymph from Kioloa, and 83 larvae and 11 nymphs from Mogo State Forest.

### 2.3. PCR of mitochondrial and nuclear amplicons

The 16S gene was amplified using the primer pair mt_16S_NGS-F (5′-TCG TGG GCA GCG TCA GAT GTG TAT AAGA -3′) and mt_16S_NGS-R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA G -3′). The 12S gene was amplified using the primer pair mt_12S-NGS-F (5′- TCG TCG GCA GCG TCA GAT GTG TAT AAGA -3′) and mt_12S_NGS-R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA G -3′). Lastly the ITS2 gene was amplified using the primer pair Nc_ITS2_NGS-F (5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GGG TCG GAT CAT ATA TCA -3′) and Nc_ITS2_NGS-R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GCA ACT TCC TCG GCA ACA -3′). PCR conditions were initial denaturation at 98 °C for 1 min, followed by 38 cycles of denaturation at 98 °C for 15 s, annealing at 61.6 °C (16S) or 63.4 °C (ITS2) or 59.8 °C (12S) for 15 s, and extension at 72 °C 15 s, with a final extension reaction at 72 °C for 5 min.

### 2.4. Sequencing of amplicons

Amplicons were prepared and sequenced on an Illumina MiSeq. An in-house amplicon barcoding and sequencing protocol was followed (prepared by Dr Stephen Doyle, see Appendix for protocol and for barcoding primers used). The DNA concentration of the final pooled library was adjusted to 2500 pM following Qubit fluorometric quantitation. Two MiSeq runs were carried out: a pilot experiment using a v2 500 cycle kit (2x250 bp paired-end), and a second experiment of v3 600 cycle kit (2x300 bp paired end), following the manufacturer instructions (Illumina MiSeq reagent kit V3 preparation guide, 2013). The final sequencing mix included 10% PhiX DNA. Sanger sequencing was also used to confirm sequences of some samples. Amplicons for Sanger sequencing were cleaned using Ampure XP beads (Beckman-Coulter) and sequenced at Macrogen Inc., Korea.

### 2.5. Sequencing analysis

MiSeq data were imported into CLC Genomics Workbench version 7.5. Adapters and primer sequences were trimmed from both Sanger and MiSeq samples using a quality score of > 20. Reads were then

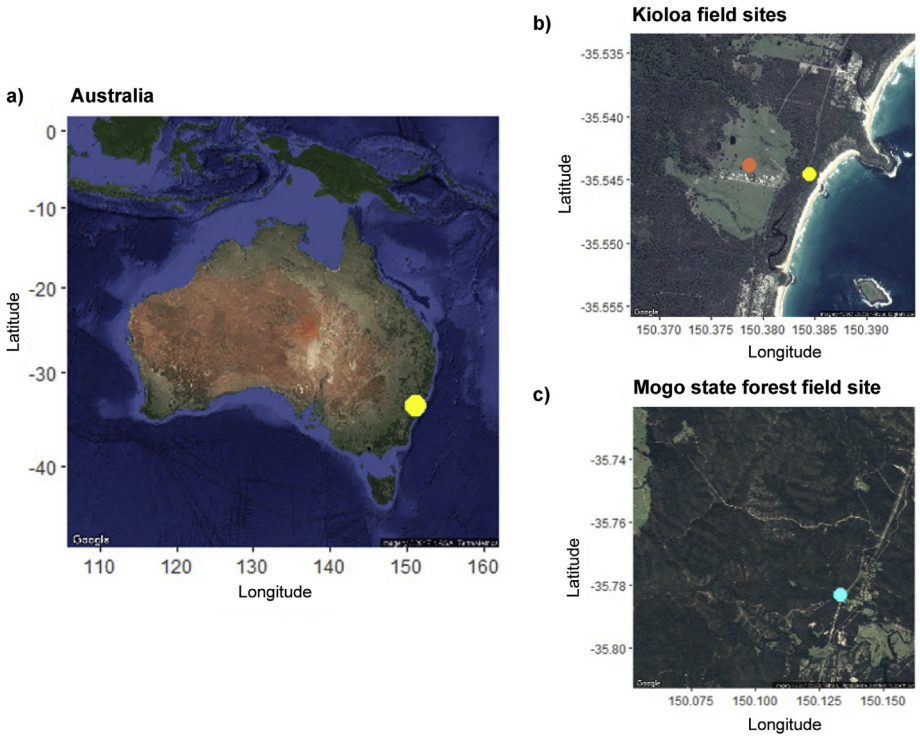


**Fig. 1.** Satellite maps of (a) Australia (b) Kioloa and (c) Mogo state forest sample collection sites with coordinates, latitude on the y axis and longitude on the x axis. (a) yellow: NSW general location of sites (b) orange: Kioloa ANU campus, yellow: Kioloa dunes (c) blue: Mogo State Forest (Kahle and Wickham, 2013). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mapped to the whole mitochondrial genome of *I. holocyclus* (NC_005293) and three ITS2 *I. holocyclus* reference sequences (GenBank accession numbers: AF208344 (Wesson et al., 1993; McLain et al., 1995), AB025591, AB025595 (Fukunaga et al., 2000)).

Prior to running the phylogenetic analysis, 12 samples were removed as they did not map to *Ixodes* spp. (e.g., suspected bacterial contaminants) or were of poor sequencing quality.

The alignments of consensus sequences for each individual tick were exported and refined in ClustalW2 (Larkin et al., 2007). Sequences from closely-related outgroups were downloaded from NCBI's GenBank database and added to the 16S alignment: *Haemaphysalis humerosa* (JX573138), *Haemaphysalis parva* (JX573136), *Rhipicephalus appendiculatus* (KC503257) and *Amblyomma triguttatum* (AB113317). The alignment was checked and further refined by eye using Mesquite v3.04 (Maddison and Maddison, 2008).

Phylogenies were estimated using maximum likelihood as implemented in the program RAxML for high-performance computing version 8.0.19 (Stamatakis, 2006, 2014) using the best of 20 replicates under the GTRCAT model of sequence evolution and 1000 long bootstrap replicates to estimate support for bipartitions. The best model for each partition was assessed using PartitionFinder version 1.1.1 (Lanfear et al., 2012), and posterior probabilities for nodes were estimated with MrBayes 3.2.5 (Ronquist et al., 2012), using 2 runs with 4 chains each and sampling every 1000 generations. Convergence between the 2 runs and stationarity were assessed using Tracer version 1.6 (Rambaut et al., 2014). The m16S Bayesian analysis was conducted with 13 million generations under GTR + I + G substitution model. The 12S Bayesian analysis was conducted with 18 million generations under the HKY + I + G substitution model. The ITS2 Bayesian analysis was conducted with 10 million generations under the K80 + G substitution model. The concatenated Bayesian analysis was conducted with 8 million generations under the above 3 substitution models, unlinked so each gene could be treated differently. We used a conservative burn-in fraction of 25%.

The phylogenetic trees were imported into R version 3.4.1 (R Core Team, 2017) and edited for publication using pegas package version 0.10 (Paradis et al., 2017), in conjunction with Geiger version 2.0.6 (Harmon et al., 2015) and Phytools version 0.6–44 (Revell, 2012).

### 2.6. Haplotype analysis

The aligned nexus files were imported into the program PopArt (Population analysis with reticulate trees) version 1.7 (Leigh and Bryant, 2015) to produce haplotype networks. PopArt does not accept missing data (Leigh and Bryant, 2015), so only individuals sequenced for all three markers were used in the final concatenated haplotype analysis (200 individuals).

Haplotype networks were produced using two algorithms: TCS network analysis (Clement et al., 2002) and median-joining network analysis (Bandelt et al., 1999). Both analyses illustrated the same topology; we present here the TCS network analyses (Clement et al., 2002).

## 3. Results

### 3.1. Phylogenetic analysis

All three trees – 12S, 16S, and ITS2 – showed a single, well-supported bipartition resulting in two clades. It was only possible to root one tree, 16S, because there were no suitable outgroup sequences that could be confidently aligned for more rapidly evolving 12S and ITS-2.

Consistent across all three markers and the concatenated analysis, *I. trichosuri* (P.n) was positioned in a separate clade from the other five voucher specimens (Fig. 2; Supplementary Figs. 4, 6, 8). The other five voucher specimens of *I. holocyclus* (Kioloa), *I. trichosuri* (R.n), *I. tasmani* (NSW), *I. tasmani* (VIC) and *I. fecialis* (NSW) were positioned together in the same clade across all markers with minimal differentiation between each species despite their obvious morphological characteristic differences (Fig. 2). As the genetic similarities in sequences among voucher specimens were unexpected, we confirmed each sequence by re-extracting DNA, amplifying, and sequencing each amplicon using Sanger sequencing.

Preliminary phylogenetic analysis of the ITS-2 marker revealed that samples 00618, 00596, 0597 and 00612 had very long branch lengths, which can reduce phylogenetic accuracy; these were removed and maximum likelihood analyses re-run (Heath et al., 2008).

When the amplicons were concatenated, the resolution of the

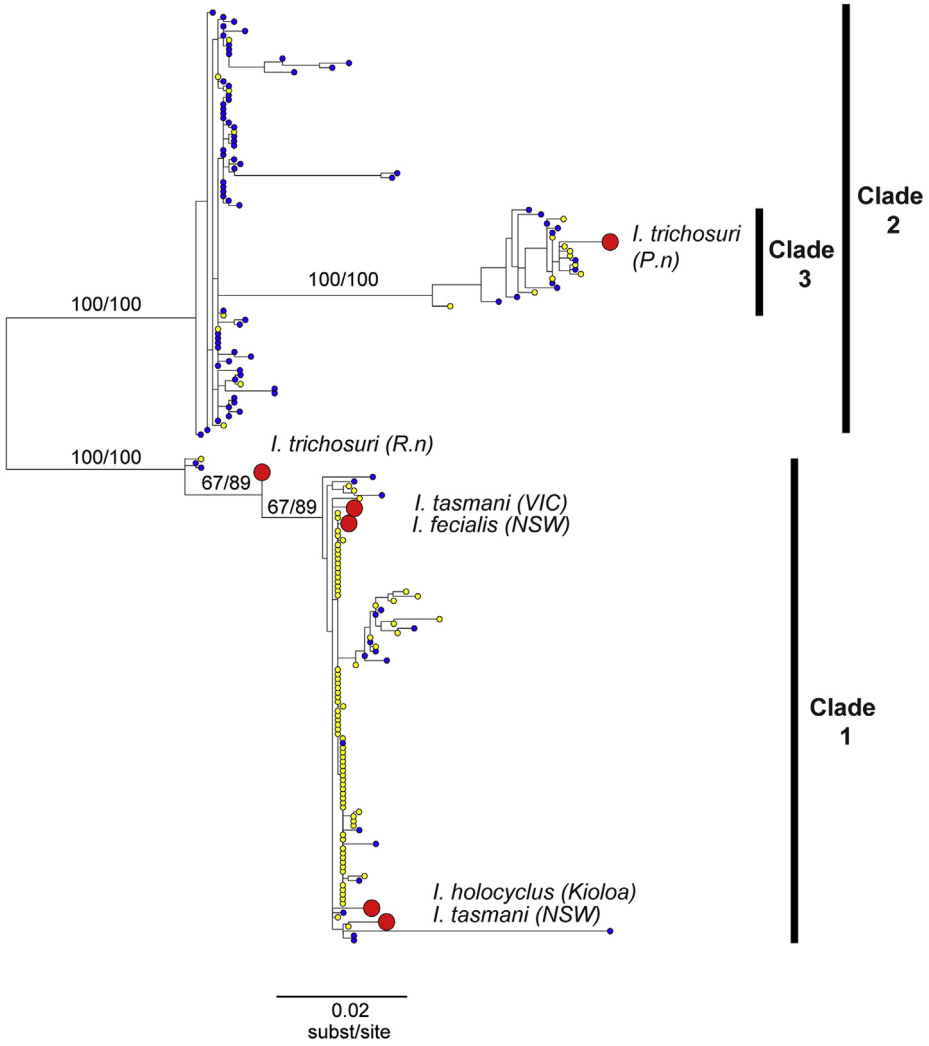


**Fig. 2.** Maximum likelihood estimate based on three markers of ticks collected at Kioloa and Mogo State Forest, NSW, Australia. Blue: Mogo State Forest (rats), yellow: Kioloa (flagging), red: identified voucher specimens. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phylogenetic tree improved. The topology of this tree is consistent (Supplementary Figs. 4, 6, 8); therefore, only the concatenated tree is presented (Fig. 2). *I. trichosuri* (P.n) was situated in a separate clade from the other five voucher specimens with strong bootstrap (100%) and Bayesian support (100%).

### 3.2. Haplotype analysis

The haplotype network analysis was conducted individually on three amplicons 12S, 16S and ITS-2 (Supplementary Figs. 5, 7, 9). The three individual haplotype networks were congruent with each other (Fig. 3) and with the results of the phylogenetic analysis. *Ixodes trichosuri* (P.n) was strongly differentiated and situated in a separate haplotype group (III) from the other five voucher specimens (II).

More Kioloa samples were present in clade I/haplotype group I with *I. holocyclus* (Kioloa), *I. tasmani* (VIC), *I. tasmani* (NSW), *I. trichosuri* (R.n), and *I. fecialis* (NSW) (Figs. 2 and 3). Haplotype group II contained no voucher specimens and was dominated by Mogo samples (Fig. 3); these represent individuals in clade II, but not clade III, in the phylogenetic tree (Fig. 2). In clade III/haplotype group III, *I. trichosuri* (P.n) was found with approximately half of the un-identified specimens from Mogo and half from Kioloa (Figs. 2 and 3).

## 4. Discussion

We collected ixodid ticks from vegetation and Australian bush rats in order to examine the phylogenetic diversity at two field sites in southern New South Wales, with the assumption that sequence diversity could be informative about species diversity. We found that *I. trichosuri* (P.n) is genetically distinguishable from *I. holocyclus* (Kioloa) and remaining unknown *Ixodes* spp., although several other morphologically defined *Ixodes* spp. were not well resolved by these amplicons. This suggests rapid evolution of morphological characteristics in *Ixodes,* leading to morphological differentiation in the absence of molecular divergence for some species.

### 4.1. Genetic differentiation among Australian ixodid ticks

We identified the species of six adult voucher specimens using morphological characteristics, including the presence or absence of the palpal spur, separation or fusion of palps 2 and 3, mouthpart type, presence or absence of a sternal plate, coxa with or without a spur, presence or absence of cornua, and the colouring of the legs (Jackson et al., 1998, 2002). These voucher specimens fell into two clades: one with a large number of unidentified larvae and *I. trichosuri* (P.n) from bandicoots, while the other specimens, *I. fecialis* (NSW), *I. tasmani (NSW), I. tasmani (VIC)* and lab-reared *I. trichosuri* (R.n) aligned in the

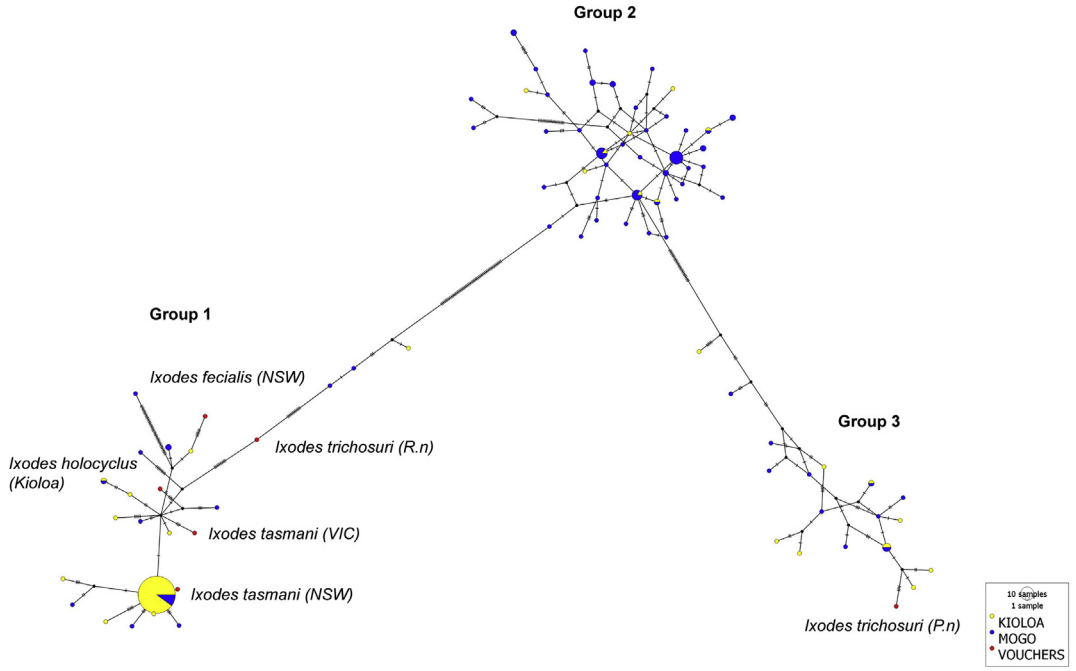


**Fig. 3.** TCS haplotype network based on 3 markers sequenced from ticks collected from Kioloa and Mogo State Forest in NSW, Australia. Samples are coloured based on their collection site, Mogo State Forest (blue) or, Kioloa (yellow). Voucher specimens are coloured red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

same clade with the paralysis tick, *I. holocyclus* (Fig. 2). The short branch lengths among voucher specimens in this second clade compared to ticks sampled as a whole was unexpected, considering the species were all morphologically distinguishable. Furthermore, the phylogenetic and haplotype analyses illustrate the potential for cryptic diversity within the genus (Fig. 3). The two specimens were morphologically identified as *I. trichosuri* are not monophyletic, and were found in distinctly separate clades, suggesting that these specimens may be cryptic species. Morphological distinctions are not yet visible in the nymphs collected for this study, but molecular divergence was greater than among morphologically identified species, suggesting that species diversity might be considerable. In the absence of additional data on interbreeding, we would conclude at a minimum that there is poor concordance between morphological and molecular diversity.

### 4.2. Differences in species diversity between sampling locations

We collected nymphs in two field sites, Kioloa and Mogo State Forest in NSW, Australia. In both phylogenetic and haplotype analyses, clade I/haplotype group I contained five of the voucher specimens and predominantly undetermined species from Kioloa, haplotype group II (which contained specimens in clade II but not clade III) contained no voucher specimens and predominantly undetermined specimens from Mogo, while clade III (nested within clade II)/ haplotype group II contained the voucher *I. trichosuri* ex *P. nasuta* and is evenly divided between undetermined specimens from Kioloa and Mogo (Figs. 2 and 3). There are several possibilities that might explain the observed differentiation in species diversity between these two locations:

(1) Geographic variation. The Kioloa and Mogo State Forest sites are only approximately 55 km apart, yet their vegetation is markedly different: open, regenerating eucalypt forest at Mogo compared with native, unimproved pasture at Kioloa. Ticks in Australia rely heavily on high humidity for their survival strategy, preferring a relative humidity of 85%, rainfall, and low temperatures (Needham and Teel, 1991; Oorebeek and Kleindorfer, 2008). Differences in vegetation may impact microenvironment humidity, and if tick species vary in off-host environmental preferences, this may in turn affect the species diversity between the two locations.

(2) Host availability. Differences in habitat and vegetation also markedly affect the hosts present at each site (Allan et al., 2003; LoGiudice et al., 2003; Oorebeek and Kleindorfer, 2008; Kilpatrick et al., 2017). Although ixodid ticks broadly will attach to many mammals, including Australian bush rats (Spratt and Haycock, 1988), kangaroos, bandicoots (Barker and Walker, 2014), domestic animals (Atwell et al., 2001), possums, livestock, and humans (Jongejan and Uilenberg, 2004; Murdoch and Spratt, 2006), there are differences in host preferences. For example, approximately 30 years ago, bandicoots were the predominate host for all life stages of *I. holocyclus* ticks in southeast coastal Qld (Doube, 1979). However, population densities of three bandicoot species, *Isoodon macrourus, Isoodon obesulus* and *P. nasuta* have since declined. Eastern grey kangaroos are now a preferred host for *I. holocyclus* (Storer et al., 2003; Jackson et al., 2007; Kolonin, 2007; Dawood et al., 2013), and the current kangaroo density at the Kioloa field site is considerably higher than at Mogo State Forest, where the bush rat density is higher. Consistent with this host preference, *I. holocyclus* and closely related ticks may be more common at the kangaroo-dominated Kioloa than they are at Mogo. This tentative explanation is tempered by the fact that ticks from Kioloa were collected exclusively by flagging, whereas ticks from Mogo were all collected from bush rats. The apparent difference in tick diversity between the two sites may, therefore, represent strong host preference of ticks at Mogo for bush rats.

(3) Life stage. The life stage – larva, nymph or adult—could also be important in determining host choice (Bonnet and Liu, 2012). Larval and nymphal ticks, which have smaller mouth parts than adults, may not be able to penetrate the skin of a kangaroo, whereas an adult may be too big to feed on a bush rat. In a study creating an artificial tick feeding assay, the membrane thickness needed to be altered depending on the life stage of the tick (Krull et al., 2017). Larvae are also small in size, and small mammals such as bush rats that have bodies close to the ground may reduce the effort required for questing larvae (Oorebeek and Kleindorfer, 2008). Adults

however, feed much longer and require a large blood meal. Unengorged adults could be questing further up the vegetation toward large mammals and are able to do so when they are fully developed (Bonnet and Liu, 2012). Thus, the observed variation may not be driven by differing tick species at the two sites, but rather differences in timing of developmental stages.

(4) Time of year. Species diversity differences between locations could reflect differences in the time of year the ticks were collected. Ticks were collected only at Kioloa in February, April, and June 2015 and at both Kioloa and Mogo in October and November 2016. In Australia, the summers are hot and the winters are relatively cold, but, more importantly, rainfall and humidity is inconsistent all year round. Spratt and Haycock (1988) observed a mild fluctuation but relatively steady tick prevalence across every month of the year on Australian bush rats. Some months there was a reduction of ticks observed on this host, but overall, ticks were active at Mogo State Forest all year round. The same is not true for other hosts; Oorebeek and Kleindorfer (2008) did not find any ticks on the Australian birds in their study from December to March, but attached ticks were observed for the remaining months of the year. Various studies have indicated a variation in responses to the climate, ecology, time of year and hosts present from different tick species (Needham and Teel, 1991; Randolph, 1998; Perret et al., 2004; Oorebeek and Kleindorfer, 2008).

## 5. Conclusions

Our results indicated that (1) four morphologically identified species were genetically closely related despite having distinct morphological features, based on selected primer sets, while one (*I. trichosuri*) was distinctly different, (2) we observed remarkable genetic diversity among the tick samples collected in a very small geographic area, and that (3) this genetic diversity was not clearly correlated with known species boundaries. Only *I. trichosuri* (P.n) collected from a bandicoot was easily distinguishable genetically from other identified ticks, and specimens identified as *I. trichosuri* were not monophyletic. We conclude that that there has been rapid evolution of morphological characteristics in species of *Ixodes,* leading to morphological differentiation in the absence of molecular divergence for some species, and possible convergence in morphological characteristics, or retention of ancestral traits, in those that are genetically distinct.

The species diversity throughout the *Ixodes* genus likely influences parasite and pathogen transmission to a range of hosts, including humans (McCoy et al., 2013). As the overlap between wilderness and human habitats increases, the zoonotic disease risks from these multi-host vectors may also increase. Determining the potential for ticks to transmit pathogens of medical and veterinary importance is hampered by challenges in species identification of the ticks concerned (Lv et al., 2014a, 2014b). Molecular methods characterising tick species diversity across different environments may be required to effectively meet these challenges.

## Conflicts of interest

The authors declare they have no conflict of interest regarding the work described in this manuscript.

## Declaration of interests

None.

## Acknowledgements

We would like to thank Dr. Stephen Doyle for his contributions in assisting with MiSeq preparation and sequencing of tick samples and the Grant Lab for their assistance and guidance. Many thanks to Andrew Robinson, ICT Research support officer, for the continuous support and updates of the LIMS-HPC resource used for our bioinformatic analyses.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.08.002.

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