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

arthropod. 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 newly described bacteria '*Ca*. Borrelia tachyglossi', '*Ca.* Neoehrlichia australis', '*Ca.* Neoehrlichia arcana' and '*Ca*. Ehrlichia ornithorhynchi'. Furthermore, three novel Anaplasmataceae species were identified including; a *Neoehrlichia* sp. in *I. australiensis* and *I. fecialis* ex quenda (*Isoodon fusciventer*) (Western Australia), an *Anaplasma* sp. from one *B. concolor* ex 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 almost 900 species described globally (Guglielmone et al., 2010). 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-Pena et al., 2013; Pfaffle et al., 2013). Metabarcoding provides an informative molecular platform 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 human-biting *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, for identification. Ticks were identified morphologically to instar and species using keys and species' descriptions (Roberts, 1970; Barker and Walker, 2014). Details of sample

101 collection are available in Supplementary File S1.

102

103 *2.2 DNA Extraction*

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

117

118 *2.3 16S amplification and library preparation for NGS sequencing*

119 Amplicons targeting a 250-320 bp product of the V1-2 hypervariable region of the 16S rRNA
120 (16S) gene were generated using the primer pair 27F-Y (Gofton et al., 2015b) and 338R
121 (Turner et al., 1999). To inhibit the amplification of abundant '*Ca*. Midichloria mitochondrii'
122 in *I. holocyclus* ticks, 3-10 µM of the CMm blocking primer was added to the PCR reaction
123 (Gofton et al., 2015a). Amplicon PCRs were carried in 25 µL reactions each containing 1 X
124 Buffer (KAPA Biosystems, USA), 1.5 mM $MgCl_2$, 0.4 µM of each forward and reverse
125 primer with MiSeq adapters, 0.4 mg/mL BSA (Fisher Biotech, Australia), 0.25 mM dNTPs

126 (Fisher Biotech, Australia), 0.5 U KAPA Taq (KAPA Biosystems, USA), and 2.0 µL of
127 undiluted genomic DNA. Samples underwent the following thermal cycling conditions;
128 initial denaturation at 95°C for 5 mins, followed by 35 cycles of denaturation at 95°C for 30
129 s, annealing at 62°C (CMm blocking primer present) or 55°C (CMm blocking primer absent)
130 for 30 s, and extension at 72°C for 45 s with a final extension at 72°C for 5 mins. Libraries
131 were then prepared using the Nextera XT DNA library preparation kit in 25 µL reactions
132 following manufacturer's recommendations. Libraries were purified using Agencourt®
133 AMPure® XP PCR purification beads (Beckman Coulter Life Sciences, USA) and pooled in
134 equimolar amounts. Final libraries were then quantified using the Qubit® 2.0 Fluorometer
135 (Thermo Fisher, Australia). Libraries were sequenced on an Illumina MiSeq™ using v2
136 chemistry (2x250 paired end).
137
138 Extraction reagent blank (n=12) and PCR no-template controls (NTC; n=7) were included in
139 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 blast (Altschul et al., 1990) on a
156 random subsample of OTUs and in the case of tick-associated microbes, GenBank accession
157 numbers and percentage similarity of top hits were recorded for 'taxa of interest' (see
158 Supplementary File S2). The profile from EXB controls and NTCs was first assessed to
159 ensure quality of sampling and absence of tick-associated bacteria. The profiles of EXB and
160 NTC were then removed bioinformatically from associated samples to eliminate potential
161 contamination and background bacteria.
162
163 Data analysis and visualisation was carried out in RStudio (RStudio Team, 2015) using
164 packages metacoder (Poisot et al., 2017), phyloseq (McMurdie and Holmes, 2013) and vegan
165 (Oksanen et al., 2019). Alpha diversity of samples was measured using observed OTUs,
166 Chao1 index, Shannon index and Simpson index. Removal of samples with a low sequencing
167 depth (<1000 assigned OTUs after data filtering), did not significantly alter alpha diversity
168 measurements among tick species (data not shown). Rarefaction curves for samples were
169 calculated to assess sequencing depth based on observed number of OTUs. Principal
170 coordinate analysis (PCoA) on weighted unifrac dissimilarity measurements was used to
171 assess the differences in microbial composition between tick species. Investigation into 'taxa
172 of interest' warranted a more rigorous assessment of sequence number than was required for
173 diversity measures in order to avoid any potential cross-contamination and machine cross-
174 talk (Dong et al., 2017; Wang et al., 2017), as such only samples with >100 sequences were
175 considered positive. Due to sample collection biases in the present study, prevalence data of

these microbes were not considered statistically relevant. Nucleotide sequences from 'taxa of interest' (Supplementary File S3) were aligned by MUSCLE (Edgar, 2004) using default parameters and aligned sequences were then imported into MEGA7 (Kumar et al., 2016) with the most appropriate nucleotide substitution model chosen based on the lowest Bayesian Information Criterion (BIC) score. Evolutionary histories were inferred using the Neighbour-Joining method based on the Tamura 3-parameter model (Tamura, 1992). Bootstrap analysis was conducted using 10,000 replicates to assess the reliability of inferred tree topologies.

3. Results

3.1 Tick-host associations

Ticks were collected from 221 wildlife hosts including 25 native and three introduced species (Fig 1, Supplementary File S1). Hosts recorded in the present study were; agile antechinus (*Antechinus agilis*), brown antechinus (*Antechinus stuartii*), spotted-tail quoll (*Dasyurus maculatus*), water rat (*Hydromys chrysogaster*), quenda (*Isoodon fusciventer*), northern brown bandicoot (*Isoodon macrourus*), western grey kangaroo (*Macropus fuliginosus*), eastern grey kangaroo (*Macropus giganteus*), red-necked wallaby (*Notamacropus rufogriseus*), red kangaroo (*Osphranter rufus*), platypus (*Ornithorhynchus anatinus*), eastern barred bandicoot (*Perameles gunnii*), long-nosed bandicoot (*Perameles nasuta*), sugar glider (*Petaurus breviceps*), eastern ring-tailed possum (*Pseudocheirus peregrinus*), black fruit-bat (*Pteropus alecto*), fruit-bat sp. (*Pteropus* sp.), bush rat (*Rattus fuscipes*), black rat (*Rattus rattus*), Tasmanian devil (*Sarcophilus harrisii*), wild pig (*Sus scrofa*), short-beaked echidna (*Tachyglossus aculeatus*), rufous-bellied pademelon (*Thylogale billardierii*), short-eared brush-tailed possum (*Trichosurus caninus*), common brush-tailed possum (*Trichosurus vulpecula*), wombat (*Vombatus ursinus*), red fox (*Vulpes vulpes*), and swamp wallaby

201 (*Wallabia bicolor*). Female ticks were the dominant instar recorded (*n*=547) followed by
202 nymphs (*n*=319) and males (*n*=153). Ticks were received from animals in the Northern
203 Territory (NT) (*n*=16), Queensland (QLD) (*n*=249), New South Wales (NSW) (*n*=316),
204 Tasmania (TAS) (*n*=136), Victoria (VIC) (*n*=44) and Western Australia (WA) (*n*=258).
205 Together, *Bothriocroton concolor* (*n*=123), *Ixodes australiensis* (*n*=210), *I. holocyclus*
206 (*n*=173) and *Ixodes tasmani* (*n*=184) accounted for over two-thirds of all ticks submitted.
207
208 *3.2 16S rRNA bacterial profiling*
209 A sub-sample of 238 ticks and 19 controls underwent 16S NGS profiling. A total of 23.9
210 million raw paired-end sequences were generated on the Illumina MiSeq. 17.9 million
211 sequences were retained after merging, and subsequent quality filtering yielded 14.8 million
212 sequences for clustering and taxonomic assignment. A total of 4,864 OTUs (average length
213 299 bases) were retained after background profiles were removed. After removal of
214 background sequences, a total of 14,328,059 bacterial sequences were assigned to tick
215 samples. Despite a high number of OTUs, only 1,535 OTUs had greater than 100 total
216 sequences from tick samples. Tick samples had an average of 60,201 assigned sequences (Fig
217 2). *Amblyomma triguttatum* had the highest alpha diversity as measured by the observed
218 number of OTUs and the chao1 index, whereas *Ixodes antechini* had the highest alpha
219 diversity as measured by the Shannon and Simpson indexes (Fig 3). Analysis of sequence
220 depth shows that in most cases observed number of OTUs, began to plateau around 50,000
221 sequences (Fig 4). An ordination plot of taxa (Fig 5) show that bacteria belonging to the
222 Proteobacteria phylum were the most abundant taxa classified. Principal coordinate analysis
223 (PCoA) of weighted Uni-Frac distances (Fig 6) demonstrate that microbiome composition
224 was largely grouped by tick species, with the most notable exception being *I. tasmani*.
225 Overall eight phyla were recorded, however after removal of low abundant and background

226 bacterial communities (identified from controls) six phyla were retained consisting of 91
227 families (Fig 7). Bacterial families identified in tick species are represented as relative
228 number of sequences in Fig 8.
229
230 *3.3 Presence of 'taxa of interest'*
231 Phylogenetic analysis of the 'taxa of interest' is represented in Fig 9, showing tick species
232 and Australian state where each OTU was identified.
233
234 A proposed novel *Anaplasma* sp. (OTU230, MK814412, 96.3% similarity) was identified in a
235 single *B. concolor* (1/20) ex echidna from NSW. A second *Anaplasma bovis*-like OTU
236 (OTU312, JN862824, 97.3% similarity) was identified in *Haemaphysalis bancrofti* (3/10) ex
237 red-necked wallaby and long-nosed bandicoot and *Haemaphysalis humerosa* (n = 1/13) tick
238 ex long-nosed bandicoot, all from NSW.
239
240 OTU5509 was assigned to the genus *Bartonella* and had a top BLAST hit of *Bartonella*
241 *bacilliformis* (LN624026, 92.1% similarity). OTU5509 was identified in two samples,
242 however was present in an extremely low number of sequences (2) in each case. Both
243 samples were *Ixodes fecialis* collected from two different quenda in WA. The low number of
244 sequences means that in the case of statistical analysis it would have been filtered out. While
245 there was no sufficient match to this sequence, it is noted that in many instances native
246 Australian *Bartonella* species are lacking sequence information for this region of the 16S
247 gene (v1-2).
248
249 Two OTUs (OTU14, CP025785, 100% similarity; and OTU4629, CP025785, 100%
250 similarity) were identified as *'Ca.* B. tachyglossi*'*. Sequences were identified in *B. concolor*

251 (8/20) ex echidnas from QLD & NSW and *H. humerosa* (1/13) ex northern brown bandicoot
252 from QLD.
253
254 A *Coxiella*-like organism (OTU12, CP032542, 95.5% similarity) was identified exclusively
255 in *B. concolor* (19/20) ex echidna from NSW and QLD. This was the second most abundant
256 OTU identified in *B. concolor*, accounting for ~20% of the overall sequences.
257 A *Coxiella* sp. (OTU15, KC170757, 100% similarity) was identified in *Haemaphysalis*
258 *longicornis* (3/4) ex red fox from NSW, this OTU represented ~61.4% of the overall assigned
259 bacterial sequences.
260
261 A novel *Ehrlichia* sp. (OTU33, AY309970, 96.3% similarity) was identified in *I. fecialis*
262 (1/13) ex quenda from WA. Other *Ehrlichia* OTUs (OTU16 and 1632) represented sequences
263 from '*Ca.* Ehrlichia ornithorhynchi' in *Ixodes ornithorhynchi* (6/10) ex platypus QLD and
264 these sequences accounted for the majority of sequences (~50.2%) from *I. ornithorhynchi*.
265
266 A *Francisella* endosymbiont (OTU5, AF001077, 98.0% similarity) was identified in *H.*
267 *humerosa* (11/13) ticks ex northern brown bandicoots from NT and QLD, and
268 *Haemaphysalis bremneri* (2/4) ex possum (species unknown) in QLD. OTU5 was the most
269 abundance sequence in *H. bremneri* and *H. humerosa*, representing 27.3% and 52.4% of the
270 assigned sequences, respectively. A second *Francisella* endosymbiont (OTU10, AB001522,
271 99% similarity) was identified in *H. bancrofti* (8/11) ex red-necked wallabies from QLD and
272 NSW, and ex a long-nosed bandicoot in NSW; and in a single *H. humerosa* (1/13) ex red-
273 necked wallaby from NSW. OTU10 was the most abundance sequences in *H. bancrofti*
274 representing 27.0% of the assigned sequences. OTU40 (AF001077, 97.6% similarity) was
275 also identified as a *Francisella*-like endosymbiont from *A. triguttatum* (6/6) ex red kangaroo

276 from WA. It was highly abundant in the ticks, accounting for 76.9% of the overall sequences.

277

278 *Midichloria* (OTU6, FM992372, 100% similarity) was identified in *I. holocyclus* (20/36) ex
279 long-nosed bandicoots from NSW and QLD, *I. fecialis* (1/13) ex red-necked wallaby NSW,
280 *H. bancrofti* (1/11) ex long-nosed bandicoot NSW, and *H. humerosa* (1/13) ex long-nosed
281 bandicoot NSW. *Midichloria* (OTU7, FM992373, 100% similarity) was identified in *I.*
282 *holocyclus* (15/36) ex long-nosed bandicoots from NSW and QLD, *I. fecialis* (1/13) ex red-
283 necked wallaby NSW and *H. bancrofti* (1/11) ex long-nosed bandicoot NSW. Overall a total
284 of 15 ticks had both OTU6 and OTU 7, which included *I. holocyclus* (13/36) ex long-nosed
285 bandicoots from NSW and QLD, *I. fecialis* (1/13) ex red-necked wallaby NSW and *H.*
286 *bancrofti* (1/11) ex long-nosed bandicoot NSW. *Midichloria* was the most abundant sequence
287 from *I. holocyclus* accounting for 36.1% of assigned sequences.

288

289 Three *Neoehrlichia* OTUs were identified. '*Ca*. Neoehrlichia arcana' (OTU25, KT203914,
290 99.3% similarity) was identified in eight ticks, *I. tasmani* (4/51), *I. holocyclus* (2/36), *H.*
291 *bancrofti* (1/11) and *H. humerosa* (1/13), ex long-nosed bandicoots NSW and QLD. '*Ca*.
292 Neoehrlichia australis' (OTU29, KT803957, 100% similarity) was identified in 10 ticks, *I.*
293 *holocyclus* (8/36) and *I. tasmani* (2/51), ex long-nosed bandicoots NSW and QLD. Three
294 ticks (*I. tasmani* and *I. holocyclu*s) were co-infected with both '*Ca*. N arcana' and '*Ca*. N.
295 australis'. A novel *Neoehrlichia* species (OTU31, MG670107, 97.3% similarity) was
296 identified in *I. fecialis* (2/13) and *I. australiensis* (2/26) ex quenda from WA, and *I. antechini*
297 (2/29) ex brown antechinus from NSW.

298

299 Due to the conserved nature of the *Rickettsia* genus at the 16S gene, reported previously
300 (Roux and Raoult, 1995; Stothard and Fuerst, 1995), resolution to species level was not

possible using the sequences obtained in the present study. *Rickettsia* sp. (OTU2, KF318168, 99.7% similarity) had a widespread distribution among samples and was detected in 61 ticks *(B. concolor, H. bancrofti, H. humerosa, I. antechini, I. australiensis, I. holocyclus* and *I. tasmani*) from four states (NSW, NT, QLD, WA). *Rickettsia* was the most abundance sequence from *B. concolor* and *I. antechini* ticks accounting for 63.3% and 12.0% of the assigned sequences, respectively

A *Rickettsiella*-like sp. (OTU1, LC388767, 92.7% similarity) 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% similarity) was identified in *I. tasmani* (11/51) ex Tasmanian devils and eastern barred bandicoot from TAS. A *Rickettsiella*-lik*e* sp. (OTU4, U97547, 98.4% similarity) 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 epidemiological data.

Overall bacterial diversity of ticks started to plateau at about 50,000 sequences, however, it is noted that in some cases this plateau was not achieved and deeper sequencing would be required in order to confidently characterise the full suite of bacterial species present. This was most evident in *A. triguttatum*, *B. auruginans*, *I. trichosuri* and *I. ornithorhynchi* and therefore we recommend a minimum of 100,000 sequences per sample for future bacterial 16S amplicon studies in these species. 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. The composition and relative proportion of bacterial taxa identified in ticks during the present

351 study are in line with previous research overseas (Zhang et al., 2014; Kurilshikov et al., 2015;
352 Sperling et al., 2017) and in Australia (Gofton et al., 2015b; Panetta et al., 2017). Molecular
353 tools continue to develop at a rapid rate with an increasing shift towards whole-genome or
354 shotgun sequencing (sequencing the full range of nucleic acids present in a metagenomic
355 sample). However, comparisons between whole-genome and amplicon sequencing continue
356 to indicate that the detection of low abundant bacteria is greatly enhanced by using targeted
357 16S amplicon bacterial profiling methods as used in the present study (Carpi et al., 2011;
358 Tessler et al., 2017).
359
360 Currently in Australia, the ecology, epidemiology, and incidence of human tick-borne
361 diseases remains largely a matter of conjecture, having received little scientific study
362 compared with many other parts of the world (Graves and Stenos, 2017). Despite significant
363 national interest, including a federal government senate inquiry (Radcliffe et al., 2016), the
364 prevailing scientific opinion concludes that Lyme borreliosis (caused by *B. burgdorferi* sensu
365 lato), for example, is absent from Australia (Chalada et al., 2016; Irwin et al., 2017). In the
366 unique Australian environment, long isolated in geological terms, it is likely that unidentified
367 tick-host life cycles have evolved that may contribute to zoonotic illness when humans
368 encroach these sylvatic ecologies and become exposed to native ticks. Indeed, it is well
369 documented that in endemic areas in Europe and North America, the causative agent of Lyme
370 borreliosis is readily identified in wildlife reservoirs (such as white-tailed deer, *Ococoileus*
371 *virginianus*, and white-footed mice, *Peromyscus leucopus*) as well as their natural tick
372 species (Bosler et al., 1984). The present study therefore is one of the first to explore the
373 concept that Australian wildlife ticks should be a promising source to identify exotic and
374 novel TBPs, and we focussed our search towards taxa known to be associated with TBDs
375 overseas ('taxa of interest'), namely *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Ehrlichia*,

376 *Francisella*, *Midichloria*, *Neoehrlichia*, *Rickettsia* and *Rickettsiella*.

377

378 A new genetic variant of *Anaplasma bovis* was recently described from questing *A.*
379 *triguttatum* ticks in WA and the nearby Barrow Island (Gofton et al., 2017). In the present
380 study we report a range expansion of a genetically similar *A. bovis* along the east coast of
381 Australia from two widespread *Haemaphysalis* tick species. As suggested by Gofton et al.,
382 (2017), this finding further supports the hypothesis that it is likely that endemic *A. bovis*
383 genotypes exist in sylvatic cycles within native Australian ticks and wildlife fauna. Further
384 research on the phylogenetic position of these *A. bovis* sequences is needed to understand
385 their likely evolutionary history and relatedness to *A. bovis* genotype Y11 identified on the
386 west coast of Australia. While three other species of *Anaplasma* (*A. marginale*, *A. centrale*
387 and *A. platys*) have been introduced to Australia (Rogers and Shiels, 1979; Callow, 1984;
388 Angus, 1996) the present study did not identify any of these species in ticks from wildlife. A
389 species of *Anaplasma* identified exclusively in *B. concolor* from echidnas sheds more light on
390 the diverse range of microbes that have been described from this specialist tick (Loh, 2018).

391

392 Although the presence of a *Bartonella* sp. was identified by only four sequences, its absence
393 in control samples means that this likely represents a true finding in female *I. fecialis*, which
394 were parasitising different individuals of the same host (quenda) in south-west WA. Recent
395 studies have shown that Australian marsupials and native rodents harbour a range of distinct
396 *Bartonella* species; '*Ca*. Bartonella antechini' has been identified in ticks (*I. antechini*) and
397 fleas (*Acanthopsylla jordani*) from mardo (*Antechinus flavipes*) in south-west WA
398 (Kaewmongkol et al., 2011c), '*Ca*. Bartonella woyliei' in woylie ticks (*I. australiensis*) and
399 fleas (*Pygiopsylla hilli*) in south-west WA, and '*Ca*. Bartonella bandicootii' in fleas
400 (*Pygiopsylla tunneyi*) from western barred bandicoots (*Perameles bougainville*) on Bernier

401 and Dorre Island (Kaewmongkol et al., 2011a). Reports of *Bartonella* spp. occurring outside
402 south-west WA include *Bartonella australis* ex eastern grey kangaroos (*Macropus giganteus)*
403 (Fournier et al., 2007). Molecular detection of *Bartonella* DNA from Australian ticks has also
404 been reported in ticks (*I. tasmani*) parasitising koalas (*Phascolarctos cinereus*) from Philip
405 Island, VIC (Vilcins et al., 2009b). Additional studies on *Bartonella* in Australia wildlife
406 include those by Gundi et al. (2009), Kaewmongkol et al. (2011a) and Dybing et al. (2016).
407 Due to the limited size and region of the 16S amplicon generated in the present study, there
408 were insufficient relevant reference sequences available from other Australian *Bartonella*
409 species for comparison. A study by Kaewmongkol et al. (2011b) into flea-derived *Bartonella*
410 from native and introduced Australian species suggests co-evolution of marsupial hosts, their
411 fleas and the *Bartonella* species.
412
413 '*Ca*. Borrelia tachyglossi' was identified in 8/20 *B. concolor* ticks from echidnas in QLD and
414 NSW, an anticipated finding given previous research into this organism (Loh et al., 2016).
415 Importantly however, the present study provides the first evidence of '*Ca*. B. tachyglossi'
416 sequences from a female *H. humerosa* tick parasitising a northern brown bandicoot from
417 QLD. In addition, no *Bothriocroton* ticks were present in the extraction batch of the positive
418 *H. humerosa* sample, and there was no evidence of *Borrelia* sequences from any controls.
419 Despite the wide geographical range of *H. humerosa* ticks, the restricted finding of '*Ca*. B.
420 tachyglossi' from QLD supports Loh et al. (2016), suggesting a restricted geographical
421 distribution along the east coast of Australia.
422
423 In tick microbiome studies overseas, *Coxiella* spp. are commonly identified (Khoo et al.,
424 2016; Machado-Ferreira et al., 2016). Additionally, recent studies on Australian ticks have
425 also identified *Coxiella* spp. in *A. triguttatum* (Cooper et al., 2013; Gofton et al., 2015b), *B.*

426 *auruginans* (Vilcins et al., 2009c), *I. holocyclus* (Cooper et al., 2013) and *R. sanguineus*
427 (Oskam et al., 2017). Interestingly the present study did not identify the widespread presence
428 of *Coxiella* spp. with only two unique OTUs identified from *B. concolor* and *H. longicornis*,
429 both of which appear to be host specific to those tick species, and the causative agent of Q-
430 Fever (*C. burnetii*) was not identified from ticks in the present study.
431
432 *Ehrlichia* sequences ('*Ca*. E. ornithorhynchi') from platypus ticks, *I. ornithorhynchi*, were
433 recently described (Gofton et al., 2018). In the present study, '*Ca*. E. ornithorhynchi' was
434 exclusively observed within *I. ornithorhynchi,* suggesting this genetically distinct *Ehrlichia*
435 species has a unique and host-specific relationship with the platypus. During this study, a
436 potentially novel species of *Ehrlichia* was identified in an *I. fecialis* female tick from a
437 quenda in WA. BLAST results show it is a close relative to an *Ehrlichia* sp. detected in
438 *Haemaphysalis* ticks from Japan (Inokuma et al., 2004). No sequences from the recently
439 described '*Ca*. E. occidentalis' (Gofton et al., 2017) were identified in the present study,
440 however it is noted *A. triguttatum* were only represented by six samples.
441
442 *Francisella*-like endosymbionts have been widely reported in ticks overseas, such as
443 *Dermacentor* spp. (Scoles, 2005), *Dermacentor occidentalis* (Gurfield et al., 2017),
444 *Haemaphysalis longicornis* (Wang et al., 2018) and *Hyalomma rufipes* (Szigeti et al., 2014),
445 and *Francisella* sequences have previously been identified in *A. fimbriatum* ticks from
446 reptiles in the Northern Territory of Australia (Vilcins et al., 2009a). In the present study, a
447 *Francisella*-like endosymbiont was identified in 100% of *A. triguttatum* ticks, and in a high
448 proportion. The high prevalence and relative abundance of the *Francisella*-like organisms in
449 *A. triguttatum* may be of medical relevance with respect to this common human-biting tick,
450 particularly given the recent report of *Francisella* bacteraemia in WA (Aravena-Román et al.,

451 2015). Importantly, as noted previously for *I. holocyclus* ticks, organisms in lower abundance
452 may be masked by plentiful endosymbionts, unless samples are sequenced deeply and/or a
453 blocking primer is used (Gofton et al., 2015a). Therefore, future bacterial profiling studies of
454 *A. triguttatum* should take these factors into consideration.
455
456 While '*Ca*. M. mitochondrii' has been documented in *I. holocyclus,* the present study
457 provides the first report of '*Ca*. M. mitochondrii' within additional species of native
458 Australian ticks (*I. fecialis, H. bancrofti* and *H. humerosa*). Despite this expansion among
459 tick species, identification of '*Ca*. M. mitochondrii' remains confined to NSW and QLD as
460 previously reported (Gofton et al., 2015a). '*Ca*. M. mitochondrii' has been detected overseas
461 in laboratory models (Cafiso et al., 2019), wildlife (Serra et al., 2018), and humans
462 (Mariconti et al., 2012).
463
464 A recent addition to the Anaplasmataceae family, '*Ca*. Neoehrlichia mikurensis' was first
465 isolated from wild rats and their ticks (*Ixodes ovatus*) in Japan (Kawahara et al., 2004). In
466 Europe, the hedgehog (*Erinaceus* spp.), a common peri-urban dweller, has been shown to
467 play an important role in life cycle of '*Ca*. N. mikurensis' (Földvári et al., 2014; Jahfari et al.,
468 2017). A high proportion of bandicoot ticks were infected with *Neoehrlichia* spp. in this
469 study, suggesting that these marsupials may play an important role in the life cycle of this
470 bacterium on the Australian continent. Bandicoots also frequently inhabit gardens and have
471 relatively close contact with humans (Carthey and Banks, 2012) closer study (including blood
472 samples and tissue biopsies) of these marsupials may provide vital information about the life
473 cycle of these microbes and the potential risk for human infection.
474
475 *Rickettsia* spp. are among the only currently recognised TBPs affecting people in Australia,

476 however, as previously outlined, the conserved nature of the 16S gene in *Rickettsia* precludes
477 rigorous species delimitation from the relatively short sequences generated in this dataset. In
478 addition to the known human pathogens (*R. australis* and *R. honei*) (Graves and Stenos,
479 2017), the presence of *Rickettsia* has been demonstrated from a variety of Australian tick
480 species. *Rickettsia gravesii* has been described from *A. triguttatum* (Li et al., 2010; Abdad et
481 al., 2017) and *Rickettsia* sequences have been previously identified in *I. tasmani* ex
482 Tasmanian devils (Vilcins et al., 2009c) and *Amblyomma fimbriatum* from reptiles (Vilcins et
483 al., 2009a).
484
485 The diversity and significance of the *Rickettsiella* genus remains largely unknown globally.
486 Phylogenetic analysis of sequence data places the genus within the Coxiellaceae family
487 (Fournier and Raoult, 2005; Leclerque and Kleespies, 2012). While the presence of these
488 organisms has been well documented in tick species around the world and in some cases they
489 have been identified as pathogenic to arthropods (Kurtti et al., 2002; Leclerque et al., 2012),
490 disease causation within the vertebrate hosts remains unknown at the present time. A study
491 by Vilcins et al. (2009c) identified *Rickettsiella* in *I. tasmani* ex koalas from Phillip Island,
492 just off the coast of south eastern Australia. A genetically similar sequence was identified in
493 the present study (OTU3) from *I. tasmani* exclusively in Tasmania.
494
495 While this is the first study to characterise the bacterial communities within native Australian
496 wildlife ticks, it is apparent that much research is still required in order to better understand
497 the tick-associated microbial life cycles and their ecologies, let alone elucidating their
498 potential for transmission and pathogenicity in vertebrate hosts, including humans.
499 Furthermore, whilst cost-effective, ticks were collected opportunistically resulting in an
500 inherent geographical bias, confining sampling largely to urban areas along the east coast of

501 the Australian continent. Nevertheless, this area is also where most humans receive tick bites,
502 although reliable data about this is also lacking.
503
504 The analysis of ticks removed from wildlife hosts comes with the inherent complication of a
505 blood meal. The host blood meal has recently been shown to affect both the tick microbiome
506 composition and the presence of pathogens (Landesman et al., 2019; Swei and Kwan, 2017).
507 Further targeted studies are needed to assess the source of microbes with respect to a tick or
508 host origin (Irwin et al., 2018). In addition, limitations in the current sequencing technologies
509 mean that high-throughput methods favour short amplicons which may not be able to
510 accurately discriminate bacterial species, as is the case with *Rickettsia* in particular (Gihring
511 et al., 2012). Furthermore, important caveats to consider in metabarcoding microbial diversity
512 analysis include PCR efficiency (including primers used and length of target amplicon),
513 variations in 16S gene copy number (Ahn et al., 2012), sequencing depth, machine cross-talk,
514 bioinformatic analysis and taxonomic assignment. While there have been some recent
515 attempts to correct these biases (Kembel et al., 2012; Rosselli et al., 2016), their application
516 to uncharacterised metagenomic samples remains limited.
517
518 **5. Conclusions**
519
520 With over 75% of emerging human infectious pathogens originating from wildlife (King,
521 2014), surveillance methods that target these species are important for investigations of
522 emerging and exotic infectious disease. Research into the interactions of wildlife hosts, ticks
523 and pathogens in Europe and North America continues to highlight the complexity of these
524 dynamic systems (Ostfeld et al., 2018; Tomassone et al., 2018). Results from the present
525 study build on recent research into Australian tick-associated microbes, further highlighting

526 the diversity of organisms present that appear related, yet distinct from their overseas
527 counterparts. With the evolutionary history of Australia's unique tick species (Beati and
528 Klompen, 2019) and wildlife fauna, it is likely that reports of their tick-associated microbes
529 (potential pathogens) will continue to reveal taxonomic differences from those described in
530 the northern hemisphere. As such, future research on emerging zoonoses should include
531 methods that are able to detect novel micro-organisms in humans and reservoirs. As
532 anthropogenic changes on the environment continue to grow in Australia, a greater emphasis
533 on wildlife disease surveillance is critical to ensure the early detection of potential infectious
534 diseases affecting humans, livestock, companion animals and wildlife (Woods et al., 2019).
535 Advances in pathogen detection and characterisation are greatly enhanced by collaboration;
536 the authors advocate for continued multidisciplinary efforts between health professionals,
537 researchers, land managers and local communities.

538 **Supporting Information**

539

540 **Data availability**

541 Associated metadata output from bioinformatic analysis and subsequent data visualisation is
542 available on FigShare repository https://doi.org/10.6084/m9.figshare.c.4608803.v1. Next-
543 generation sequencing data can be accessed from NCBI Sequence Read Archive under
544 BioProject: PRJNA559059 (BioSample accession numbers: SAMN12512474 –
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546

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558

559 **Compliance with ethical standards**

560 This study was conducted under the compliance of the Australian Code for the Responsibility
561 Conduct of Research (2007) and Australian Code for the Care and Use of Animals for
562 Scientific Purposes, 2013. Tick collection was carried out opportunistically with the approval

563 from the Murdoch University Animal Ethics Committee.

564

565 **Figure Captions**

566 **Fig 1.** Chord diagram showing tick-wildlife associations recorded in the present study. The
567 wildlife hosts recorded are represented on the lower half of the plot, and tick species across
568 the top. Host records that could not be assigned to species level are not represented in this
569 plot. A complete list of records are available in Supplementary File S1. Silhouette wildlife
570 images sourced from phylopic.org.

571 **Fig 2.** Sequence information from 16S rRNA bacterial profiling of ticks on the Illumina
572 MiSeq platform after bioinformatic analysis; (a) number sequences assigned to 97%
573 operational taxonomic units (OTUs) and (b) number of sequences per tick.

574 **Fig 3.** Alpha diversity of bacterial communities in ticks parasitising Australian wildlife
575 measured as; observed number of operational taxonomic units (OTUs), Chao1 index,
576 Shannon index and Simpson index. Tick species abbreviated to; *A. triguttatum* (*A. tri*), *B.*
577 *aurugians* (*B. aur*), *B. concolor* (*B. con*), *H. bancrofti* (*H. ban*), *H. bremneri* (*H. bremneri*),
578 *H. humerosa* (*H. hum*), *H. longicornis* (*H. lon*), *I. antechini* (*I. ant*), *I. australiensis* (*I. aus*), *I.*
579 *fecialis* (*I. fec*), *I. holocyclus* (*I. hol*), *I. myrmecobii* (*I. myr*), *I. ornithorhynchi* (*I. orn*), *I.*
580 *tasmani* (*I. tas*), and *I. trichosuri* (*I. tri*).

581 **Fig 4.** Rarefaction plot of 16S rRNA bacterial sequences clustered into 97% operational
582 taxonomic units (OTUs) from ticks parasitising Australian wildlife.

583 **Fig 5.** Non-metric multidimensional scaling (NMDS) plot of operational taxonomic units
584 (OTUs) based on Bray-Curtis dissimilarity matrix. Taxa filtered to only display OTUs that
585 were present at least twice in >10% of tick samples.

586 **Fig 6.** Principal coordinate analysis (PCoA) plot of the weighted UniFrac distance matrix.

587 Plots shows beta-diversity of tick bacterial communities, displayed by tick species and instar.
588 Operational taxonomic units (OTUs) that represented <100 sequences in a tick sample were
589 disregarded.

590 **Fig 7.** Heat tree of overall microbiome composition of ticks parasitising Australian wildlife.
591 Taxa is shown to family level with colour scale representing number of operational
592 taxonomic units (OTUs).

593 **Fig 8.** Stacked barplot of bacterial composition (shown at family level) from Australian ticks
594 represented as percentage of assigned sequences. Taxa at the family level that represented
595 <10% of the relative sequences within each tick species were grouped as "Low abundant".
596 Sequences not able to be accurately assigned to family taxa are displayed as "Unclassified".

597 **Fig 9.** Neighbour-joining phylogenetic tree displaying taxa of interest prevalence and
598 distribution among tick samples. Geographic data relating to tick collection is represented by
599 state; New South Wales (NSW), Northern Territory (NT), Queensland (QLD), Tasmania
600 (TAS) and Western Australia (WA). Evolutionary histories were inferred based on the
601 Tamura 3-parameter model with bootstrap analysis (10,000 replicated). Tick samples were
602 considered positive for taxa of interest if >100 sequences present. Information on number of
603 sequences, taxonomic identity (as inferred from NCBI BLAST analysis with nucleotide
604 database), and sequences can be sound in supplementary information (Supplementary File S2
605 & S3).

606 **Supplementary Files**

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

609 **Supplementary File S2.** Number of sequences relating to the taxa of interest in tick samples.
610 The top hit from NCBI BLAST results showing Genbank accession number, percent identity
611 and E-value are given, and final evaluation of taxonomic assignment.

612 **Supplementary File S3.** Fasta sequences for operational taxonomic units (OTUs) used in the
613 taxa of interest investigation.

614 **References**

Abdad, M.Y., Abou Abdallah, R., Karkouri, K., Beye, M., Stenos, J., Owen, H., Unsworth, N., Robertson, I., Blacksell, S.D., Nguyen, T.T., Nappez, C., Raoult, D., Fenwick, S., Fournier, P.E., 2017. *Rickettsia gravesii* sp. nov.: a novel spotted fever group rickettsia in Western Australian *Amblyomma triguttatum triguttatum*. Int. J. Syst. Evol. Microbiol. 67(9), 3156–3161.

Ahantarig, A., Trinachartvanit, W., Baimai, V., Grubhoffer, L., 2013. Hard ticks and their bacterial endosymbionts (or would be pathogens). Folia Microbiol. (Praha) 58(5), 419–28.

Ahn, J.H., Kim, B.Y., Song, J., Weon, H.Y., 2012. Effects of PCR cycle number and DNA polymerase type on the 16S rRNA gene pyrosequencing analysis of bacterial communities. J. Microbiol. 50(6), 1071–1074.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J Mol Biol 515(3), 403-410.

Andreotti, R., Perez deLeon, A.A., Dowd, S.E., Guerrero, F.D., Bendele, K.G., Scoles, G.A., 2011. Assessment of bacterial diversity in the cattle tick *Rhipicephalus* (*Boophilus*) *microplus* through tag-encoded pyrosequencing. BMC Microbiol. 11(1), 6.

Angus, B.M., 1996. The history of the cattle tick *Boophilus microplus* in Australia and achievement in its control. Int. J. Parasitol. 26(12), 1341–1355.

Aravena-Román, M., Merritt, A., Inglis, T.J., 2015. First case of *Francisella* bacteraemia in Western Australia. New Microbes New Infect. 8, 75–77.

Barker, S.C., Walker, A.R., 2014. Ticks of Australia. The species that infest domestic animals and humans. Zootaxa 3816, 1-144

Beati, L., Klompen, H., 2019. Phylogeography of Ticks (Acari: Ixodida). Annu. Rev. Entomol. 64, 379–397.

Bokulich, N.A., Kaehler, B.D., Rideout, B.A., Dillon, M., Bolyen, E., Knight, R., Huttley, G.A., Caporaso, J.G., 2018. Optimizing taxonomic classification of marker gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 6, 90.

Bonnet, S., Michelet, L., Moutailler, S., Cheval, J., Hebert, C., Vayssier-Taussat, M., Eloit, M., 2014. Identification of parasitic communities within European ticks using next-generation sequencing. PLoS Negl. Trop. Dis. 8(3), e2753.

Bonnet, S.I., Binetruy, F., Hernández-Jarguín, A.M., Duron, O., 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. Front. Cell. Infec. Microbiol. 7, 236.

Bosler, E.M., Ormiston, B.G., Coleman, J.L., Hanrahan, J.P., Benach, J.L., 1984. Prevalence of the Lyme Disease spirochete in populations of white-tailed deer and white-footed Mice. Yale J. Biol. Med. 57(4), 651–659.

Burnard, D., Shao, R., 2019. Mitochondrial genome analysis reveals intraspecific variation within Australian hard tick species. Ticks Tick Borne Dis. 10(3), 677–681.

Cafiso, A., Sassera, D., Romeo, C., Serra, V., Hervet, C., Bandi, C., Plantard, O., Bazzocchi, C., 2019. *Midichloria mitochondrii*, endosymbiont of *Ixodes ricinus*: evidence for the transmission to the vertebrate host during the tick blood meal. Ticks Tick Borne Dis. 10(1), 5–12.

Callow, L.L., 1984. Animal Health in Australia. Volume 5: Protozoal and Rickettsial Diseases. Australian Government Publishing Services, Canberra, Australia.

Carthey, A.J.R., Banks, P.J., 2011. When does an alien become a native species? A vulnerable native mammal recognizes and responds to its long-term alien predator. PLoS One

7(2), e31804.
Carpi, G., Cagnacci, F., Wittekindt, N.E., Zhao, F., Qi, J., Tomsho, L.P., Drautz, D.I., Rizzoli, A., Schuster, S.C., 2011. Metagenomic profile of the bacterial communities associated with *Ixodes ricinus* ticks. PLoS One 6(10), e25604.
Chalada, M.J., Stenos, J., Bradbury, R.S., 2016. Is there a Lyme-like disease in Australia? Summary of findings to date. One Health 2, 42–54.
Chalada, M.J., Stenos, J., Vincent, G., Barker, D., Bradbury, R.S., 2018. A molecular survey of tick-borne pathogens from ticks collected in central Queensland, Australia. Vector Borne Zoonotic Dis 18, 151–163.
Cooper, A., Stephens, J., Ketheesan, N., Govan, B., 2013. Detection of Coxiella burnetii DNA in Wildlife and Ticks in Northern Queensland, Australia. Vector Borne Zoonotic Dis. 13(3), 12–16.
de la Fuente, J., Antunes, S., Bonnet, S., Cabezas-Cruz, A., Domingos, A.G., Estrada-Pena, A., Johnson, N., Kocan, K.M., Mansfield, K.L., Nijhof, A.M., Papa, A., Rudenko, N., Villar, M., Alberdi, P., Torina, A., Ayllon, N., Vancova, M., Golovchenko, M., Grubhoffer, L., Caracappa, S., Fooks, A.R., Gortazar, C., Rego, R.O.M., 2017. Tick-pathogen interactions and vector competence: Identification of molecular drivers for tick-borne diseases. Front. Cell. Infect. Microbiol. 7, 114.
DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072.
Dobson, A.D.M., Finnie, T.J.R., Randolph, S.E., 2011. A modified matrix model to describe the seasonal population ecology of the European tick *Ixodes ricinus*. J. Appl. Ecol. 48(4), 1017–1028.
Dong, X., Kleiner, M., Sharp, C.E., Thorson, E., Li, C., Liu, D., Strous, M., 2017. Fast and simple analysis of MiSeq amplicon sequencing data with MetaAmp. Front. Microbiol. 8, 1461.
Dybing, N.A., Jacobson, C., Irwin, P., Algar, D., Adams, P.J., 2016. *Bartonella* species identified in rodent and feline hosts from island and mainland Western Australia. Vector Borne Zoonotic Dis. 16(4), 238–44.
Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10(1), 996–968.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26(19), 2640–2461.
Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32(5), 1792–1797.
Edgar, R.C., Flyvbjerg, H., 2015. Error filtering, pair assembly and error correction for next-generation sequencing reads. Bioinformatics 31(21), 3476–82.
Estrada-Pena, A., Gray, J., Kahl, 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, 29.
Ferrell, A., Brinkerhoff, R., 2018. Using landscape analysis to test hypotheses about drivers of tick abundance and infection prevalence with *Borrelia burgdorferi*. Int. J. Environ. Res. Public Health 15(4), 737.
Földvári, G., Jahfari, S., Rigó, K., Jablonszky, M., Szekeres, S., Majoros, G., Tóth, M., Molnár, V., Coipan, E.C., Sprong, H., 2014. *Candidatus* Neoehrlichia mikurensis and

Anaplasma phagocytophilum in Urban Hedgehogs. Emerg. Infect. Dis. 20(3), 496–498.

Fournier, P., Taylor, C., Rolain, J.M., Barrassi, L., Smith, G., Raoult, D., 2007. *Bartonella australis* sp. nov. from kangaroos, Australia. Emerg. Infect. Dis. 13(12), 1961–1963.

Fournier, P.-E., Raoult, D., 2005. Genus II. Rickettsiella Philip 1956, in: Garrity, G.M., Brenner, D.J., Krieg, N.R., Staley, J.T. (Eds.), Bergey's Manual of Systematic Bacteriology, Part B. Springer, New York, pp. 241–247.

Gihring, T.M., Green, S.J., Schadt, C.W., 2012. Massively parallel rRNA gene sequencing exacerbates the potential for biased community diversity comparisons due to variable library sizes. Environ. Microbiol. 14(2), 285–290.

Gofton, A.W., Doggett, S.L., Ratchford, A., Oskam, C.L., Paparini, A., Ryan, U.M., Irwin, P.J., 2015b. Bacterial profiling reveals novel "Ca. Neoehrlichia", *Ehrlichia*, and *Anaplasma* species in Australian human-biting ticks. PLoS One 10(12), e0145449.

Gofton, A.W., Loh, S.M., Barbosa, A.D., Paparini, A., Gillett, A., Macgregor, J., Oskam, C.L., Ryan, U.M., Irwin, P.J., 2018. A novel *Ehrlichia* species in blood and *Ixodes ornithorhynchi* ticks from platypuses (*Ornithorhynchus anatinus*) in Queensland and Tasmania, Australia. Ticks Tick Borne Dis. 9(2), 435–442.

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.M., Irwin, P.J., 2015a. Inhibition of the endosymbiont "*Candidatus* Midichloria mitochondrii" during 16S rRNA gene profiling reveals potential pathogens in Ixodes ticks from Australia. Parasit. Vectors 8, 345.

Gofton, A.W., Waudby, H.P., Petit, S., Greay, T.L., Ryan, U.M., Irwin, P.J., 2017. Detection and phylogenetic characterisation of novel *Anaplasma* and *Ehrlichia* species in *Amblyomma triguttatum* subsp. from four allopatric populations in Australia. Ticks Tick Borne Dis. 8(5), 749–756.

Graves, S.R., Stenos, J., 2017. Tick-borne infectious diseases in Australia. Med. J. Aust. 206(7), 320–324.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Pena, A., Horak, I.G., Shao, R., Barker, S.C., 2010. The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. Zootaxa 2528(1-4), 27.54.

Gundi, V.A., Taylor, C., Raoult, D., La Scola, B., 2009. *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., identified in Australian rats. Int. J. Syst. Evol. Microbiol. 59(Pt 12), 2956–2961.

Gurfield, N., Grewal, S., Cua, L.S., Torres, P.J., Kelley, S.T., 2017. Endosymbiont interference and microbial diversity of the Pacific coast tick, *Dermacentor occidentalis*, in San Diego County, California. PeerJ 5, e3202.

Inokuma, H., Beppu, T., Okuda, M., Shimada, Y., Sakata, Y., 2004. Detection of Ehrlichial DNA in *Haemaphysalis* ticks recovered from dogs in Japan that is slosely related to a novel *Ehrlichia* sp. found in cattle yicks from Tibet, Thailand, and Africa. J. Clin. Microbiol. 42(3), 1353–1355.

Irwin, P.J., Egan, S., Greay, T., Oskam, C.L., 2018. Bacterial tick-associated infections in Australia: current studies and future direction. Microbiol. Aust. 39(4), 200–202.

Irwin, P.J., Robertson, I.D., Westman, M.E., Perkins, M. Straubinger, R.K., 2017. Searching for Lyme borreliosis in Australia: results of a canine sentinel study. Parasit. Vectors 10(1), 114.

Jahfari, S., Ruyts, S.C., Frazer-Mendelewska, E., Jaarsma, R., Verheyen, K., Sprong, H., 2017. Melting pot of tick-borne zoonoses: the European hedgehog contributes to the

maintenance of various tick-borne diseases in natural cycles urban and suburban areas. Parasit. Vectors 10(1), 134.
Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P., 2008. Global trends in emerging infectious diseases. Nature 451(7181), 990–993.
Kaewmongkol, G., Kaewmongkol, S., Burmej, H., Bennett, M.D., Fleming, P.A., Adams, P.J., Wayne, A.F., Ryan, U., Irwin, P.J., Fenwick, S.G., 2011a. Diversity of *Bartonella* species detected in arthropod vectors from animals in Australia. Comp. Immunol. Microbiol. Infect. Dis. 34(5), 411–417.
Kaewmongkol, G., Kaewmongkol, S., McInnes, L.M., Burmej, H., Bennett, M.D., Adams, P.J., Ryan, U., Irwin, P.J., Fenwick, S.G., 2011b. Genetic characterization of flea-derived *Bartonella* species from native animals in Australia suggests host-parasite co-evolution. Infect. Genet. Evol. 11(8), 1868–1872.
Kaewmongkol, G., Kaewmongkol, S., Owen, H., Fleming, P.A., Adams, P.J., Ryan, U., Irwin, P.J., Fenwick, S.G., 2011c. Candidatus Bartonella antechini: a novel *Bartonella* species detected in fleas and ticks from the yellow-footed antechinus (*Antechinus flavipes*), an Australian marsupial. Vet. Microbiol. 149(3-4), 517–521.
Kawahara, M., Rikihisa, Y., Isogai, E., Takahashi, M., Misumi, H., Suto, C., Shibata, S., Zhang, C., Tsuji, M., 2004. Ultrastructure and phylogenetic analysis of "*Candidatus* Neoehrlichia mikurensis" in the family Anaplasmataceae, isolated from wild rats and found in *Ixodes ovatus* ticks. Int. J. Syst. Evol. Microbiol. 54(Pt 5), 1837–1843.
Kembel, S.W., Wu, M., Eisen, J.A., Green, J.L., 2012. Incorporating 16S gene copy number information improves estimates of microbial diversity and abundance. PLoS Comput. Biol. 8(10), e1002743.
Khoo, J.J., Lim, F.S., Chen, F., Phoon, W.H., Khor, C.S., Pike, B.L., Chang, L.Y., AbuBakar, S., 2016. *Coxiella* detection in ticks from wildlife and livestock in Malaysia. Vector Borne Zoonotic Dis. 16(12), 744–751.
King, L.J., 2014. Combating the triple threat: the need for a one health approach, in: Atlas, R.M., Maloy, S. (Eds.), One Health: People, Animals and the Environment. American Society for Microbiology, Washington, pp. 3–16.
Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33(7), 1870–1874.
Kurilshikov, A., Livanova, N.N., Fomenko, N.V., Tupikin, A.E., Rar, V.A., Kabilov, M.R., Livanov, S.G., Tikunova, N.V., 2015. Comparative metagenomic profiling of symbiotic bacterial communities associated with I*xodes persulcatus*, *Ixodes pavlovskyi* and *Dermacentor reticulatus* ticks. PLoS One 10(7), e0131413.
Kurtti, T.J., Palmer, A.T., Oliver, J.H., 2002. *Rickettsiella*-like bacteria in *Ixodes woodi* (Acari: Ixodidae). J. Med. Entomol. 39(3), 534–540.
Kwan, J.Y., Griggs, R., Chicana, B., Miller, C., Swei, A., 2017. Vertical vs. horizontal transmission of the microbiome in a key disease vector, *Ixodes pacificus*. Mol. Ecol. 26(23), 6578–6589.
Landesman, W.J., Mulder, K., Allan, B.F., Bashor, L.A., Keesing, F., LoGiudice, K., Ostfeld, R.S., 2019. Potential effects of blood meal host on bacterial community composition in *Ixodes scapularis* nymphs. Ticks Tick Borne Dis. 10(3), 523-527.
Leclerque, A., Kleespies, R.G., 2012. A *Rickettsiella* bacterium from the hard tick, *Ixodes woodi*: molecular taxonomy combining multilocus sequence typing (MLST) with significance testing. PLoS One 7(5), e38062.
Leclerque, A., Kleespies, R.G., Schuster, C., Richards, N.K., Marshall, S.D.G., Jackson, T.A.,

2012. Multilocus sequence analysis (MLSA) of '*Rickettsiella costelytrae'* and '*Rickettsiella pyronotae*', intracellular bacterial entomopathogens from New Zealand. J. Appl. Microbiol. 113(5), 1228–1237.

Li, A.Y., Adams, P.J., Abdad, M.Y., Fenwick, S.G., 2010. High prevalence of *Rickettsia gravesii* sp. nov. in *Amblyomma triguttatum* collected from feral pigs. Vet. Microbiol. 146(1-2), 59–62.

LoGiudice, K., Ostfeld, R.S., Schmidt, K.A., Keesing, F., 2003. The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk. Proc. Natl. Acad. Sci. U.S.A. 100(2), 567–571.

Loh, S.M., 2018. Identification and characterisation of microorganisms in Australian wildlife tick (PhD thesis). Murdoch University, Perth, Western Australia.

Loh, S.M., Gofton, A.W., Lo, N., Gillett, A., Ryan, U.M., Irwin, P.J., Oskam, C.L., 2016. Novel *Borrelia* species detected in echidna ticks, *Bothriocroton concolor*, in Australia. Parasites & Vectors 9, 339.

Lydecker, H.W., Banks, P.B., Hochuli, D.F., 2019. Counting ticks (Acari: Ixodida) on hosts is complex: a review and comparison of methods. J. Med. Entomol. tjz094.

Machado-Ferreira, E., Vizzoni, V.F., Balsemao-Pires, E., Moerbeck, L., Gazeta, G.S., Piesman, J., Voloch, C.M., Soares, C.A., 2016. *Coxiella* symbionts are widespread into hard ticks. Parasitol. Res. 115(12), 4691–4699.

Mariconti, M., Epis, S., Gaibani, P., Dalla Valle, C., Sassera, D., Tomao, P., Fabbi, M., Castelli, F., Marone, P., Sambri, V., Bazzocchi, C., Bandi, C., 2012. Humans parasitized by the hard tick *Ixodes ricinus* are seropositive to *Midichloria mitochondrii*: is *Midichloria* a novel pathogen, or just a marker of tick bite? Pathog. Glob. Health 106(7), 391–396.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8(4), e61217.

Mediannikov, O., Fenollar, F., 2014. Looking in ticks for human bacterial pathogens. Microb. Pathog. 77, 142–8.

Merrill, M.M., Boughton, R.K., Lord, C.C., Sayler, K.A., Wight, B., Anderson, W.M., Wisely, S.M., 2018. Wild pigs as sentinels for hard ticks: A case study from south-central Florida. Int. J. Parasitol. Parasites Wildl. 7(2), 161–170.

Morens, D.M., Fauci, A.S., 2012. Emerging infectious diseases in 2012: 20 years after the institute of medicine report. MBio 3(6), e00494-12.

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2019. Vegan: community ecology package. R package version 2.5-5

Oskam, C.L., Gofton, A.W., Greay, T.L., Yang, R., Doggett, S., Ryan, U.M., Irwin, P.J., 2017. Molecular investigation into the presence of a *Coxiella* sp. in *Rhipicephalus sanguineus* ticks in Australia. Vet. Microbiol. 201, 141–145.

Ostfeld, R.S., Brunner, J.L., 2015. Climate change and *Ixodes* tick-borne diseases of humans. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 370(1665), 20140051.

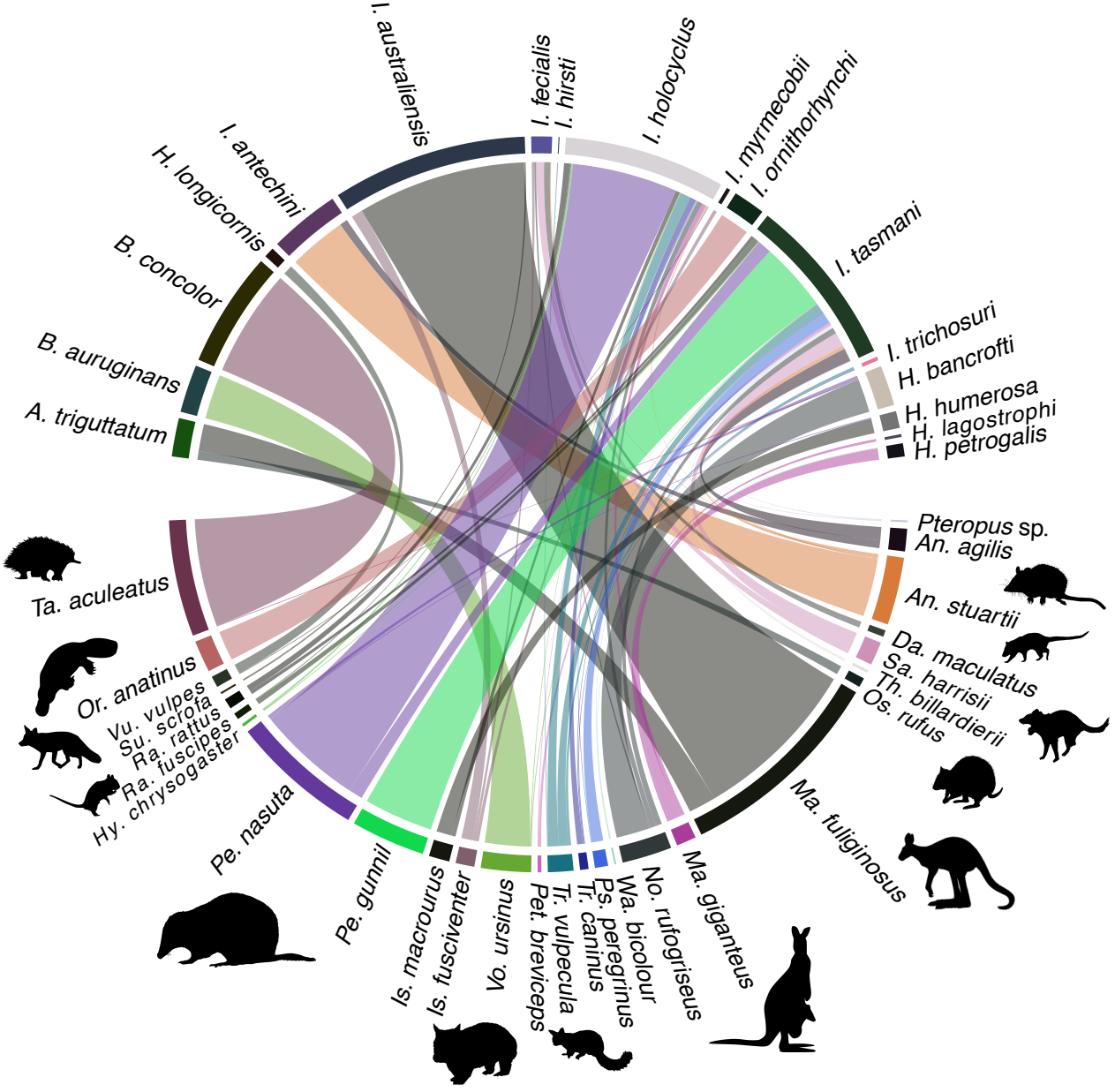
Ostfeld, R.S., Levi, T., Keesing, F., Oggenfuss, K., Canham, C.D., 2018. Tick-borne disease risk in a forest food web. Ecology 99,(7) 1562–1573.

Panetta, J.L., Šíma, R., Calvani, N.E.D., Hajdušek, O., Chandra, S., Panuccio, J., Šlapeta, J., 2017. Reptile-associated *Borrelia* species in the goanna tick (*Bothriocroton undatum*) from Sydney, Australia. Parasit. Vectors 10(1), 616.

Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. 32(6), 897–928.

Pfäffle, M., Littwin, N., Muders, S.V., Petney, T.N., 2013. The ecology of tick-borne diseases. Int. J. Parasitol. 43(12-13), 1059–1077.
Poisot, T., Foster, Z.S.L., Sharpton, T.J., Grünwald, N.J., 2017. Metacoder: An R package for visualization and manipulation of community taxonomic diversity data. PLOS Comput. Biol. 13(2), e1005404.
Ponnusamy, L., Gonzalez, A., Treuren, W.V., Welss, S., Parobek, C.M., Jullano, J.J., Knight, R., Roe, R.M., Apperson, C.S., Meshnick, S.R., 2014. Diversity of *Rickettsiales* in the microbiome of the lone star tick, *Amblyomma americanum*. Appl. Environ. Microbiol. 80(1), 354–359.
Radcliffe, J., Rusjakovski, N., Forkert, J., Jones, M., Stewart, C., 2016. Growing evidence of an emerging tick-borne disease that causes a Lyme-like illness for many Australian patients. Community Affairs References Committee, Canberra, Australia.
Rinker, D.C., Pitts, R.J., Zwiebel, L.J., 2016. Disease vectors in the era of next generation sequencing. Genome Biol. 17(1), 95.
Roberts, F.S.H., 1970. Australian Ticks. CSIRO, Melbourne.
Rogers, R.J., Shiels, I.A., 1979. Epidemiology and control of anaplasmosis in Australia. J. S. Afr. Vet. Assoc. 50(4), 363–366.
Rosselli, R., Romoli, O., Vitulo, N., Vezzi, A., Campanaro, S., de Pascale, F., Schiavon, R., Tiarca, M., Poletto, F., Concheri, G., Valle, G., Squartini, A., 2016. Direct 16S rRNA-seq from bacterial communities: a PCR-independent approach to simultaneously assess microbial diversity and functional activity potential of each taxon. Sci. Rep. 6, 32165.
Roux, V., Raoult, D., 1995. Phylogenetic analysis of the genus *Rickettsia* by 16S rDNA sequencing. Res. Microbiol. 146(5), 385–396.
RStudio Team, 2015. RStudio: Integrated Development for R. RStudio Inc, Boston, MA.
Scoles, G.A., 2004. Phylogenetic analysis of the *Francisella*-like endosymbionts of *Dermacentor* ticks. J. Med. Entomol. 41(3), 277–286.
Serra, V., Cafiso, A., Formenti, N., Verheyden, H., Plantard, O., Bazzocchi, C., Sassera, D., 2018. Molecular and serological evidence of the presence of *Midichloria mitochondrii* in roe deer (*Capreolus capreolus*) in France. J. Wildl. Dis. 54(3), 597–600.
Sperling, J.L., Silva-Brandão, K.L., Brandão, M.M., Lloyd, V.K., Dang, S., Davis, C.S., Sperling, F.A.H., Magor, K.E., 2017. Comparison of bacterial 16S rRNA variable regions for microbiome surveys of ticks. Ticks Tick Borne Dis. 8(4), 453–461.
Špitalská, E., Sparagano, O., Stanko, M., Schwarzová, K., Špitalský, Z., Škultéty, Ľ., Havlíková, S.F., 2018. Diversity of *Coxiella*-like and *Francisella*-like endosymbionts, and *Rickettsia* spp., *Coxiella burnetii* as pathogens in the tick populations of Slovakia, Central Europe. Ticks Tick Borne Dis. 9(5), 1207–1211.
Stothard, D.R., Fuerst, P.A., 1995. Evolutionary analysis of the spotted fever and thyphus groups of *Rickettsia* using 16S rRNA gene sequences. Syst. Appl. Microbiol. 18(1), 52–61.
Sumrandee, C., Baimai, V., Trinachartvanit, W., Ahantarig, A., 2016. Molecular detection of *Rickettsia*, *Anaplasma*, *Coxiella* and *Francisella* bacteria in ticks collected from Artiodactyla in Thailand. Ticks Tick Borne Dis. 7(5), 678-689.
Swei, A., Kwan, J.Y., 2017. Tick microbiome and pathogen acquisition altered by host blood meal. ISME J. 11(3), 813–816.
Szigeti, A., Kreizinger, Z., Hornok, S., Abichu, G., Gyuranecz, M., 2014. Detection of *Francisella*-like endosymbiont in *Hyalomma rufipes* from Ethiopia. Ticks Tick Borne

Dis. 5(6), 818–820.
Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Mol. Biol. Evol. 9(4), 678–687.
Tessler, M., Neumann, J.S., Afshinnekoo, E., Pineda, M., Hersch, R., Velho, L.F.M., Segovia, B.T., Lansac-Toha, F.A., Lemke, M., DeSalle, R., Mason, C.E., Brugler, M.R., 2017. Large-scale differences in microbial biodiversity discovery between 16S amplicon and shotgun sequencing. Sci. Rep. 7(1), 6589.
Tomassone, L., Berriatua, E., De Sousa, R., Duscher, G.G., Mihalca, A.D., Silaghi, C., Sprong, H., Zintl, A., 2018. Neglected vector-borne zoonoses in Europe: Into the wild. Vet. Parasitol. 251, 17–26.
Turner, S., Pryer, K.M., Miao, V.P., Palmer, J.D., 1999. Investigating deep phylogenetic relationships among cyanbacteria and plastids by small subunit rRNA sequence analysis. J Eukaryot Microbiol 46(4), 327–338.
Vayssier-Taussat, M., Kazimirova, M., Hubalek, Z., Hornok, S., Farkas, R., Cosson, J.F., Bonnet, S., Vourch, G., Gasqui, P., Mihalca, A.D., Plantard, O., Silaghi, C., Cutler, S., Rizzoli, A., 2015. Emerging horizons for tick-borne pathogens: from the 'one pathogen-one disease' vision to the pathobiome paradigm. Future Microbiol. 10(12), 2033–2043.
Vilcins, I.-M., Fournier, P., Old, J.M., Deane, E., 2009a. Evidence for the presence of *Francisella* and spotted fever group *Rickettsia* DNA in the tick *Amblyomma fimbriatum* (Acari: Ixodidae), Northern Territory Australia. J. Med. Entomol. 46(4), 926–33.
Vilcins, I.M., Kosoy, M., Old, J.M., Deane, E.M., 2009b. *Bartonella*-like DNA detected in *Ixodes tasmani* ticks (Acari: Ixodida) infesting Koalas (*Phascolarctos cinereus*) in Victoria, Australia. Vector Borne Zoonotic Dis. 9(5), 499–503.
Vilcins, I.M., Old, J.M., Deane, E., 2009c. Molecular detection of *Rickettsia*, *Coxiella* and *Rickettsiella* DNA in three native Australian tick species. Exp. Appl. Acarol. 49(3), 229–242.
Wang, B., Wan, L., Wang, A., Li, L.M., 2017. An adaptive decorrelation method removes Illumina DNA base-calling errors caused by crosstalk between adjacent clusters. Sci. Rep. 7, 41348.
Wang, Y., Mao, L., Sun, Y., Wang, Z., Zhang, Jiayong, Zhang, Jibo, Peng, Y., Xia, L., 2018. A Novel *Francisella*-Like endosymbiont in *Haemaphysalis longicornis* and *Hyalomma asiaticum*, China. Vector Borne Zoonotic Dis. 18(12), 669–676.
Woods, R., Reiss, A., Cox-Witton, K., Grillo, T., Peters, A., 2019. The importance of wildlife disease monitoring as part of global surveillance for zoonotic diseases: the role of Australia. Trop. Med. Infect. Dis. 4(1), 29.
Zhang, X., Yang, Z., Lu, B., Ma, X., Zhang, C., Xu, H., 2014. The composition and transmission of microbiome in hard tick, I*xodes persulcatus*, during blood meal. Ticks Tick Borne Dis. 5(6), 864–870.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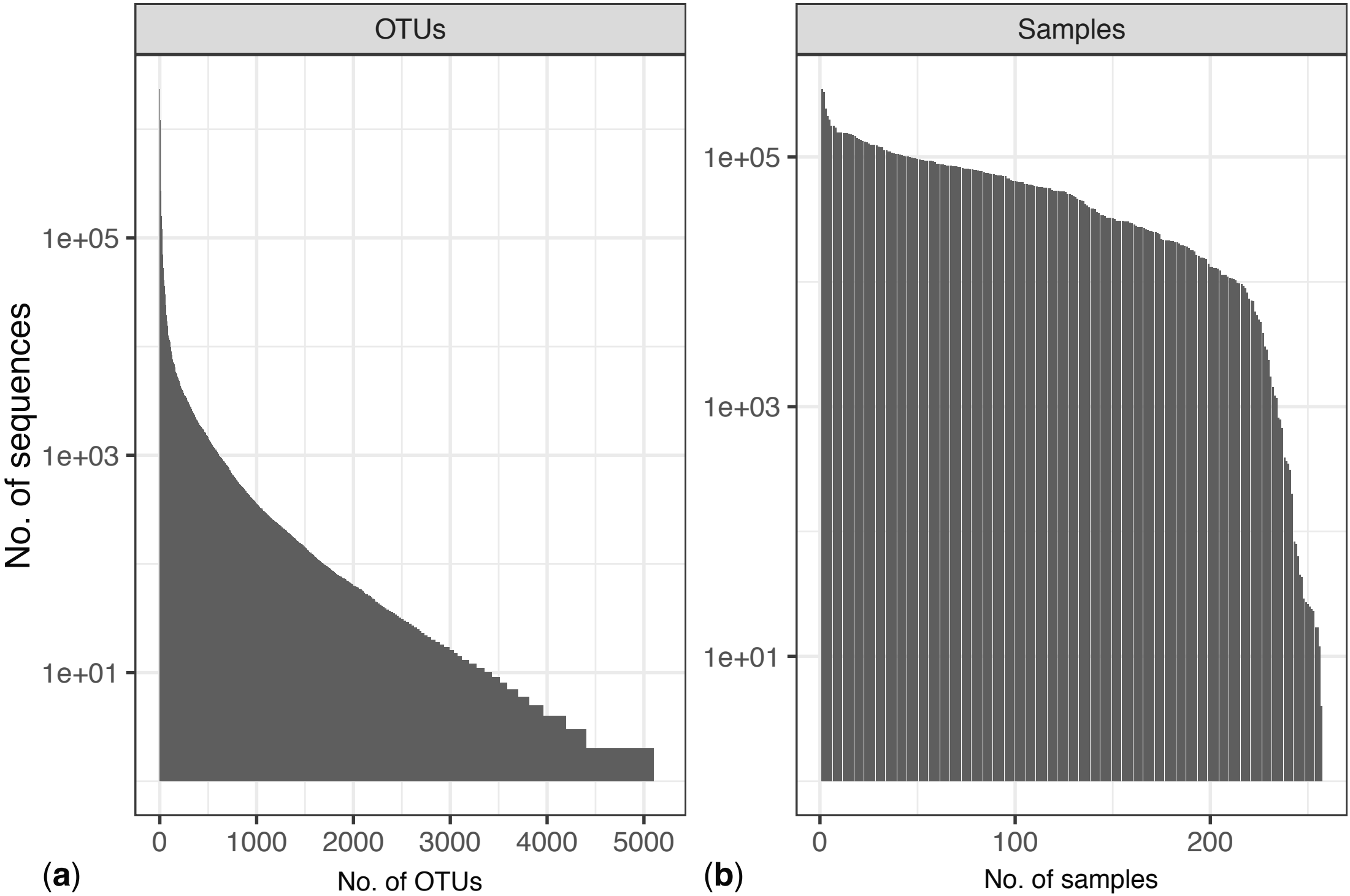
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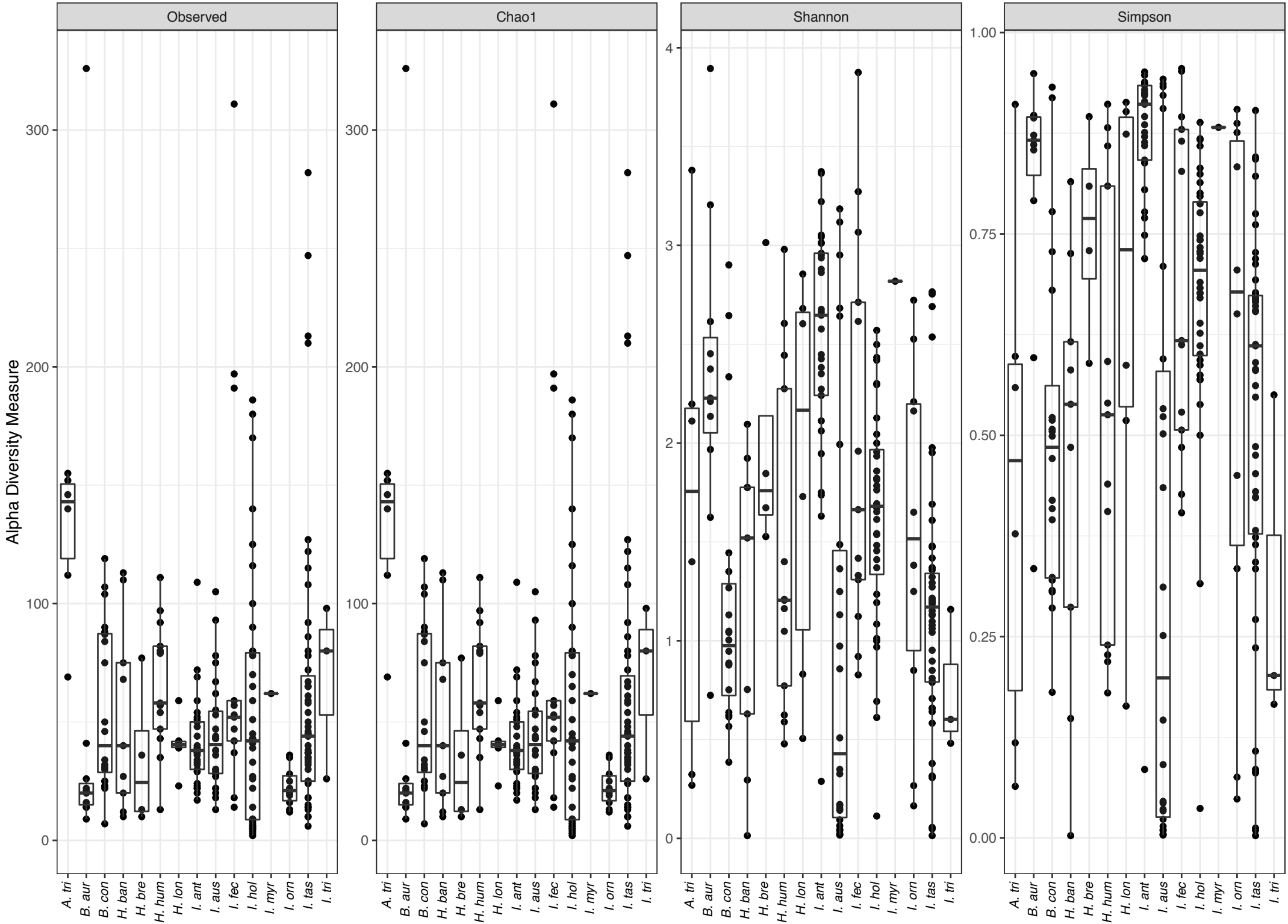
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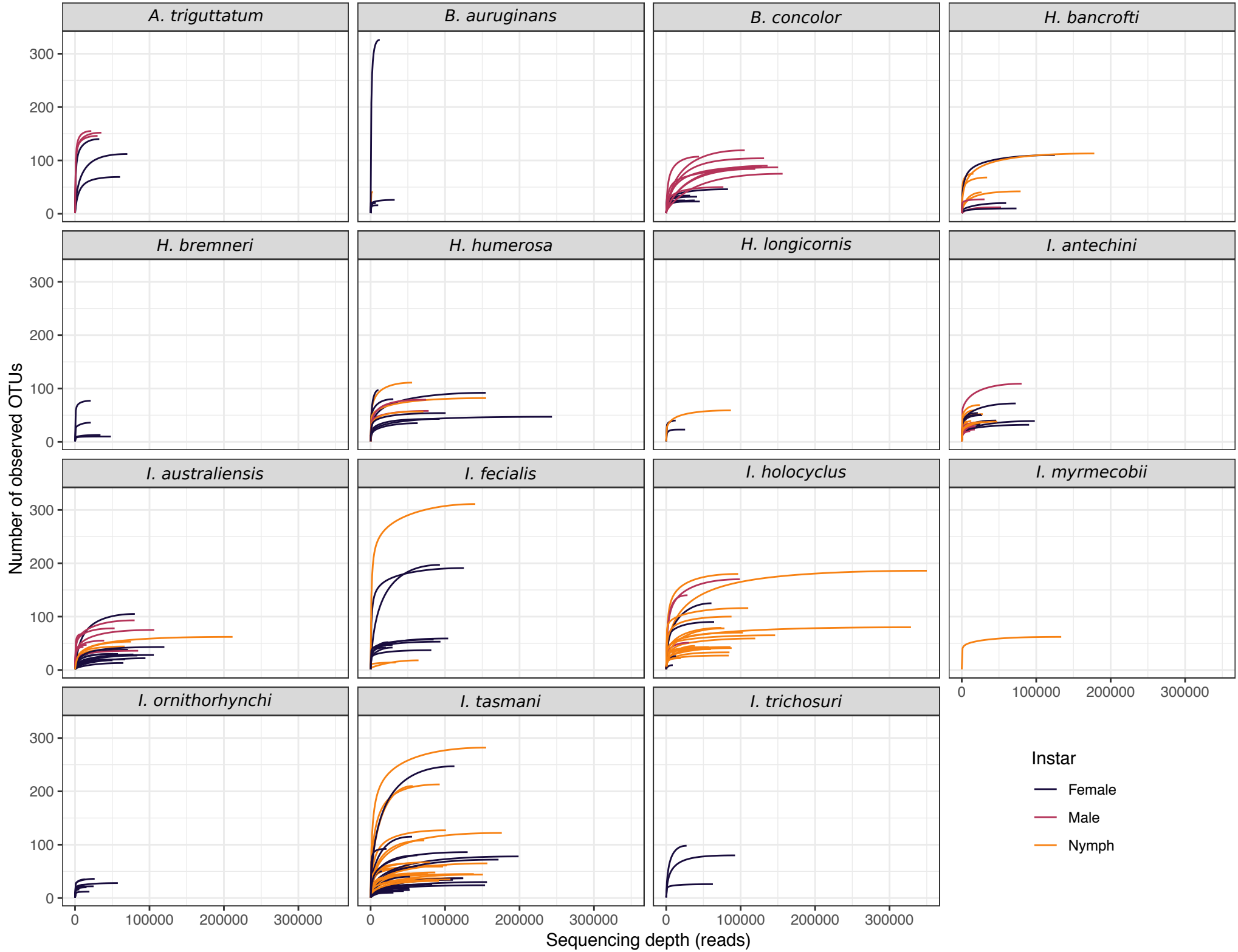
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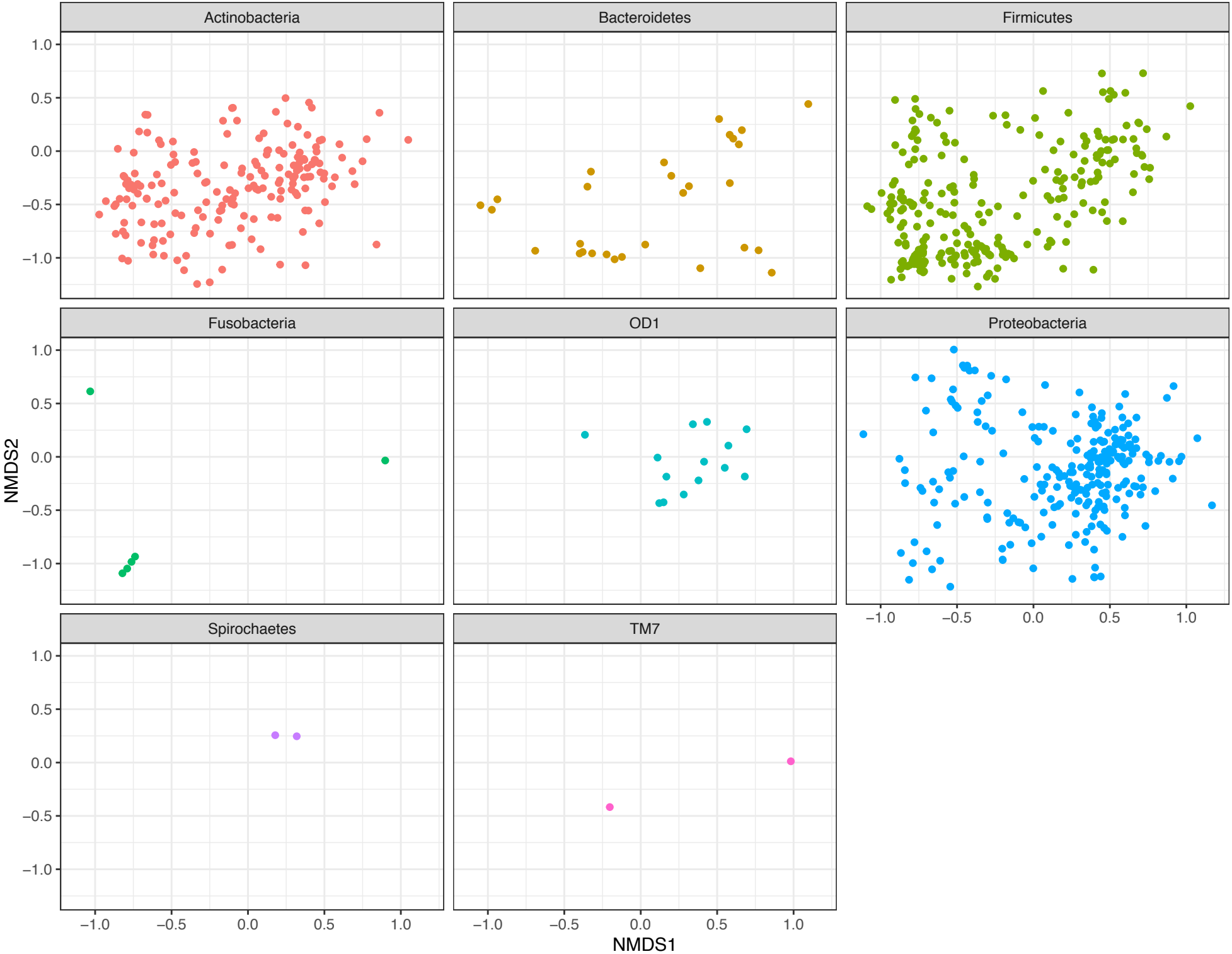
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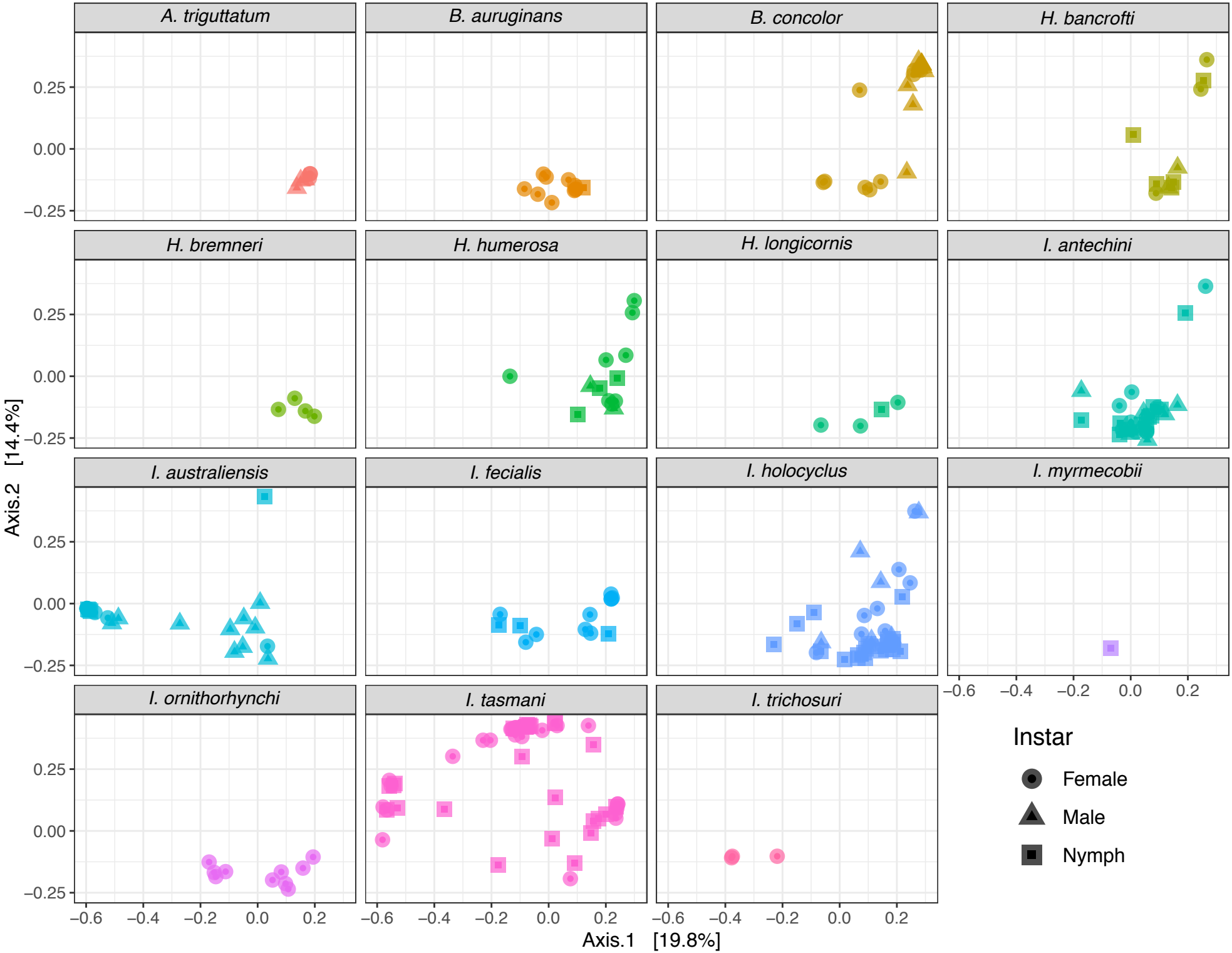
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I. ornithorhynchi
I. tasmani
I. trichosuri
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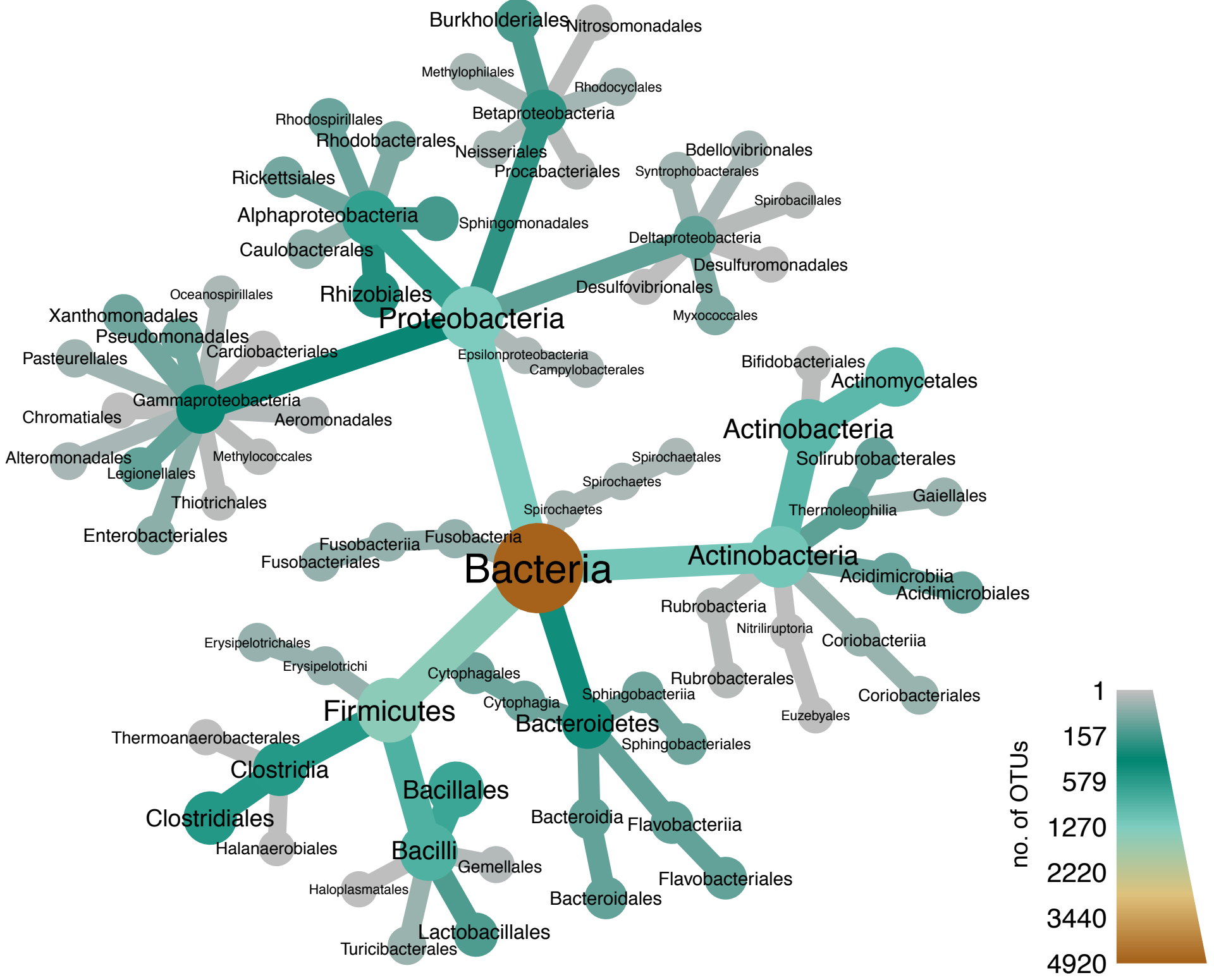
Male
Nymph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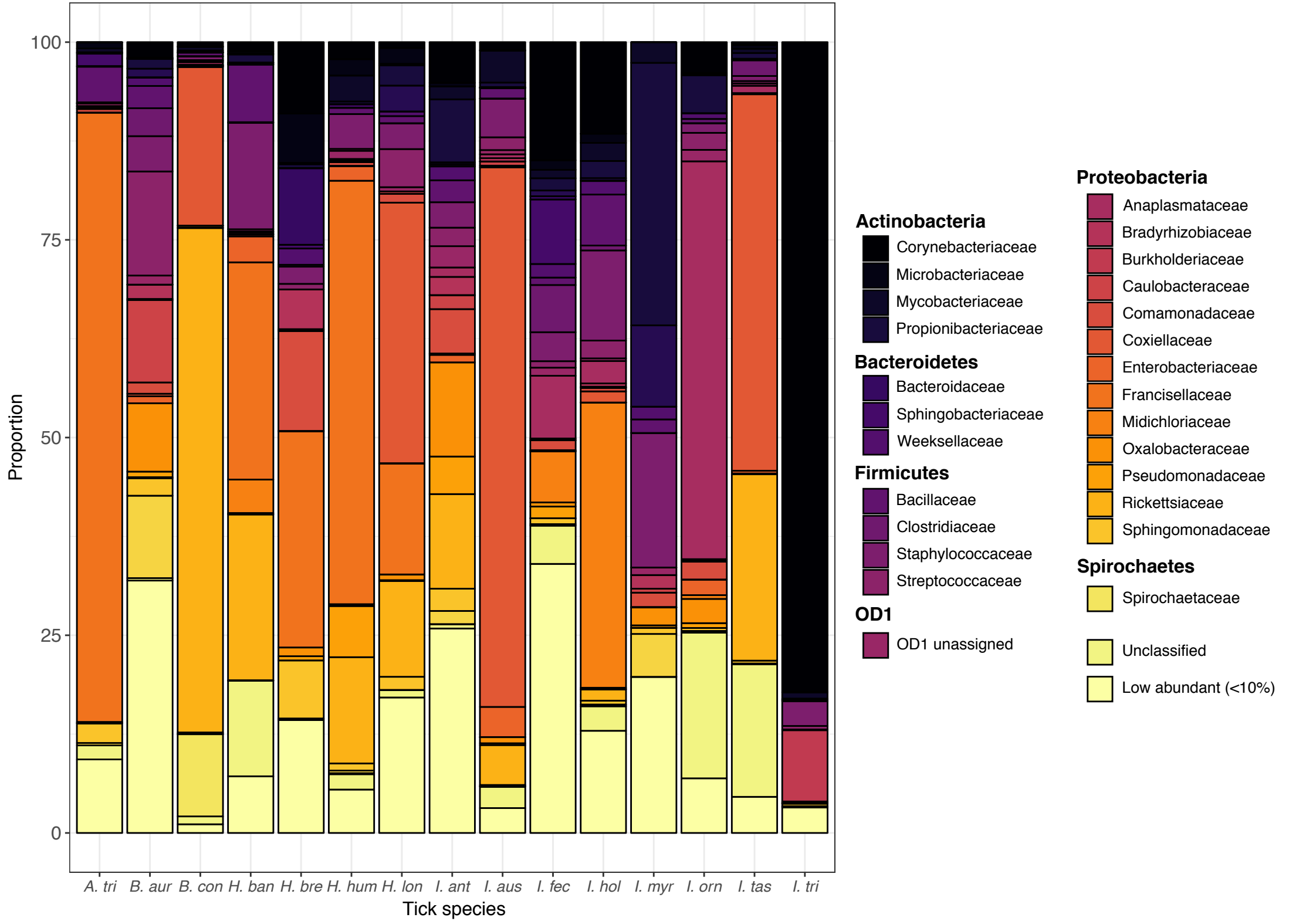
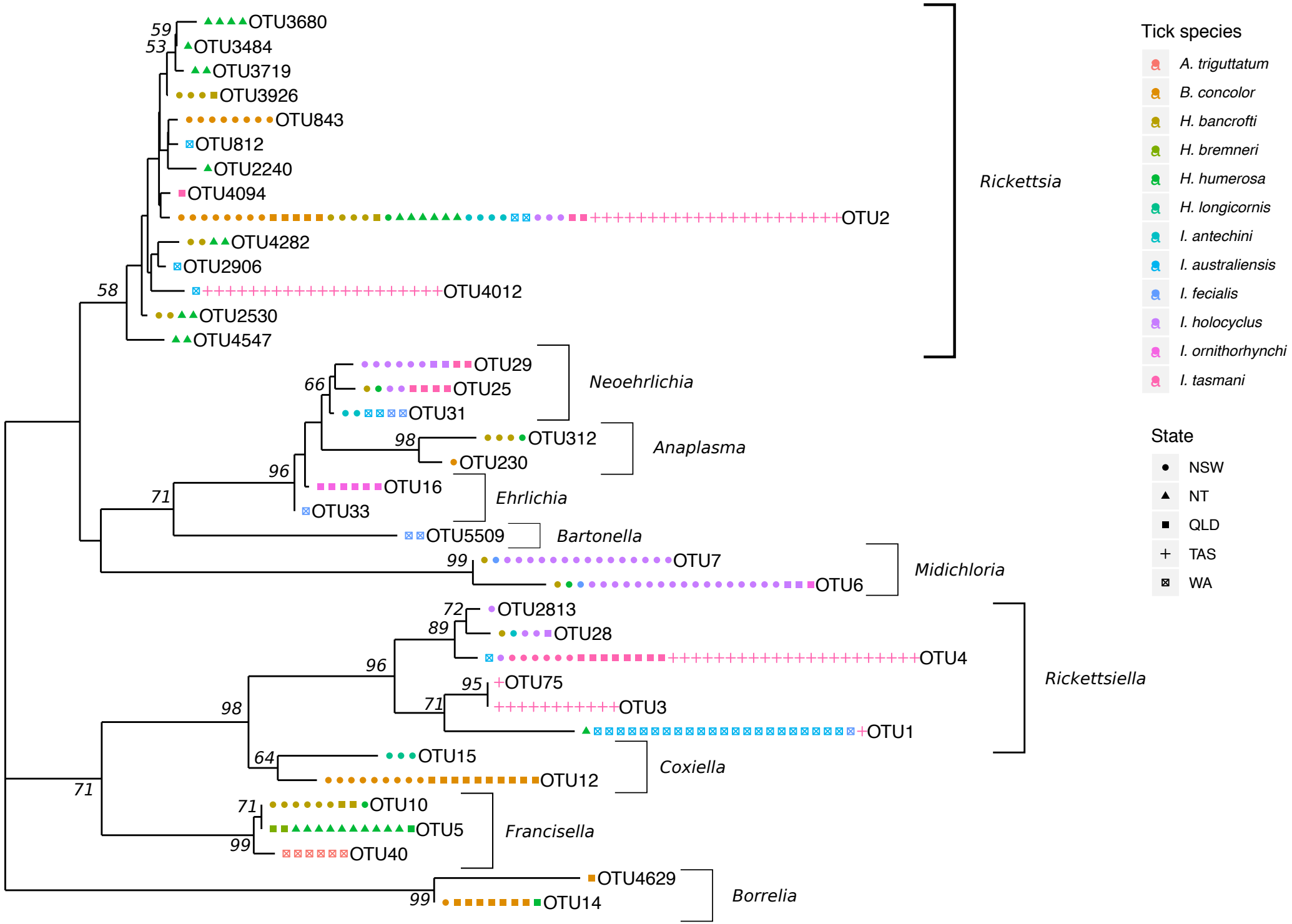
4920


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
59
53
58
Rickettsia
OTU29
OTU25
OTU31
66
Neoehrlichia
OTU312
OTU230
98
Anaplasma
96
OTU16
OTU33
71
Ehrlichia
OTU5509
Bartonella
99
OTU7
OTU6
Midichloria
OTU2813
OTU28
72
89
OTU4
96
OTU75
95
OTU3
71
OTU1
Rickettsiella
98
OTU15
OTU12
64
Coxiella
71
OTU10
OTU5
OTU40
71
99
Francisella
OTU4629
OTU14
99
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