Running title: Bacterial community profiling highlights complex diversity and novel organisms in wildlife ticks.

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Abstract

Ticks (Acari: Ixodida) transmit a greater variety of pathogens than any other blood-feeding group of arthropods. While numerous microbes have been identified inhabiting Australian Ixodidae, some of which are related to globally important tick-borne pathogens, little is known about the bacterial communities within ticks collected from Australian wildlife. In this study, 1,019 ticks were identified on 221 hosts spanning 27 wildlife species. Next-generation

sequencing was used to amplify the V1-2 hypervariable region of the bacterial 16S rRNA gene from 238 ticks; *Amblyomma triguttatum* (n=6), *Bothriocroton auruginans* (n=11), *Bothriocroton concolor* (n=20), *Haemaphysalis bancrofti* (n=10), *Haemaphysalis bremneri* (n=4), *Haemaphysalis humerosa* (n=13), *Haemaphysalis longicornis* (n=4), *Ixodes antechini* (n=29), *Ixodes australiensis* (n=26), *Ixodes fecialis* (n=13), *Ixodes holocyclus* (n=37), *Ixodes myrmecobii (*n=*1), Ixodes ornithorhynchi* (n=10), *Ixodes tasmani* (n=51) and *Ixodes trichosuri* (n=3). After bioinformatic analyses, over 14 million assigned bacterial sequences revealed the presence of recently described bacteria '*Candidatus* Borrelia tachyglossi', '*Candidatus* Neoehrlichia australis', '*Candidatus* Neoehrlichia arcana' and '*Candidatus* Ehrlichia ornithorhynchi'. Furthermore, three novel Anaplasmataceae species were identified in the present study including; a *Neoehrlichia* sp. in *I. australiensis* and *I. fecialis* collected from quenda (*Isoodon fusciventer*) (Western Australia), an *Anaplasma* sp. from one *B. concolor* from echidna (*Tachyglossus aculeatus*) (New South Wales), and an *Ehrlichia* sp. from a single *I. fecialis* parasitising a quenda (WA). This study highlights the diversity of bacterial genera harboured within wildlife ticks, which may prove to be of medical and/or veterinary importance in the future.

Keywords: Microbiome; ticks; Ixodida; wildlife; marsupials; Anaplasmataceae

1. Introduction

Current estimates suggest that approximately 17% of all infectious diseases of humans are vector-borne (Rinker et al., 2016) and global trends show that vector-borne diseases (VBDs) are rising at a rapid rate (Jones et al., 2008; Morens and Fauci, 2012). The complex interplay between pathogen, vector, host(s) and the environment make VBDs particularly challenging to understand. In addition, factors such as climate change (Ostfeld and Brunner, 2015), land

use (Ferrell and Brinkerhoff, 2018), feral animal populations (Merrill et al., 2018) and the microclimate within a landscape (Dobson et al., 2011) can further influence the prevalence and distribution of VBDs.

Ticks (Acari: Ixodida) comprise a group of haematophagous (blood feeding) arthropods with over 900 species described globally (Guglielmone et al., 2014; Mans et al., 2019). Ticks are known to transmit various pathogens; however, they also harbour a range of endosymbiont and commensal species (Špitalská et al., 2018). The epidemiology of recognised tick-borne diseases (TBDs) in the northern hemisphere demonstrates that wildlife serve as sentinels and can be used to monitor the presence and distribution of tick-borne pathogens (TBPs). Importantly, research has shown that some wildlife species act as dilution hosts for certain TBPs, whereas others may act as amplification hosts (LoGiudice et al., 2003).

The complexity of TBDs means that studies are increasingly shifting away from isolated species-specific studies toward ecosystem-based, collaborative research (Estrada-Peña et al., 2013; Pfäffle et al., 2013). Metabarcoding provides an informative molecular tool to characterise the bacterial diversity in ticks. Worldwide, next-generation sequencing (NGS)-based analyses have been applied to a range of tick species that are important from medical and veterinary perspectives, including *Amblyomma americanum* (Ponnusamy et al., 2014), *Ixodes ricinus* (Bonnet et al., 2014), and *Rhipicephalus microplus* (Andreotti et al., 2011).

Metabarcoding studies of the bacterial microbiome of Australian ticks have been reported only recently, with the first bacterial profiling by NGS conducted on the human-biting tick *Ixodes holocyclus* (Gofton et al., 2015a). These authors identified a highly abundant endosymbiont '*Candidatus* Midichloria mitochondrii' (CMm) and after blocking the

amplification of this organism, a greater bacterial diversity was revealed, including a number of novel microbes (Gofton et al., 2015a,b). Critically, in contrast to many parts of the world where multiple TBPs have been elucidated within well-studied tick-host-environment ecologies, there is a relative dearth of such information available for Australia. With this in mind, the aims of our study were to survey the bacterial communities present in ticks collected from Australian wildlife and to investigate their genetic relatedness to 'taxa of interest', i.e. tick-associated pathogenic and endosymbiotic organisms (Parola and Raoult, 2001; Mediannikov and Fenollar, 2014; Sumrandee et al., 2016). 'Taxa of interest' in the present study were defined as genera within alphaproteobacteria, gammaproteobacteria and spirochaetes known to be transmitted by ticks in other parts of the world, specifically; *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*, *Francisella*, *Midichloria*, *Neoehrlichia*, *Rickettsia* and *Rickettsiella*. (Ahantarig et al., 2013; Vayssier-Taussat et al., 2015; Bonnet et al., 2017; de la Fuente et al., 2017).

2. Materials and methods

2.1 Sample collection and identification

1,019 ticks were sourced opportunistically from wildlife in Australia by veterinarians, veterinary nurses, wildlife carers, researchers and via submissions from members of the public, and preserved in 70% ethanol before being shipped to Murdoch University, Western Australia (WA), for identification. Ticks were identified morphologically to life stage and species using keys and species' descriptions (Roberts, 1970; Barker and Walker, 2014). Details of sample collection are available in Supplementary File S1.

2.2 DNA Extraction

101 A sub-sample of ticks (*n*=238) was chosen for DNA extraction and bacterial profiling. Ticks
102 were selected to represent as many tick species, hosts, geographical regions and life stages as
103 possible within the study. Prior to DNA extraction, individual ticks were surface-sterilised in
104 10% hypochlorite solution, rinsed in 70% ethanol and DNA-free PBS, and then air-dried.
105 Total genomic DNA (gDNA) was extracted using the Qiagen DNeasy Blood and Tissue kit
106 (Qiagen, Germany) following the manufacturer's recommendations with the following
107 modifications; ticks were placed in a 2 mL safe lock Eppendorf tube with a 5 mm steel bead,
108 frozen in liquid nitrogen for 1 minute and homogenised by shaking at 40 Hz in a Tissue Lyser
109 LT (Qiagen, Germany). The volume of elution buffer AE was adjusted to 200 µL for
110 engorged females, 100 µL for unengorged adults and 40 µL for nymphs. A double elution
111 was carried out to increase gDNA yield for unengorged adults and nymphs. Sterile and DNA-
112 free equipment and tubes were used for each step. Extraction reagents blank (EXB) controls
113 were performed alongside tick extractions to assess background bacterial communities.
114

115 *2.3 16S amplification and library preparation for NGS sequencing*

116 Amplicons targeting a 250-320 bp product of the V1-2 hypervariable region of the 16S rRNA
117 (16S) gene were generated using the primer pair 27F-Y (Gofton et al., 2015b) and 338R
118 (Turner et al., 1999). Previous research identified that paralysis tick, *I. holocyclus*, has a
119 highly abundant bacterial species ('*Ca*. M. mitochondrii'), which masks the diversity of
120 bacteria in 16S metabarcoding studies (see Gofton et al. 2015a). Therefore, a blocking primer
121 developed by Gofton et al. (2015a) was used to inhibit the amplification of '*Ca*. M.
122 mitochondrii' in *I. holocyclus* ticks. Depending on tick life stage and level of engorgement, 3-
123 10 µM of the CMm blocking primer was added to the PCR reaction. Amplicon PCRs were
124 conducted in 25 µL reactions each containing 1 X Buffer (KAPA Biosystems, USA), 1.5 mM
125 $MgCl_2$, 0.4 µM of each forward and reverse primer with MiSeq adapters, 0.4 mg/mL BSA

126 (Fisher Biotech, Australia), 0.25 mM dNTPs (Fisher Biotech, Australia), 0.5 U KAPA Taq
127 (KAPA Biosystems, USA), and 2.0 µL of undiluted genomic DNA. Samples underwent the
128 following thermal cycling conditions; initial denaturation at 95°C for 5 mins, followed by 35
129 cycles of denaturation at 95°C for 30 s, annealing at 62°C (CMm blocking primer present) or
130 55°C (CMm blocking primer absent) for 30 s, and extension at 72°C for 45 s with a final
131 extension at 72°C for 5 mins. Libraries were then prepared using the Nextera XT DNA
132 library preparation kit in 25 µL reactions following manufacturer's recommendations.
133 Libraries were purified using Agencourt® AMPure® XP PCR purification beads (Beckman
134 Coulter Life Sciences, USA) and pooled in equimolar amounts. Final libraries were then
135 quantified using the Qubit® 2.0 Fluorometer (Thermo Fisher, Australia). Libraries were
136 sequenced on an Illumina MiSeq™ using v2 chemistry (2x250 paired end).
137
138 Extraction reagents blanks (n=12) and PCR no-template controls (NTC; n=7) were included
139 in all stages of the workflow. All pre- and post-PCR procedures were performed in physically
140 separate dedicated laboratories and sterile protocols were maintained through library
141 preparation in order to minimise amplicon contamination.
142
143 *2.4 Bioinformatics and statistical analysis*
144 Raw fastq files were downloaded from the Illumina BaseSpace Sequence Hub for analysis in
145 a custom pipeline using USEARCH (Edgar, 2010). Raw paired-end sequences were merged
146 in USEARCH v10, a minimum of 50 nucleotide (nt) overlap and maximum number of
147 mismatches increased to 15 nt due to long overlap of paired-end sequences. Only sequences
148 with perfect primer sequences were retained, primer sequences and distal bases were trimmed
149 using USEARCH v8.0. Sequences were quality filtered in USEARCH v10, allowing a <1%
150 expected error rate and singletons were discarded (Edgar and Flyvbjerg, 2015). Sequences

151 were then clustered into operational taxonomic units (OTUs) of 97% similarity using the
152 UPARSE algorithm (Edgar, 2013) in USEARCH v10. Taxonomy was assigned in QIIME2
153 v2018.4 using the q2-feature-classifier (Bokulich et al., 2018) with reference to a trained
154 Greengenes database (DeSantis et al., 2006) (release May 2013) using the primer pair 27F-Y/
155 338R. Taxonomic assignments were confirmed using NCBI MegaBLAST (Morgulis et al.,
156 2008) on a random subsample of OTUs and in the case of tick-associated microbes, GenBank
157 accession numbers and percentage identity of top hits were recorded for 'taxa of interest'.
158 The profiles from EXB controls and NTCs were first assessed to ensure quality of sampling
159 and absence of tick-associated bacteria. The following criteria were used to assess inclusion
160 of sequences: all OTUs that appeared exclusively in controls were removed; OTUs that had a
161 higher relative sequence abundance in controls compared to tick samples were removed; and
162 OTUs were removed that appeared in over half of controls (i.e. at least eight) that had a
163 taxonomic identity associated with environmental bacteria (e.g. members of the phyla
164 Acidobacteria and Cyanobacteria). In addition, potential cross-contamination during library
165 preparation or 'cross-talk' at the sequencing level was assessed by inspecting the presence of
166 expected tick-associated bacteria (e.g. members of the obligate intracellular bacterial families
167 Anaplasmataceae and Midichloriaceae) in controls. The profiles of EXB controls and NTCs
168 were then removed bioinformatically from associated samples to eliminate background
169 bacteria.
170
171 Data analysis and visualisation was carried out in RStudio (RStudio Team, 2015) using
172 packages metacoder (Poisot et al., 2017), phyloseq (McMurdie and Holmes, 2013) and vegan
173 (Oksanen et al., 2019). Alpha diversity of samples was measured using observed OTUs,
174 Chao1 index, Shannon index and Simpson index. Removal of samples with a low sequencing
175 depth (<1000 assigned OTUs after data filtering), did not significantly alter alpha diversity

176 measurements among tick species (data not shown). Rarefaction curves for samples were
177 calculated to assess sequencing depth based on observed number of OTUs. Principal
178 coordinate analysis (PCoA) on weighted unifrac dissimilarity measurements was used to
179 assess the differences in microbial composition between tick species. Investigation into 'taxa
180 of interest' warranted a more rigorous assessment of sequence number than was required for
181 diversity measures in order to avoid any potential cross-contamination and machine cross-
182 talk (Dong et al., 2017; Wang et al., 2017), as such only samples with >100 sequences were
183 considered positive. Taxonomic assignment to 'taxa of interest' was assessed based on NCBI
184 MegaBLAST top hit of named organism. Where precent identity was ≤97%, the terminology
185 of nearest named Genus-like was employed. Due to sample collection biases in the present
186 study, prevalence data of these microbes were not considered statistically relevant.
187 Nucleotide sequences from 'taxa of interest' were aligned by MUSCLE (Edgar, 2004) using
188 default parameters and aligned sequences were then imported into MEGA7 (Kumar et al.,
189 2016) with the most appropriate nucleotide substitution model chosen based on the lowest
190 Bayesian Information Criterion (BIC) score. Evolutionary histories were inferred using the
191 Neighbour-Joining method based on the Tamura 3-parameter model (Tamura, 1992).
192 Bootstrap analysis was conducted using 10,000 replicates to assess the reliability of inferred
193 tree topologies.
194

195 **3. Results**

196

197 *3.1 Tick-host associations*

198 Ticks were collected from 221 wildlife hosts including 24 native and three introduced species
199 (Fig. 1, Supplementary File S1). Hosts recorded in the present study included; agile
200 antechinus (*Antechinus agilis*), brown antechinus (*Antechinus stuartii*), spotted-tail quoll

201 (*Dasyurus maculatus*), water rat (*Hydromys chrysogaster*), quenda (*Isoodon fusciventer*),
202 northern brown bandicoot (*Isoodon macrourus*), western grey kangaroo (*Macropus*
203 *fuliginosus*), eastern grey kangaroo (*Macropus giganteus*), red-necked wallaby
204 (*Notamacropus rufogriseus*), red kangaroo (*Osphranter rufus*), platypus (*Ornithorhynchus*
205 *anatinus*), eastern barred bandicoot (*Perameles gunnii*), long-nosed bandicoot (*Perameles*
206 *nasuta*), sugar glider (*Petaurus breviceps*), eastern ring-tailed possum (*Pseudocheirus*
207 *peregrinus*), black fruit-bat (*Pteropus alecto*), fruit-bat sp. (*Pteropus* sp.), bush rat (*Rattus*
208 *fuscipes*), black rat (*Rattus rattus*), Tasmanian devil (*Sarcophilus harrisii*), wild pig (*Sus*
209 *scrofa*), short-beaked echidna (*Tachyglossus aculeatus*), rufous-bellied pademelon
210 (*Thylogale billardierii*), short-eared brush-tailed possum (*Trichosurus caninus*), common
211 brush-tailed possum (*Trichosurus vulpecula*), wombat (*Vombatus ursinus*), red fox (*Vulpes*
212 *vulpes*), and swamp wallaby (*Wallabia bicolor*). Female ticks were the dominant life stage
213 recorded (*n*=547) followed by nymphs (*n*=319) and males (*n*=153). Ticks were received from
214 animals in the Northern Territory (NT) (*n*=16), Queensland (QLD) (*n*=249), New South
215 Wales (NSW) (*n*=316), Tasmania (TAS) (*n*=136), Victoria (VIC) (*n*=44) and Western
216 Australia (WA) (*n*=258). Together, *Bothriocroton concolor* (*n*=123), *Ixodes australiensis*
217 (*n*=210), *I. holocyclus* (*n*=173) and *Ixodes tasmani* (*n*=184) accounted for over two-thirds of
218 all ticks submitted.
219

220 *3.2 16S rRNA bacterial profiling*

221 A sub-sample of 238 ticks and 19 controls underwent 16S NGS profiling. A total of 23.9
222 million raw paired-end sequences were generated on the Illumina MiSeq. 17.9 million
223 sequences were retained after merging, and subsequent quality filtering yielded 14.8 million
224 sequences for clustering and taxonomic assignment. A total of 4,864 OTUs (average length
225 299 bases) were retained after background profiles were removed. After removal of

background sequences, a total of 14,328,059 bacterial sequences were assigned to tick samples. Despite a high number of OTUs, only 1,535 OTUs had greater than 100 total sequences from tick samples. Tick samples had an average of 60,201 assigned sequences (see Supplementary File S2). *Amblyomma triguttatum* had the highest median alpha diversity as measured by the observed number of OTUs and the chao1 index, whereas *Ixodes antechini* had the highest median alpha diversity as measured by the Shannon and Simpson indexes (Fig. 2). Rarefaction analysis of the sequence depth shows that the observed number of OTUs plateaued at 50,000 sequences (Fig. 3). After the removal of sequences from controls, eight phyla were retained. An ordination plot of OTUs (Fig. 4) show that bacteria belonging to the Proteobacteria phylum were the most abundant and diverse taxa classified, followed by Firmicutes and Actinobacteria. Bacterial families identified in tick species, represented as relative number of sequences in Fig. 5, show 26 dominant taxa. While bacterial composition varied between tick species, sequences from the members of Coxiellaceae, Francisellaceae, and Rickettsiaceae families (Phylum: Proteobacteria) were the most abundant. Although sample sizes varied among tick species, beta diversity analysis incorporating abundance (sequence number) and taxonomic relatedness, showed evidence of different bacterial communities among tick species (Supplementary File S3).

3.3 Presence of 'taxa of interest'

In total, 37 OTUs were identified as 'taxa of interest' in the present study. Their taxonomic identity and abundance (number of sequences) in each sample is available in Supplementary File S4 (see Supplementary File S5 for fasta file of sequences). Phylogenetic analysis of the 'taxa of interest' is represented in Fig. 6, showing tick species and Australian states and territory, where each OTU was identified.

A proposed novel *Anaplasma* sp. (OTU230, MK814412, 96.3% identity) was identified in a single *B. concolor* (1/20) collected from (ex) echidna from NSW. A second *Anaplasma bovis*-like OTU (OTU312, JN862824, 97.3% identity) was identified in *Haemaphysalis bancrofti* (3/10) ex red-necked wallaby and long-nosed bandicoot and *Haemaphysalis humerosa* (n = 1/13) tick ex long-nosed bandicoot, all from NSW.

OTU5509 was assigned to the genus *Bartonella* and had a top BLAST hit of *Bartonella bacilliformis* (LN624026, 92.1% identity). OTU5509 was identified in two samples, however, was present in an extremely low number of sequences (2) in each case. Both samples were *I. fecialis* collected from two different quenda in WA. The low number of sequences means that in the case of statistical analysis, this OTU would have been filtered out. While there was no sufficient match to this sequence, it is noted that in many instances native Australian *Bartonella* species are lacking sequence information for this region of the 16S gene (V1-2).

Two OTUs (OTU14, CP025785, 100% identity; and OTU4629, CP025785, 100% identity) were identified as *'Ca.* B. tachyglossi*'*. Sequences were identified in *Bothriocroton concolor* (8/20) ex echidnas from QLD & NSW and *H. humerosa* (1/13) ex northern brown bandicoot from QLD.

A *Coxiella*-like organism (OTU12, CP032542, 95.5% identity) was identified exclusively in *B. concolor* (19/20) ex echidna from NSW and QLD. This was the second most abundant OTU identified in *B. concolor*, accounting for ~20% of the overall sequences.
A *Coxiella* sp. (OTU15, KC170757, 100% identity) was identified in *Haemaphysalis longicornis* (3/4) ex red fox from NSW and, OTU represented ~61.4% of the overall assigned

bacterial sequences.

A novel *Ehrlichia* sp. (OTU33, AY309970, 96.3% identity) was identified in *I. fecialis* (1/13) ex quenda from WA. Other *Ehrlichia* OTUs (OTU16 and 1632) represented sequences from '*Ca.* Ehrlichia ornithorhynchi' in *Ixodes ornithorhynchi* (6/10) ex platypus QLD and these sequences accounted for the majority of sequences (~50.2%) from *I. ornithorhynchi*.

A *Francisella* endosymbiont (OTU5, AF001077, 98.0% identity) was identified in *H. humerosa* (11/13) ticks ex northern brown bandicoots from NT and QLD, and *Haemaphysalis bremneri* (2/4) ex possum (species unknown) in QLD. OTU5 was the most abundance sequence in *H. bremneri* and *H. humerosa*, representing 27.3% and 52.4% of the assigned sequences, respectively. A second *Francisella* endosymbiont (OTU10, AB001522, 99% identity) was identified in *H. bancrofti* (8/11) ex red-necked wallabies from QLD and NSW, and ex a long-nosed bandicoot in NSW; and in a single *H. humerosa* (1/13) ex red-necked wallaby from NSW. OTU10 was the most abundance sequences in *H. bancrofti* representing 27.0% of the assigned sequences. OTU40 (AF001077, 97.6% identity) was also identified as a *Francisella*-like endosymbiont from *A. triguttatum* (6/6) ex red kangaroo from WA. It was highly abundant in these ticks, accounting for 76.9% of the overall sequences.

Midichloria (OTU6, FM992372, 100% identity) was identified in *I. holocyclus* (20/36) ex long-nosed bandicoots from NSW and QLD, *I. fecialis* (1/13) ex red-necked wallaby NSW, *H. bancrofti* (1/11) ex long-nosed bandicoot NSW, and *H. humerosa* (1/13) ex long-nosed bandicoot NSW. *Midichloria* (OTU7, FM992373, 100% identity) was identified in *I. holocyclus* (15/36) ex long-nosed bandicoots from NSW and QLD, *I. fecialis* (1/13) ex red-necked wallaby NSW and *H. bancrofti* (1/11) ex long-nosed bandicoot NSW. Overall a total

301 of 15 ticks had both OTU6 and OTU 7, which included *I. holocyclus* (13/36) ex long-nosed
302 bandicoots from NSW and QLD, *I. fecialis* (1/13) ex red-necked wallaby NSW and *H.*
303 *bancrofti* (1/11) ex long-nosed bandicoot NSW. While the CMm blocking primer was
304 incorporated into the PCRs conducted on *I. holocyclus, Midichloria* sequence were still
305 observed in 2/9 females, 3/6 males and 16/21 nymphs. *Midichloria* was the most abundant
306 sequence from *I. holocyclus* accounting for 36.1% of assigned sequences.
307
308 Three *Neoehrlichia* OTUs were identified. '*Ca*. Neoehrlichia arcana' (OTU25, KT203914,
309 99.3% identity) was identified in eight ticks, *I. tasmani* (4/51), *I. holocyclus* (2/36), *H.*
310 *bancrofti* (1/11) and *H. humerosa* (1/13), ex long-nosed bandicoots NSW and QLD. '*Ca*.
311 Neoehrlichia australis' (OTU29, KT803957, 100% identity) was identified in 10 ticks, *I.*
312 *holocyclus* (8/36) and *I. tasmani* (2/51), ex long-nosed bandicoots NSW and QLD. Three
313 ticks (*I. tasmani* and *I. holocyclu*s) were co-infected with both '*Ca*. N arcana' and '*Ca*. N.
314 australis'. A novel *Neoehrlichia* species (OTU31, MG670107, 97.3% identity) was identified
315 in *I. fecialis* (2/13) and *I. australiensis* (2/26) ex quenda from WA, and *I. antechini* (2/29) ex
316 brown antechinus from NSW.
317
318 Due to the conserved nature of the *Rickettsia* genus at the 16S gene, reported previously
319 (Roux and Raoult, 1995; Stothard and Fuerst, 1995), resolution to species level was not
320 possible using the sequences obtained in the present study. *Rickettsia* sp. (OTU2, KF318168,
321 99.7% identity) had a widespread distribution among samples and was detected in 61 ticks
322 *(B. concolor, H. bancrofti, H. humerosa, I. antechini, I. australiensis, I. holocyclus* and *I.*
323 *tasmani*) from three states and one territory (NSW, NT, QLD, WA). *Rickettsia* was the most
324 abundant sequence from *B. concolor* and *I. antechini* ticks accounting for 63.3% and 12.0%
325 of the assigned sequences, respectively

A *Rickettsiella*-like sp. (OTU1, LC388767, 92.7% identity) was identified in *I. australiensis* (22/26) and was the most abundant sequence accounting for 76.8% of assigned sequences. A *Rickettsiella*-like sp. (OTU3, EU430251, 95.1% identity) was identified in *I. tasmani* (11/51) ex Tasmanian devils and eastern barred bandicoot from TAS. A *Rickettsiella*-like sp. (OTU4, U97547, 98.4% identity) was identified in, *I. tasmani* (36/51) ex bandicoots, possum and a sugar glider from NSW, TAS and QLD; *I. australiensis* (1/26) ex western grey kangaroo from WA; and *I. holocyclus* (1/36) ex long nosed bandicoot NSW. OTU4 accounted for 30.2% of the overall sequences assigned for *I. tasmani.*

4. Discussion

Currently there is a scarcity of detailed information about the life cycles of Australian ticks, however it is generally assumed that, as with other hard tick species around the world, the majority will exhibit a three-host life cycle (Barker and Walker, 2014). Despite these limitations, the opportunistic sampling strategy used in this study provides an economical way to survey a wide range of tick and wildlife fauna across a range of geographical areas across Australia. The paucity of larvae from this data set is also a limitation of this type of sampling. Tick larval stages, which may indicate the presence of transovarially-transmitted organisms (Kwan et al., 2017), are difficult to see with the naked eye, and in situations where collection of ticks is not the main objective, they are easily overlooked (Lydecker et al., 2019). Furthermore, the opportunistic nature of the sampling precluded assessment of the infestation rates of the various hosts. In future, if TBPs of humans and the wildlife themselves are identified, further extensive surveys of tick-pathogen-wildlife ecologies will require more targeted and comprehensive approaches in order to gather sufficient relevant

351 epidemiological data.

352

353 As with previous studies (Swei and Kwan, 2016; Zolnik et al., 2016) alpha diversity
354 measures were highly variable both within and between tick species. This may be driven by a
355 variety of factors such as; starting material (i.e. size/life stage of tick), extraction methods,
356 library preparation, normalisation of DNA input concentrations for sequencing, batch
357 sequencing effects and bioinformatics (Greay et al. 2018). Within the present study,
358 sequencing depth was likely most impacted by sample input type and normalisation of DNA
359 concentrations, as all samples went through the same library preparation and bioinformatic
360 analysis. Overall, bacterial diversity of ticks started to plateau by 50,000 sequences however,
361 it is noted that in some cases this plateau was not achieved and deeper sequencing would be
362 required in order to confidently characterise the full suite of bacterial taxa present. This was
363 most evident in *A. triguttatum*, *B. auruginans*, *I. trichosuri* and *I. ornithorhynchi* and
364 therefore we recommend a minimum of 100,000 sequences per sample for future bacterial
365 16S amplicon studies in these species to be confident that the complete bacterial community
366 has been sampled. Studies that investigate the shift of the core microbiome (i.e. most
367 abundant bacteria taxa) relative to a given parameter may not require the same depth of
368 sequencing, as seen in previous studies investigating the effect of temperature (Thapa et al.,
369 2019) and life stage (Andreotti et al., 2011).

370

371 After bioinformatic analysis, including stringent quality filtering, a large number of OTUs
372 remained with relatively low number of reads. This has been noted in previous studies of the
373 tick microbiome (Budachetri et al., 2016; Zolnik et al., 2016) and in the broader field of high-
374 throughput microbiome studies (Pollock et al., 2018). Depending on bioinformatic analysis
375 and quality filtering, the number of OTUs and therefore diversity can vary greatly among

studies (Greay et al., 2018). In addition, current practice in microbiome studies is to normalise count data, however models show that this can oversimplify the data (McMurdie and Holmes, 2014).

Ordination analysis demonstrated that tick species was the strongest predictor of bacterial composition. Beta-diversity analysis showed that the common marsupial tick, *I. tasmani*, exhibited a variable bacterial composition. This diversity may be explained by the wide geographic distribution of this tick in Australia, its own high genetic diversity (Burnard and Shao, 2019), and its ability to parasitise many marsupial species. In the present study, bacterial profiling of *I. tasmani* included ticks from three states (NSW, QLD, TAS) and seven host species. With this in mind, we suggest that future studies should combine careful taxonomic status identity with data on microbial communities of *I. tasmani*. Although sample sizes were limited in 'host-specialist' ticks *B. auruginans* (wombats), *H. bremneri* (possums), *I. antechini* (antechinus) and *I. ornithorhynchi* (platypus), these specimens showed less diversity of bacterial communities between samples.

An important caveat on the relative diversity of bacterial communities in the present study was the use of a blocking primer inhibiting the amplification of '*Ca*. M. mitochondrii'. The inclusion of this blocking primer it vital to explore the full bacterial community of *I. holocyclus* (see Gofton et al., 2015), however it does impact the analysis and interpretation of the data. Due to the inhibition of this bacteria, there is an inherent bias in the bacterial community, and comparisons in alpha and beta-diversity measures must account for this. In the case of highly abundant organisms, the use of a blocking primer assay will likely not completely inhibit amplification, and thus a reduced level of the organisms may be still detected, as was the case in the present study. In this instance, the use of diversity measures

401 that rely on presence/absence data will not be affected by this granted there is still some level
402 of detection (i.e. number of species observed for alpha diversity and Jaccard index for beta-
403 diversity). The use of more advanced, and usually preferred, diversity indexes may be
404 impacted by the manipulation of bacterial composition, and care must be taken when
405 comparing results with other studies (Greay et al., 2018). Despite these limitations, the use of
406 blocking primers has shown to be vital in the context of investigating known tick-borne
407 pathogens and novel related taxa from tick samples. In the present study we have
408 characterised the bacterial communities of ten tick species for the first time. This information
409 provides the first step of focusing future research and where this established technique should
410 be applied.
411
412 Members of the Proteobacteria represented the most diverse and abundant (as determine by
413 number of sequences) in Australian hard ticks analysed in the present study. This finding is
414 consistent with previous microbiome studies from tick species, such as *Ixodes scapularis*
415 (Sperling et al., 2017; Thapa et al., 2019), *Ixodes persulcatus* (Zhang et al., 2014; Kurilshikov
416 et al., 2015) and *Amblyomma americanum* (Fryxell and DeBruyn, 2016). Additionally the
417 identification of novel bacterial taxa in native Australian ticks species is consistent with
418 recent findings (Gofton et al., 2015b; Panetta et al., 2017).
419
420 Currently in Australia, the ecology, epidemiology, and incidence of human TBDs remains
421 largely a matter of conjecture, having received little scientific study compared with many
422 other parts of the world (Graves and Stenos, 2017). Despite significant national interest,
423 including a federal government senate inquiry (Radcliffe et al., 2016), the prevailing
424 scientific opinion concludes that Lyme borreliosis (caused by *B. burgdorferi* sensu lato), for
425 example, is absent from Australia (Chalada et al., 2016; Irwin et al., 2017). In the unique

426 Australian environment, long isolated in geological terms, it is likely that unidentified tick-
427 host life cycles have evolved, given the endemic wildlife (including tick fauna) present
428 (Long, 2017; Beati and Klompen, 2019). It is possible that these cycles may contribute to
429 zoonotic illness when humans encroach these sylvatic ecologies and become exposed to
430 native ticks. Indeed, it is well documented that in endemic areas in Europe and North
431 America, the causative agent of Lyme borreliosis is readily identified in wildlife (such as
432 white-tailed deer, *Odocoileus virginianus*, and white-footed mice, *Peromyscus leucopus*) as
433 well as their natural tick species (Bosler et al., 1984). The present study therefore is one of
434 the first to explore the concept that Australian wildlife ticks should be a promising source to
435 identify exotic and novel TBPs, and we focussed our search towards taxa known to be
436 associated with TBDs overseas ('taxa of interest'), namely *Anaplasma*, *Bartonella*, *Borrelia*,
437 *Coxiella*, *Ehrlichia*, *Francisella*, *Midichloria*, *Neoehrlichia*, *Rickettsia* and *Rickettsiella*.
438

439 A new genetic variant of *Anaplasma bovis* was recently described from questing *A.*
440 *triguttatum* ticks in WA and the nearby Barrow Island (Gofton et al., 2017). In the present
441 study we report a range expansion of a genetically similar *A. bovis* along the east coast of
442 Australia from two widespread *Haemaphysalis* species. As suggested by Gofton et al.,
443 (2017), this finding further supports the hypothesis that it is likely that endemic *A. bovis*
444 genotypes exist in sylvatic cycles within native Australian ticks and wildlife fauna. Further
445 research on the phylogenetic position of these *A. bovis* sequences is needed to understand
446 their likely evolutionary history and relatedness to *A. bovis* genotype Y11 identified in
447 Western Australia. While three other species of *Anaplasma* (*A. marginale*, *A. centrale* and *A.*
448 *platys*) have been introduced to Australia (Rogers and Shiels, 1979; Callow, 1984; Angus,
449 1996), the present study did not identify any of these species in ticks from wildlife. This is
450 likely due to the absence of the cattle tick (*R.* (*B.*) *australis*) and the brown dog tick (*R.*

451 *sanguineus*) specimens from wildlife hosts in the present study. A species of *Anaplasma*
452 identified exclusively in *B. concolor* from echidnas sheds more light on the diverse range of
453 microbes that have been described from this specialist tick (Loh, 2018).
454
455 Although the presence of a *Bartonella* sp. was identified by only four sequences, its absence
456 in control samples means that this likely represents a true finding in female *I. fecialis*, which
457 were parasitising different individuals of the same host (quenda) in south-west WA. Recent
458 studies have shown that Australian marsupials and native rodents harbour a range of distinct
459 *Bartonella* species; '*Ca*. Bartonella antechini' has been identified in ticks (*I. antechini*) and
460 fleas (*Acanthopsylla jordani*) from mardo (*Antechinus flavipes*) in south-west WA
461 (Kaewmongkol et al., 2011c), '*Ca*. Bartonella woyliei' in woylie ticks (*I. australiensis*) and
462 fleas (*Pygiopsylla hilli*) in south-west WA, and '*Ca*. Bartonella bandicootii' in fleas
463 (*Pygiopsylla tunneyi*) from western barred bandicoots (*Perameles bougainville*) on Bernier
464 and Dorre Island (Kaewmongkol et al., 2011a). Reports of *Bartonella* spp. occurring outside
465 south-west WA include *Bartonella australis* ex eastern grey kangaroos (*Macropus giganteus)*
466 (Fournier et al., 2007). Molecular detection of *Bartonella* DNA from Australian ticks has also
467 been reported in ticks (*I. tasmani*) parasitising koalas (*Phascolarctos cinereus*) from Philip
468 Island, VIC (Vilcins et al., 2009b). Additional studies on *Bartonella* in Australia wildlife
469 include those by Gundi et al. (2009), Kaewmongkol et al. (2011a) and Dybing et al. (2016).
470 Due to the limited size and region of the 16S amplicon generated in the present study, there
471 were insufficient relevant reference sequences available from other Australian *Bartonella*
472 species for comparison. A study by Kaewmongkol et al. (2011b) into flea-derived *Bartonella*
473 from native and introduced Australian species suggests co-evolution of marsupial hosts, their
474 fleas and the *Bartonella* species.
475

476 ‘*Ca*. Borrelia tachyglossi’ was identified in 8/20 *B. concolor* ticks from echidnas in QLD and
477 NSW, an anticipated finding given previous research into this organism (Loh et al., 2016).
478 Importantly however, the present study provides the first evidence of ‘*Ca*. B. tachyglossi’
479 sequences from a female *H. humerosa* tick parasitising a northern brown bandicoot from
480 QLD. In addition, no *Bothriocroton* ticks were present in the extraction batch of the positive
481 *H. humerosa* sample, and there was no evidence of *Borrelia* sequences from any controls.
482 Despite the wide geographical range of *H. humerosa* ticks, the restricted finding of ‘*Ca*. B.
483 tachyglossi’ from QLD supports Loh et al. (2016), suggesting a restricted geographical
484 distribution along the east coast of Australia.
485
486 In tick microbiome studies overseas, *Coxiella* spp. are commonly identified (Khoo et al.,
487 2016; Machado-Ferreira et al., 2016). Additionally, recent studies on Australian ticks have
488 also identified *Coxiella* spp. in *A. triguttatum* (Cooper et al., 2013; Gofton et al., 2015b), *B.*
489 *auruginans* (Vilcins et al., 2009c), *I. holocyclus* (Cooper et al., 2013) and *R. sanguineus*
490 (Oskam et al., 2017). Interestingly the present study did not identify the widespread presence
491 of *Coxiella* spp. with only two unique OTUs identified from *B. concolor* and *H. longicornis*,
492 both of which appear to be host specific to those tick species, and the causative agent of Q-
493 Fever (*C. burnetii*) was not identified from ticks in the present study.
494
495 *Ehrlichia* sequences (‘*Ca*. E. ornithorhynchi’) from platypus ticks, *I. ornithorhynchi*, were
496 recently described (Gofton et al., 2018). In the present study, ‘*Ca*. E. ornithorhynchi’ was
497 exclusively observed within *I. ornithorhynchi,* suggesting this genetically distinct *Ehrlichia*
498 species has a unique and host-specific relationship with the platypus. During this study, a
499 potentially novel species of *Ehrlichia* was identified in an *I. fecialis* female tick from a
500 quenda in WA. BLAST results show it is a close relative to an *Ehrlichia* sp. detected in

501 *Haemaphysalis* ticks from Japan (Inokuma et al., 2004). No sequences from the recently
502 described '*Ca*. E. occidentalis' (Gofton et al., 2017) were identified in the present study,
503 however it is noted *A. triguttatum* were only represented by six samples.
504
505 *Francisella*-like endosymbionts have been widely reported in ticks overseas, such as
506 *Dermacentor* spp. (Scoles, 2005), *Dermacentor occidentalis* (Gurfield et al., 2017),
507 *Haemaphysalis longicornis* (Wang et al., 2018) and *Hyalomma rufipes* (Szigeti et al., 2014),
508 and *Francisella* sequences have previously been identified in *A. fimbriatum* ticks from
509 reptiles in the Northern Territory of Australia (Vilcins et al., 2009a). In the present study, a
510 *Francisella*-like endosymbiont was identified in 100% of *A. triguttatum* ticks, and in a high
511 proportion. The high prevalence and relative abundance of the *Francisella*-like organisms in
512 *A. triguttatum* may be of medical relevance with respect to this common human-biting tick,
513 particularly given the recent report of *Francisella* bacteraemia in WA (Aravena-Román et al.,
514 2015). Importantly, as noted previously for *I. holocyclus* ticks, organisms in lower abundance
515 may be masked by abundant endosymbionts, unless samples are sequenced deeply and/or a
516 blocking primer is used (Gofton et al., 2015a). Therefore, future bacterial profiling studies of
517 *A. triguttatum* should take these factors into consideration.
518
519 While '*Ca*. M. mitochondrii' has been documented in *I. holocyclus,* the present study
520 provides the first report of '*Ca*. M. mitochondrii' within additional species of native
521 Australian ticks (*I. fecialis, H. bancrofti* and *H. humerosa*). Despite this expansion among
522 tick species, identification of '*Ca*. M. mitochondrii' remains confined to NSW and QLD as
523 previously reported (Gofton et al., 2015a). '*Ca*. M. mitochondrii' has been detected overseas
524 in laboratory models (Cafiso et al., 2019), wildlife (Serra et al., 2018), and humans
525 (Mariconti et al., 2012). Despite the use of a CMm blocking primer by Gofton et al. (2015a)

526 in the library preparation of *I. holocyclus*, the bacteria was still identified in the majority of
527 ticks (21/36); however its abundance was greatly reduced to an average of 26.1% of reads per
528 sample in comparison to previous research demonstrating a relative abundance of 98.2%
529 (Gofton et al., 2015a).
530
531 A recent addition to the Anaplasmataceae family, '*Ca*. Neoehrlichia mikurensis' was first
532 isolated from wild rats and their ticks (*Ixodes ovatus*) in Japan (Kawahara et al., 2004). In
533 Europe, the hedgehog (*Erinaceus* spp.), a common peri-urban dweller, has been shown to
534 play an important role in the life cycle of '*Ca*. N. mikurensis' (Földvári et al., 2014; Jahfari et
535 al., 2017). A high proportion of bandicoot ticks were infected with *Neoehrlichia* spp. in this
536 study, suggesting that these marsupials may play an important role in the life cycle of this
537 bacterium on the Australian continent. Bandicoots also frequently inhabit gardens and have
538 relatively close contact with humans (Carthey and Banks, 2012). Closer study of these
539 marsupials may provide vital information about the life cycle of these microbes and the
540 potential risk for human infection.
541
542 *Rickettsia* spp. are among the only currently recognised TBPs affecting people in Australia,
543 however, as previously outlined, the conserved nature of the 16S gene in *Rickettsia* precludes
544 rigorous species delimitation from the relatively short sequences generated in this dataset. In
545 addition to the known human pathogens (*R. australis* and *R. honei*) (Graves and Stenos,
546 2017), the presence of *Rickettsia* has been demonstrated from a variety of Australian tick
547 species. *Rickettsia gravesii* has been described from *A. triguttatum* (Li et al., 2010; Abdad et
548 al., 2017) and *Rickettsia* sequences have been previously identified in *I. tasmani* ex
549 Tasmanian devils (Vilcins et al., 2009c) and *Amblyomma fimbriatum* from reptiles (Vilcins et
550 al., 2009a).

551

552 The diversity and significance of the *Rickettsiella* genus remains largely unknown globally.
553 Phylogenetic analysis of sequence data places the genus within the Coxiellaceae family
554 (Fournier and Raoult, 2005; Leclerque and Kleespies, 2012). While the presence of these
555 organisms has been well documented in tick species around the world and in some cases, they
556 have been identified as pathogenic to arthropods (Kurtti et al., 2002; Leclerque et al., 2012),
557 disease causation within the vertebrate hosts remains unknown at the present time. A study
558 by Vilcins et al. (2009c) identified *Rickettsiella* in *I. tasmani* ex koalas from Phillip Island,
559 just off the coast of south eastern Australia. A genetically similar sequence was identified in
560 the present study (OTU3) from *I. tasmani* exclusively in Tasmania.
561

562 While this is the first study to characterise the bacterial communities within native Australian
563 wildlife ticks, it is apparent that much research is still required in order to better understand
564 the tick-associated microbial life cycles and their ecologies, let alone elucidating their
565 potential for transmission and pathogenicity in vertebrate hosts, including humans.
566 Furthermore, whilst cost-effective, ticks were collected opportunistically resulting in an
567 inherent geographical bias, confining sampling largely to urban areas along the east coast of
568 the Australian continent. Nevertheless, this area is also where most humans receive tick bites,
569 although reliable data about this is also lacking. In addition, the present study suggests that
570 'taxa of interest' are largely restricted to a combination of geographical location and tick
571 species. For example, '*Ca*. Midichloria' was identified in *H. humerosa* and *I. fecialis* from
572 NSW only and was absent in samples from NT for *H. humerosa* and WA and TAS for *I.*
573 *fecialis.*
574

575 The analysis of ticks removed from wildlife hosts comes with the inherent complication of a

576 blood meal. The host blood meal has been recently shown to influence both the tick
577 microbiome composition and the presence of pathogens (Landesman et al., 2019; Swei and
578 Kwan, 2017). Further targeted studies are needed to assess the source of microbes with
579 respect to a tick or host origin (Irwin et al., 2018). In addition, limitations in the current
580 sequencing technologies mean that high-throughput methods favour short amplicons which
581 may not be able to accurately discriminate bacterial species, as is the case with *Rickettsia* in
582 particular (Gihring et al., 2012). Furthermore, important caveats to consider in metabarcoding
583 microbial diversity analysis include PCR efficiency (including primers used and length of
584 target amplicon), variations in 16S gene copy number (Ahn et al., 2012), sequencing depth,
585 machine cross-talk, bioinformatic analysis and taxonomic assignment. While there have been
586 some recent attempts to correct these biases (Kembel et al., 2012; Rosselli et al., 2016), their
587 application to uncharacterised metagenomic samples remains limited. While 16S
588 metabarcoding continues to be vital in bacterial biodiversity discovery (Carpi et al., 2011;
589 Tessler et al., 2017), future molecular studies incorporating metagenomics and
590 metatranscriptomics, to detect actively expressed genes (Cabezas-Cruz et al., 2018; Greay et
591 al., 2018), will be useful in characterising the full suite of micro-organisms present in
592 Australian ticks. A multi-disciplinary approach incorporating cell culture, in vitro tick studies
593 and morphological techniques (e.g. fluorescence microscopy) will also be needed to assess
594 the potential for transmissibility and pathogenicity of these novel tick-associated organisms.
595

596 **5. Conclusions**

597

598 With over 75% of emerging human infectious pathogens originating from wildlife (King,
599 2014), surveillance methods that target these species are important for investigations of
600 emerging and exotic infectious disease. Research into the interactions of wildlife hosts, ticks

601 and pathogens in Europe and North America continues to highlight the complexity of these
602 dynamic systems (Ostfeld et al., 2018; Tomassone et al., 2018). Results from the present
603 study build on recent research into Australian tick-associated microbes, further highlighting
604 the diversity of organisms present that appear related, yet distinct from their overseas
605 counterparts. With the evolutionary history of Australia's unique tick species (Beati and
606 Klompen, 2019) and wildlife fauna, it is likely that reports of their tick-associated microbes
607 (potential pathogens) will continue to reveal taxonomic differences from those described in
608 the northern hemisphere. As such, future research on emerging tick-borne zoonoses should
609 include methods that are able to detect novel micro-organisms in humans and reservoirs and
610 include a variety of sample types such as blood, tissue (e.g. skin, spleen etc.) and tick from
611 both the host and environment (questing). As anthropogenic changes to the environment
612 continue to grow in Australia, a greater emphasis on wildlife disease surveillance is critical to
613 ensure the early detection of potential infectious diseases affecting humans, livestock,
614 companion animals and wildlife (Woods et al., 2019). Advances in pathogen detection and
615 characterisation are greatly enhanced by collaboration; the authors advocate for continued
616 multidisciplinary efforts between health professionals, researchers, land managers and local
617 communities.
618

619 **Supporting Information**

620

621 **Data availability**

622 Associated metadata output from bioinformatic analysis and subsequent data visualisation is
623 available on FigShare repository https://doi.org/10.6084/m9.figshare.c.4608803.v1. Next-
624 generation sequencing data can be accessed from NCBI Sequence Read Archive under
625 BioProject: PRJNA559059 (BioSample accession numbers: SAMN12512474 –

626 SAMN12512711).

627

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639

640 **Compliance with ethical standards**

641 This study was conducted under the compliance of the Australian Code for the Responsibility
642 Conduct of Research (2007) and Australian Code for the Care and Use of Animals for
643 Scientific Purposes, 2013. Tick collection was carried out opportunistically with the approval
644 from the Murdoch University Animal Ethics Committee.

645

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647

648 **Legends to Figures**

649 **Fig. 1.** Chord diagram showing tick-wildlife associations recorded in the present study. The
650 wildlife hosts recorded are represented on the lower half of the plot, and tick species across
651 the top. Thickness of bar relative to number of ticks. Host records that could not be assigned
652 to species level are not represented in this plot. A complete list of records is available in
653 Supplementary File S1. Introduced wildlife species are denotes with an asterisk. Silhouette
654 wildlife images sourced from phylopic.org.

655 **Fig. 2.** Alpha diversity of bacterial communities in ticks parasitising Australian wildlife
656 measured as; observed number of operational taxonomic units (OTUs), Chao1 index,
657 Shannon index and Simpson index. Tick species abbreviated to; *A. triguttatum* (*A. tri*), *B.*
658 *aurugians* (*B. aur*), *B. concolor* (*B. con*), *H. bancrofti* (*H. ban*), *H. bremneri* (*H. bremneri*),
659 *H. humerosa* (*H. hum*), *H. longicornis* (*H. lon*), *I. antechini* (*I. ant*), *I. australiensis* (*I. aus*), *I.*
660 *fecialis* (*I. fec*), *I. holocyclus* (*I. hol*), *I. myrmecobii* (*I. myr*), *I. ornithorhynchi* (*I. orn*), *I.*
661 *tasmani* (*I. tas*), and *I. trichosuri* (*I. tri*).

662 **Fig 3.** Rarefaction plot of 16S rRNA bacterial sequences clustered into 97% operational
663 taxonomic units (OTUs) from ticks parasitising Australian wildlife.

664 **Fig 4.** Non-metric multidimensional scaling (NMDS) plot of operational taxonomic units
665 (OTUs) based on Bray-Curtis dissimilarity matrix. Taxa filtered to only display OTUs that
666 were present at least twice in >10% of tick samples.

667 **Fig 5.** Stacked barplot of bacterial composition (shown at family level) from Australian ticks
668 represented as percentage of assigned sequences. Taxa at the family level that represented
669 <10% of the relative sequences within each tick species were grouped as "Low abundant".
670 Sequences not able to be accurately assigned to family taxa are displayed as "Unclassified".

671 **Fig 6.** Neighbour-joining phylogenetic tree displaying taxa of interest prevalence and

distribution among tick samples. Geographic data relating to tick collection is represented by state and territory; New South Wales (NSW), Northern Territory (NT), Queensland (QLD), Tasmania (TAS) and Western Australia (WA). Evolutionary histories were inferred based on the Tamura 3-parameter model with bootstrap analysis (10,000 replicates) (bootstrap values >60 are displayed). Tick samples were considered positive for taxa of interest if >100 sequences present. Information on number of sequences, taxonomic identity (as inferred from NCBI MegaBLAST analysis with nucleotide database), and sequences can be found in supplementary information (Supplementary File S4 & S5).

Supplementary Files

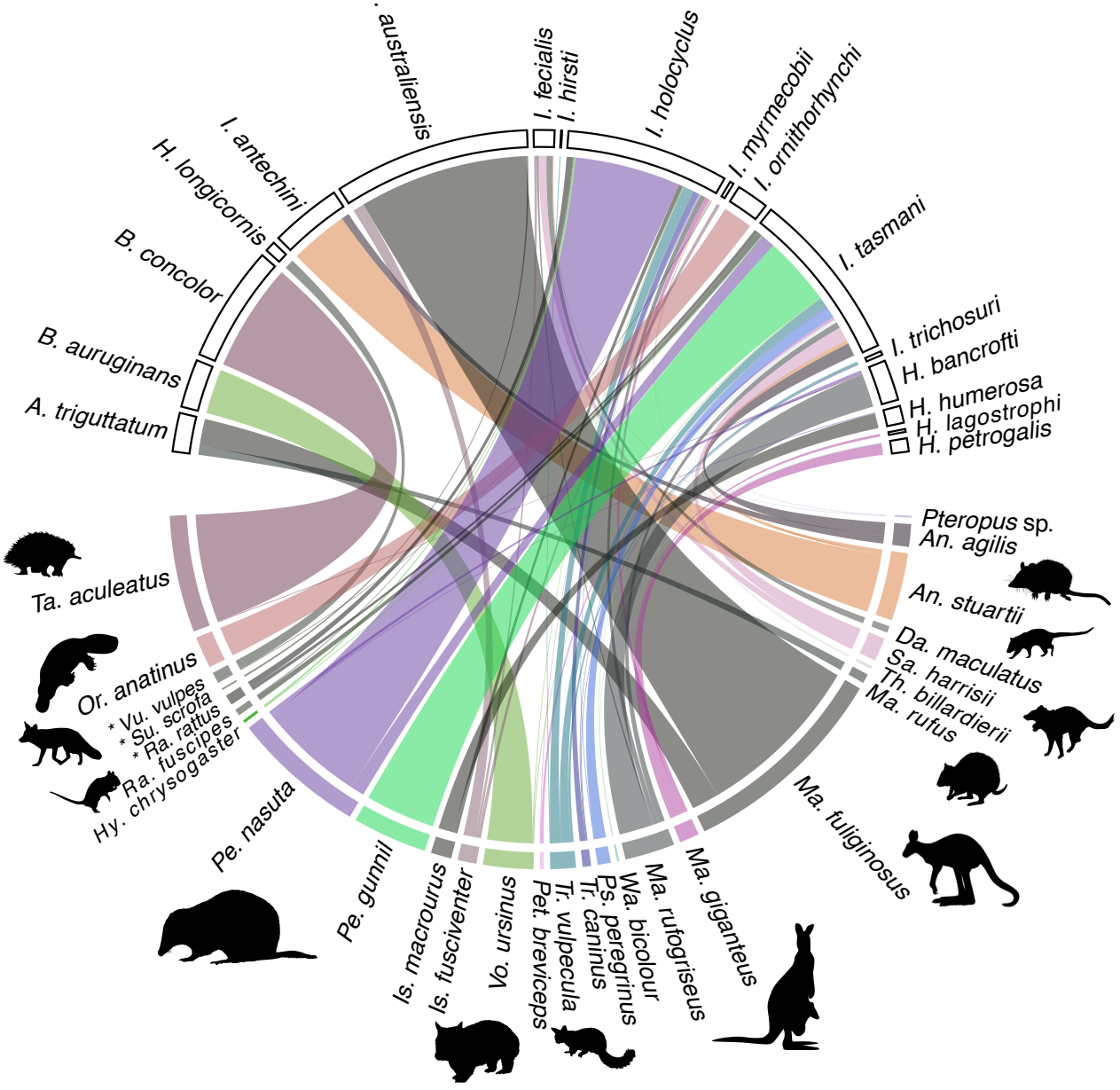
Supplementary File S1. Sample metadata of tick-wildlife records identified from the present study.

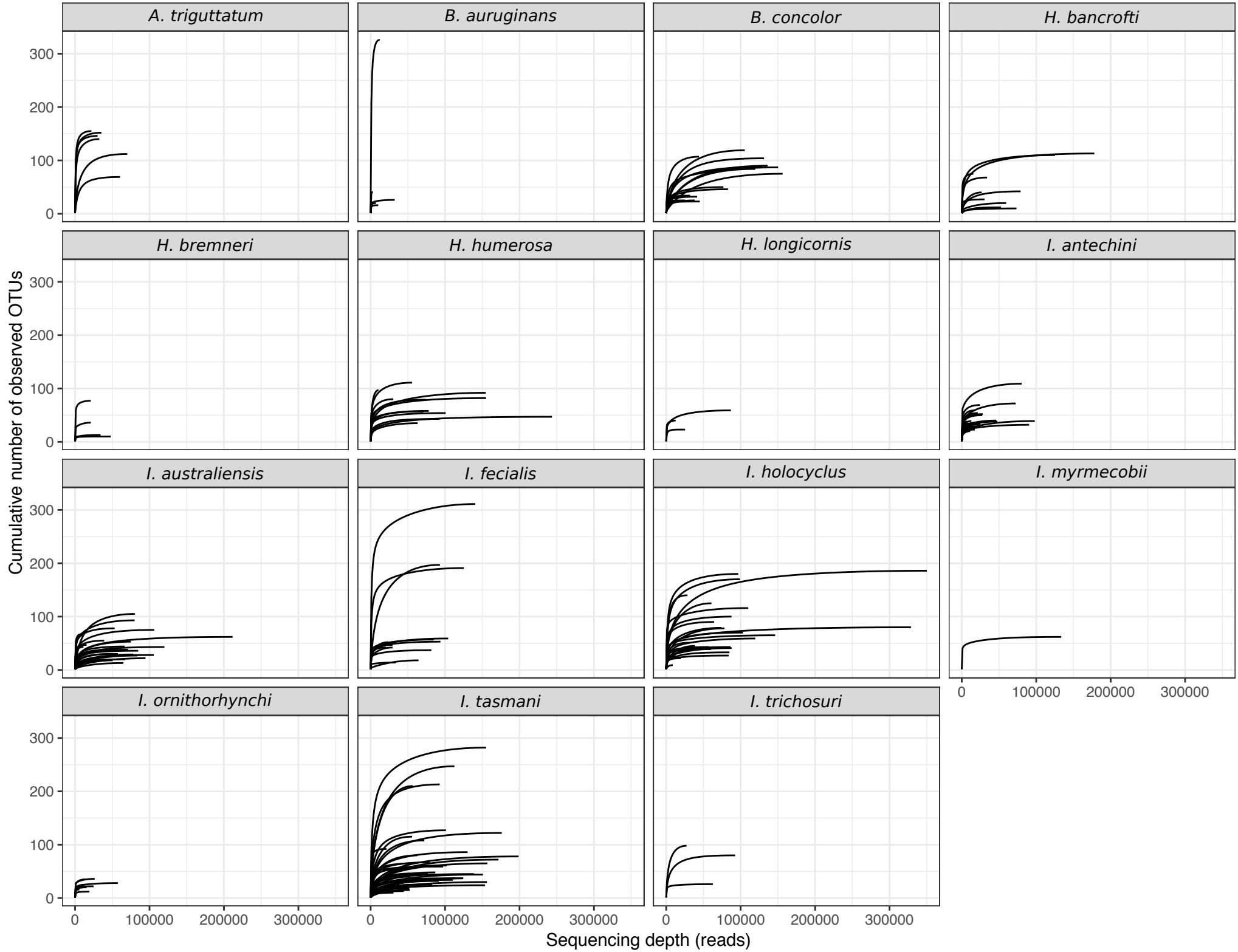
Supplementary File S2. Sequence information from 16S rRNA bacterial profiling of ticks on the Illumina MiSeq platform after bioinformatic analysis; (a) number sequences assigned to 97% operational taxonomic units (OTUs) and (b) number of sequences per sample.

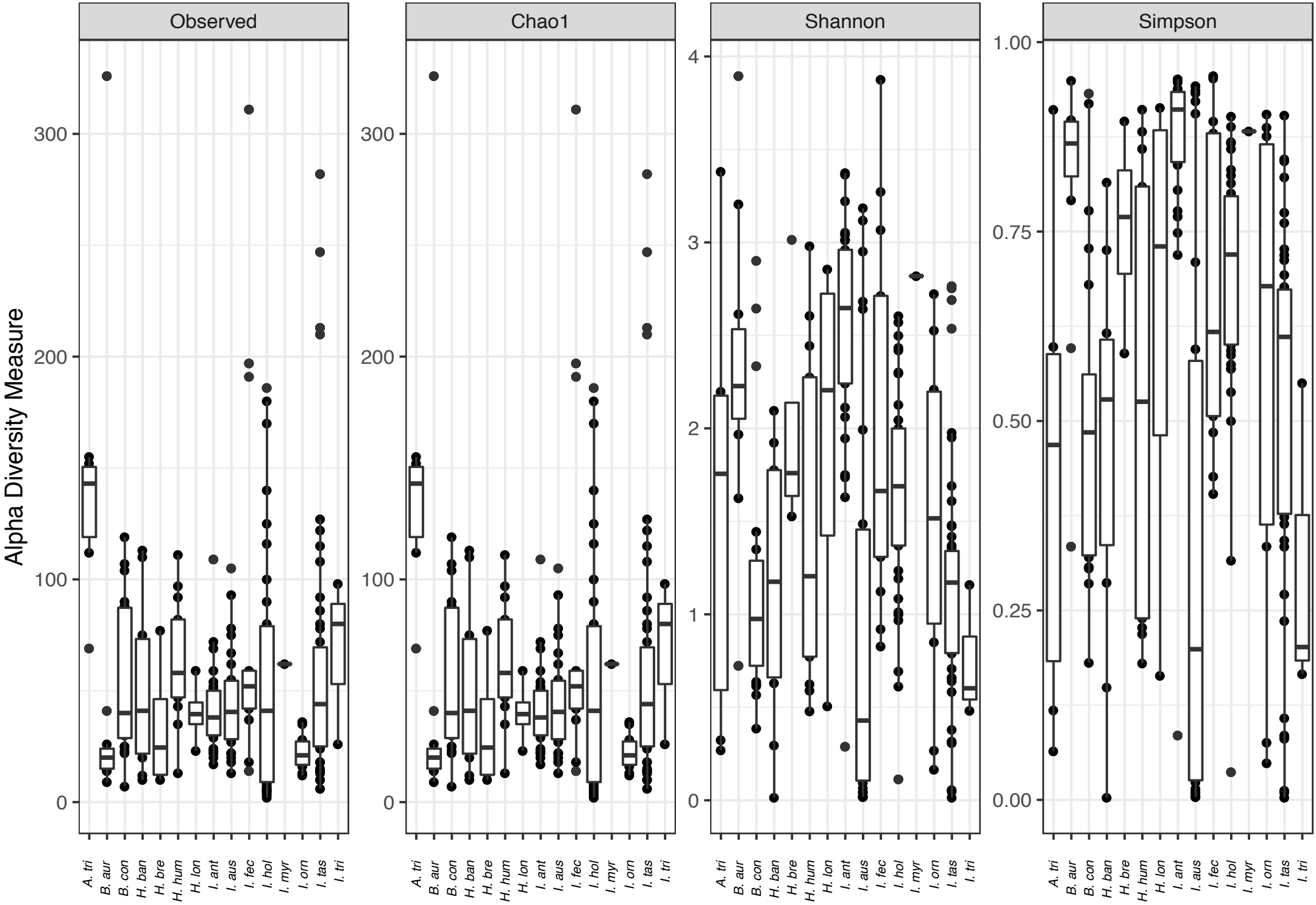
Supplementary File S3. Principal coordinate analysis (PCoA) plot of the weighted UniFrac distance matrix. Plots show beta diversity of tick bacterial communities, displayed by tick species and life stage. Operational taxonomic units (OTUs) that represented <100 sequences in a tick sample were removed.

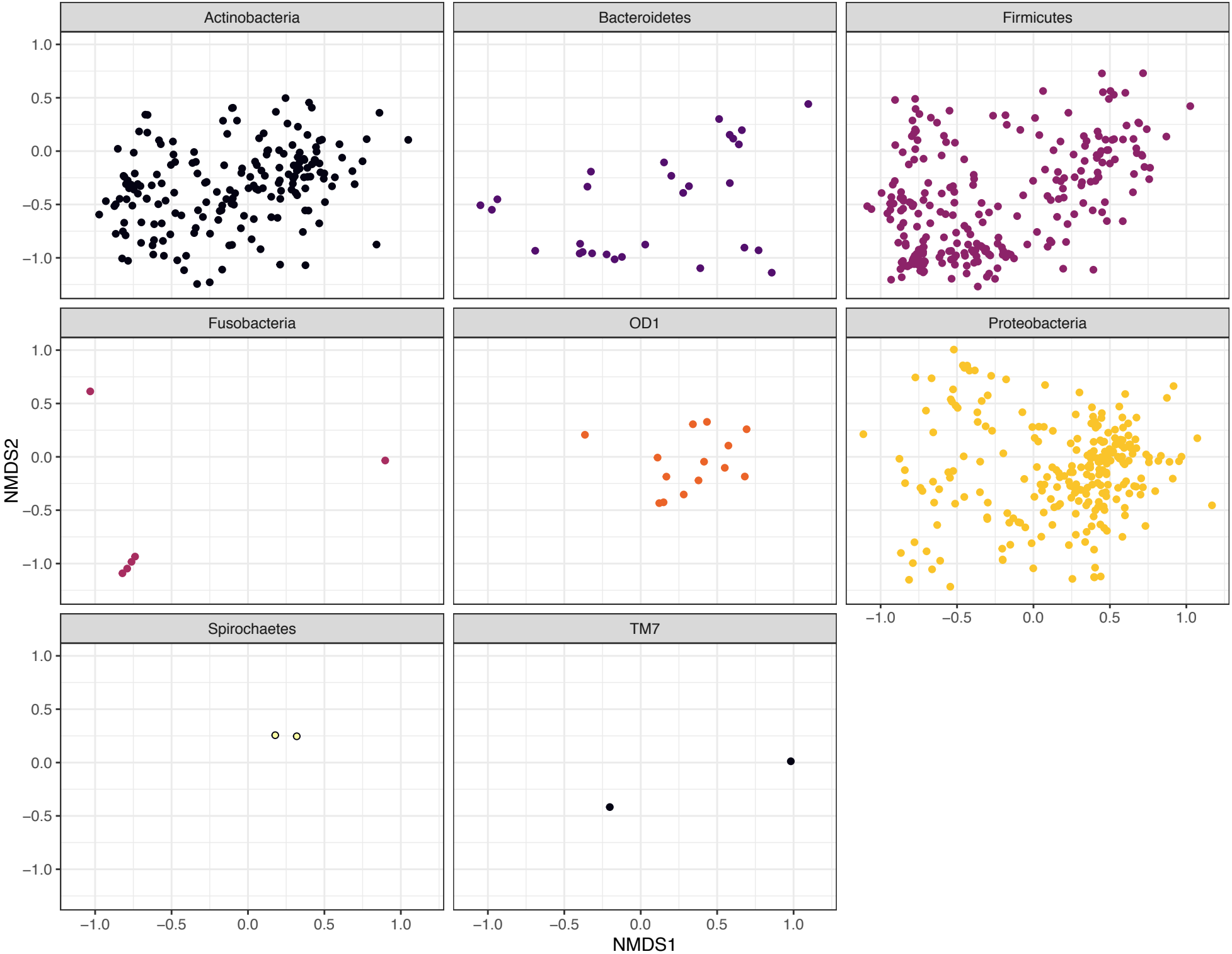
Supplementary File S4. Number of sequences relating to the taxa of interest in tick samples. The top hit from NCBI BLAST results showing GenBank accession number, percent identity and E-value are given, and final evaluation of taxonomic assignment.

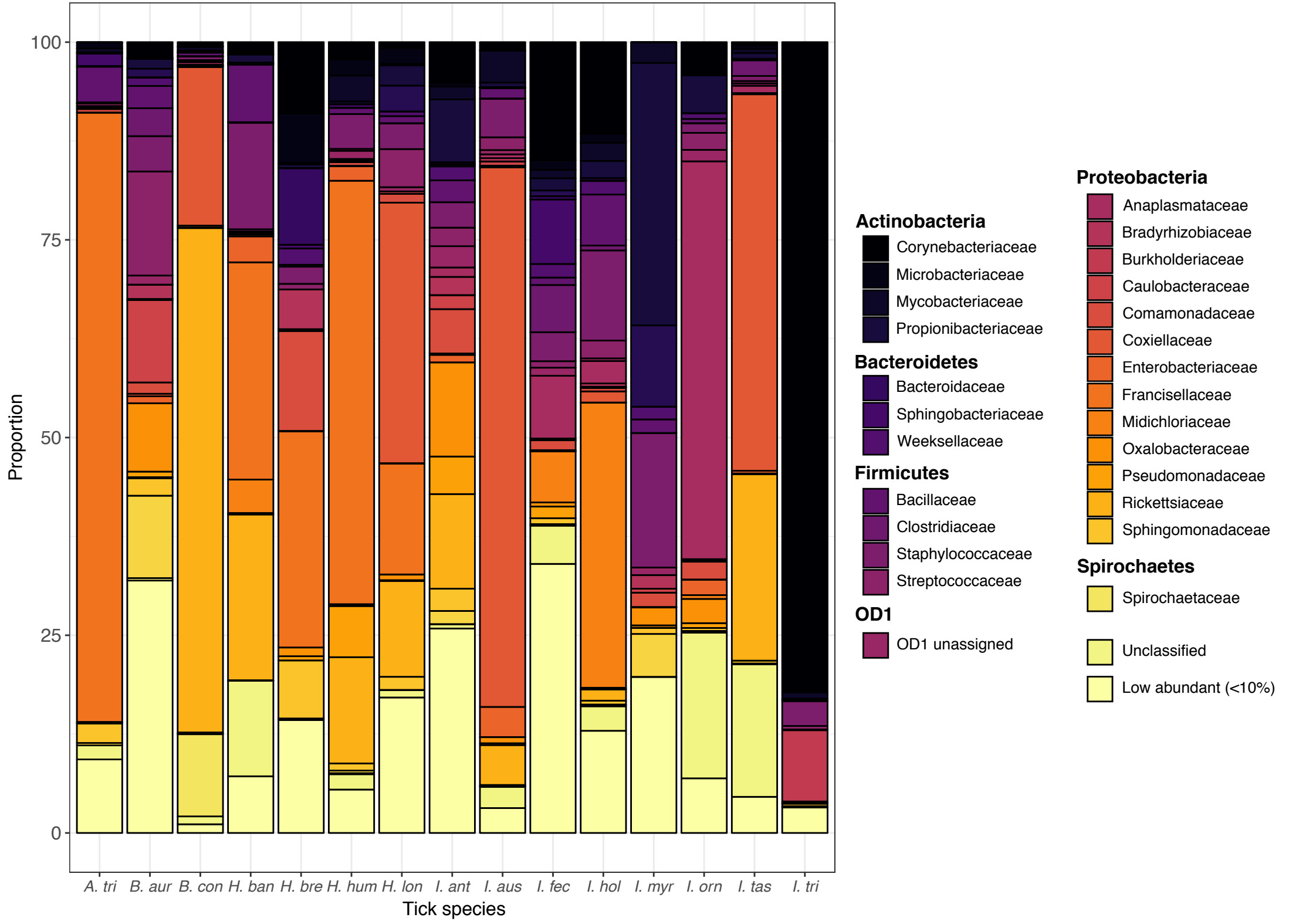
Supplementary File S5. Fasta sequences for operational taxonomic units (OTUs) used in the taxa of interest investigation.

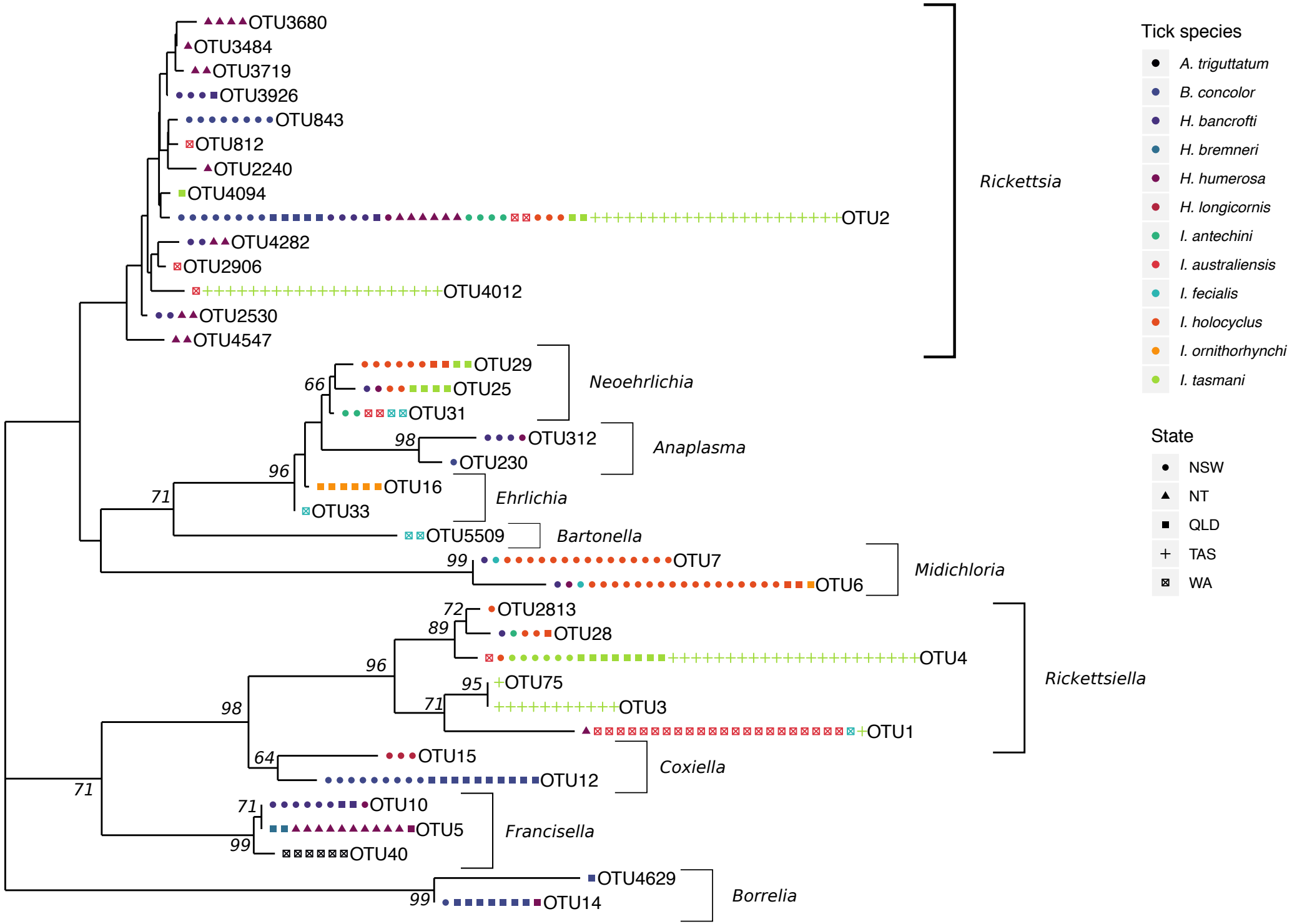

I. australiensis
I. fecialis
I. hirsti
I. holocyclus
I. myrmecobii
I. ornithorhynchi
I. tasmani
I. trichosuri
H. bancrofti
H. humerosa
H. lagostrophi
H. petrogalis
Pteropus sp.
An. agilis
An. stuartii
Da. maculatus
Sa. harrisii
Th. billardierii
Ma. rufus
Ma. fuliginosus
Ma. giganteus
Ma. rufogriseus
Wa. bicolour
Ps. peregrinus
Tr. caninus
Tr. vulpecula
Pet. breviceps
Vo. ursinus
Is. fusciventer
Is. macrourus
Pe. gunnii
Pe. nasuta
Hy. chrysogaster
Ra. fuscipes
Ra. rattus
* Su. scrofa
* Vu. vulpes
Or. anatinus
Ta. aculeatus
A. triguttatum
B. auruginans
B. concolor
H. longicornis
I. antechini


A. triguttatum
B. auruginans
B. concolor
H. bancrofti
H. bremneri
H. humerosa
H. longicornis
I. antechini
I. australiensis
I. fecialis
I. holocyclus
I. myrmecobii
I. ornithorhynchi
I. tasmani
I. trichosuri
Cumulative number of observed OTUs
Sequencing depth (reads)
0
100
200
300
0
100000
200000
300000


Observed
Chao1
Shannon
Simpson
Alpha Diversity Measure
0
100
200
300
0
1
2
3
4
0.00
0.25
0.50
0.75
1.00
A. tri
B. aur
B. con
H. ban
H. bre
H. hum
H. lon
I. ant
I. aus
I. fec
I. hol
I. myr
I. orn
I. tas
I. tri


Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
OD1
Proteobacteria
Spirochaetes
TM7
NMDS2
NMDS1
1.0
0.5
0.0
−0.5
−1.0


100
75
50
25
0
Proportion
A. tri
B. aur
B. con
H. ban
H. bre
H. hum
H. lon
I. ant
I. aus
I. fec
I. hol
I. myr
I. orn
I. tas
I. tri
Tick species
Actinobacteria
Corynebacteriaceae
Microbacteriaceae
Mycobacteriaceae
Propionibacteriaceae
Bacteroidetes
Bacteroidaceae
Sphingobacteriaceae
Weeksellaceae
Firmicutes
Bacillaceae
Clostridiaceae
Staphylococcaceae
Streptococcaceae
OD1
OD1 unassigned
Proteobacteria
Anaplasmataceae
Bradyrhizobiaceae
Burkholderiaceae
Caulobacteraceae
Comamonadaceae
Coxiellaceae
Enterobacteriaceae
Francisellaceae
Midichloriaceae
Oxalobacteraceae
Pseudomonadaceae
Rickettsiaceae
Sphingomonadaceae
Spirochaetes
Spirochaetaceae
Unclassified
Low abundant (<10%)


OTU3680
OTU3484
OTU3719
OTU3926
OTU843
OTU812
OTU2240
OTU4094
OTU2
OTU4282
OTU2906
OTU4012
OTU2530
OTU4547
Rickettsia
OTU29
OTU25
OTU31
66
Neoehrlichia
OTU312
OTU230
98
Anaplasma
96
OTU16
OTU33
71
Ehrlichia
OTU5509
Bartonella
99
OTU7
OTU6
Midichloria
72
OTU2813
89
OTU28
OTU4
96
95
OTU75
71
OTU3
OTU1
Rickettsiella
98
64
OTU15
OTU12
Coxiella
71
71
OTU10
OTU5
99
OTU40
Francisella
OTU4629
99
OTU14
Borrelia
Tick species
A. triguttatum
B. concolor
H. bancrofti
H. bremneri
H. humerosa
H. longicornis
I. antechini
I. australiensis
I. fecialis
I. holocyclus
I. ornithorhynchi
I. tasmani
State
NSW
NT
QLD
TAS
WA