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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 and Tick-Borne Diseases, 8(5), 749–756. https://doi.org/10.1016/j.ttbdis.2017.05.009
Document Version: Author's Version

Published Version: https://doi.org/10.1016/j.ttbdis.2017.05.009

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PII: S1877-959X(17)30157-7
DOI: http://dx.doi.org/doi:10.1016/j.ttbdis.2017.05.009
Reference: TTBDIS 835

To appear in:

Received date: 4-4-2017
Revised date: 16-5-2017
Accepted date: 22-5-2017

Please cite this article as: Gofton, Alexander W., Waudby, Helen P., Petit, Sophie, Greay, Telleasha L., Ryan, Una M., Irwin, Peter J., Detection and phylogenetic characterisation of novel Anaplasma and Ehrlichia species in Amblyomma triguttatum subsp.from four allopatric populations in Australia.Ticks and Tick-borne Diseases http://dx.doi.org/10.1016/j.ttbdis.2017.05.009

Detection and phylogenetic characterisation of novel *Anaplasma* and *Ehrlichia* species in *Amblyomma triguttatum* subsp. from four allopatric populations in Australia

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Abstract

Anaplasma and *Ehrlichia* spp. are tick-borne pathogens that can cause severe disease in domestic animals, and several species are responsible for emerging zoonoses in the northern hemisphere. Until recently, the only members of these genera reported in Australia (*A. marginale*, *A. centrale*, and *A. platys*) were introduced from other continents, through the importation of domestic animals and their associated ticks. However, unique *Anaplasma* and *Ehrlichia* 16S rRNA gene sequences were recently detected for the first time in native Australian ticks, particularly in *Amblyomma triguttatum* subsp. ticks from southwest Western Australia (WA). We used molecular techniques to survey *Am. triguttatum* subsp. ticks from four allopatric populations in southern and western Australia for *Anaplasma* and *Ehrlichia* species, and described here the phylogeny of these novel organisms. An *A. bovis* variant (genotype Y11) was detected in ticks from two study sites; Yanchep National Park (12/280, 4.3%) and Barrow Island (1/69, 1.4%). Phylogenetic analysis of 16S rRNA and *groEL* gene sequences concluded that *A. bovis* genotype Y11 is a unique genetic variant, distinct from other *A. bovis* isolates worldwide. Additionally, a novel *Ehrlichia* species was detected in *Am. triguttatum* subsp. from three of the four study sites; Yanchep National Park (18/280, 6.4%),

Bungendore Park (8/46, 17.4%), and Innes National Park (9/214, 4.2%), but not from Barrow Island. Phylogenetic analysis of 16S, *groEL*, *gltA*, and *map1* gene sequences revealed that this *Ehrlichia* sp. is most closely related to, but clearly distinct from, *E. ruminantium* and *Ehrlichia* sp. Panola Mountain. We propose to designate this new species '*Candidatus* Ehrlichia occidentalis'. *Anaplasma bovis* genotype Y11 and *'Candidatus* E. occidentalis' are the first *Anaplasma* and *Ehrlichia* species to be recorded in native Australian ticks.

Abbreviations

Ca: *Candidatus*; 16S: 16S rRNA gene; WA: Western Australia; SA: South Australia; *groEL*: 60kDa heat shock chaperonin gene; *gltA*: citrate synthase gene; *map1*: major antigenic protein 1 gene; SNP: single nucleotide polymorphism; Mtn: Mountain; NP: National Park

Keywords: *Ehrlichia*; ; ; ; , *Anaplasma*, Australia, *Amblyomma triguttatum*, tick-borne disease**1. Introduction**

Members of the bacterial genera *Anaplasma*, *Ehrlichia*, and '*Candidatus* (*Ca*.) Neoehrlichia' (Family Anaplasmataceae) are obligate intracellular mammalian pathogens that are vectored by ixodids (hard ticks) (Rar and Golovljova, 2011). In nature, these bacteria persist in wildlife reservoir hosts and circulate in mammal-tick-mammal transmission cycles, where ticks act only as vectors and not as reservoirs (Rar and Golovljova, 2011). In mammals, *Anaplasma*, *Ehrlichia*, and '*Ca.* Neoehrlichia' species invade haematopoietic or endothelial cells, where they form and multiply within intracytoplasmic vacuoles (Rar and Golovljova, 2011; Rikihisa, 1991).

Many wildlife reservoirs can sustain asymptomatic or subclinical *Anaplasma* and *Ehrlichia* infections; however, transmission (via tick-bite) to naïve hosts such as humans and domestic animals can result in serious illness, including anaemia, fever, headache, muscle

pains, nausea, rash, and in severe cases, death (Rar and Golovljova, 2011; Rikihisa, 1991). Several species, such as *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, and '*Ca*. Neoehrlichia mikurensis', are responsible for emerging zoonotic diseases (Colwell et al., 2011; Paddock and Childs, 2003; Parola et al., 2005; Silaghi et al., 2015; Stuen, 2007).

Until recently, it was unknown whether Australia had any native *Anaplasma*, *Ehrlichia*, or '*Ca*. Neoehrlichia' species (Angus, 1996). Since the arrival of Europeans on the Australian continent nearly 250 years ago, three *Anaplasma* species (*A. marginale*, *A. centrale*, and *A. platys*) have been introduced through the importation of domestic animals and their associated ticks (*Haemaphysalis longicornis*, *Rhipicephalus australis*, and *R. sanguineus* sensu lato) (Angus, 1996; Callow, 1984). However, in Australia, these introduced *Anaplasma* species have only been reported in domestic animals, are only transmitted by introduced ixodids, and to the authors' knowledge have never been detected in native wildlife or enzootic ixodids (Angus, 1996; Callow, 1984).

Australia has unique tick and mammal fauna that have co-evolved in relative isolation since the breakup of the Gondwana landmass ~100 million years ago (Upchurch, 2008). Indeed, phylogenetic analysis of ixodids has shown some Australian species to be evolutionarily distant from northern hemisphere ixodids (Xu et al., 2003). Importantly, Australia is free from all members of the *Ixodes ricinus/persulcatus* species complex (Barker and Walker, 2014; Roberts, 1970), responsible for the transmission of well-described pathogens, including *Anaplasma* and *Ehrlichia* spp., in other parts of the world.

Recently, bacterial profiling studies of native Australian ixodids based on next-generation bacterial 16S rRNA gene (16S) metabarcoding, have revealed novel *Anaplasma*, *Ehrlichia*, and '*Ca*. Neoehrlichia' species that appear to be unique to Australia (Gofton et al., 2015a; Gofton et al., 2015b). For example, two new species, '*Ca*. Neoehrlichia australis' and '*Ca*. Neoehrlichia arcana', were recently identified in the Australian paralysis tick, *Ixodes holocyclus*, in eastern Australia (Gofton et al., 2015a; Gofton et al., 2015b), and further characterised by PCR and Sanger sequencing (Gofton et al., 2015a; Gofton et al., 2016).

Phylogenetic analyses of these '*Ca*. Neoehrlichia' species demonstrated that they are closely related to the emerging zoonotic pathogen '*Ca*. Neoehrlichia mikurensis' that occurs in Europe and Asia (Gofton et al., 2016). '*Candidatus* Neoehrlichia australis' and '*Ca*. Neoehrlichia arcana' were also highly prevalent (8.7% and 3.1%, respectively) in *I. holocyclus* populations from its entire enzootic range (Gofton et al., 2015a; Gofton et al., 2016).

In Gofton et al. (2015a), novel *Anaplasma* and *Ehrlichia* 16S gene sequences were identified in the ornate kangaroo tick, *Amblyomma* (*Am.*) *triguttatum* subsp., from southwest Western Australia (WA). Preliminary analysis of partial 16S sequences from these bacteria suggested they are closely related to *A. bovis* and *E. ruminantium*, respectively, both of which have never been reported in Australia. Although a preliminary analysis of 16S gene sequences has been performed, detailed studies to confirm the taxonomic status of the novel *Anaplasma* and *Ehrlichia* from *Am. triguttatum* subsp. ticks are lacking.

The Australian native ixodid *Am. triguttatum* has several subspecies that are distributed through most of the Australian continent, and parasitise a wide variety of native and introduced fauna. In its enzootic range, this tick frequently bites domestic and companion animals, and is one of the most common ticks to bite people, especially in southwest WA (Gofton, et al. 2015a; Greay et al. 2016).

In the present study, molecular techniques were used to identify and characterise *Anaplasma* and *Ehrlichia* species in four allopatric populations of questing *Am. triguttatum* subsp. ticks from southern, south-western, and north-western Australia.

2. Methods

2.1 Tick collection and identification

Two subspecies of *Am. triguttatum* (*Am. t. triguttatum* and *Am. t. ornatissimum*) were collected from four allopatric populations across South and Western Australia; Yanchep National Park (NP) (n=280), Bungendore Park (n=46), Innes NP (n=214), and Barrow Island

(*n*=69) (Fig. 1). A summary of *Am. triguttatum* subsp. life stages collected at each location is presented in Table 1.

Questing *Am. t. triguttatum* were collected from Yanchep NP and Bungendore Park from October-November 2016 by flagging the ground and low-lying vegetation with a 1 m^2 white flannel cloth stretched between two wooden dowels. Carbon dioxide (dry ice) baits were also used to attract ticks to flagging sites. Questing *Am. t. triguttatum* were collected from Innes NP on the southern tip of Yorke Peninsula, South Australia (SA) in January-December 2006, as part of a survey investigating the seasonal density and distribution of this tick in the area (Waudby and Petit, 2007). Questing *Am. t. ornatissimum* (*n*=69) were collected opportunistically from the ground at the Barrow Island Nature Reserve from May-June 2016.

After collection, ticks were placed directly into 70% ethanol and stored at ambient temperature until molecular analysis. Ticks were morphologically identified into species, life stage, and sex under a stereomicroscope using standard keys for Australian ticks (Barker and Walker, 2014; Roberts, 1970).

2.2 DNA extraction and PCR

Prior to DNA extraction, the ticks' external surface was decontaminated in 10% sodium hypochlorite, washed in 70% ethanol, rinsed in sterile and DNA-free PBS, and air-dried. Individual ticks were then snap-frozen in liquid nitrogen for 1 min, and pulverised in a 2 ml microtube containing a 5 mm steel bead by beating at 40 Hz for 1 min. DNA was purified from tick homogenates using the DNeasy Blood and Tissue kit (QIAGEN) following the manufacturer's recommendations. Extraction reagent blank controls were included alongside DNA extractions. DNA extraction, PCR setup and DNA handling procedures were all performed in physically contained and separate dedicated laboratory areas, and PCR and post-PCR procedures were performed in separate dedicated laboratories.

All *Am. triguttatum* subsp. DNA samples were screened for *Anaplasma* and *Ehrlichia* DNA using several PCR assays targeting the phylogenetically informative 16S, 17 kDa heat

shock protein (*groEL*), citrate synthase (*gltA*), and Major Antigenic Protein 1 (*map1*) genes (Table S1) (Anderson et al., 1991; Gofton et al., 2016; Kawahara et al., 2004; Liz et al., 2000; Loftis et al., 2006; Paddock et al., 1997; Rar et al., 2010; Weisburg et al., 1991). All PCR assays were performed in 25 µl volumes containing PCR buffer (KAPA Biosystems), 2.5 mM $MgCl_2$, 1 mM dNTPs, 400 nM of each primer, and 0.5 U KAPA Taq DNA polymerase (KAPA Biosystems). Primary PCRs used 2 µl of genomic DNA as template, and nested and hemi-nested PCRs used 1 µl of primary product as a template. All PCRs included no-template controls. All PCRs were performed with an initial denaturation at 95°C for 3 min, followed by denaturation, annealing, and extension cycles as outlined in Table S1, followed by a final extension at 72°C for 5 min.

All PCR products were electrophoresed through 1-2% agarose gels stained with SYBR safe (Invitrogen), and amplicons of the correct length were excised from the gels and purified with the QIAquick gel extraction kit (QIAGEN) following the manufacturers recommendations. Resulting purified amplicons were sequenced with both PCR primers on an ABI 3730 96 DNA Analyser using Big Dye v3.1 terminators (Life Technologies).

2.3 Phylogenetic analysis

16S, *groEL*, *gltA*, and *map1* nucleotide sequences were aligned with sequences from related *Anaplasma* and *Ehrlichia* species retrieved from GenBank. Sequences were aligned with MAFFT (Katoh et al., 2002), trimmed to remove terminal gaps, and alignments were refined with MUSCLE (Edgar, 2004). Statistical selection of the most suitable nucleotide substitution model based on the Bayesian information criteria as performed with jModelTest2 (Darriba et al., 2012; Guindon and Gascuel, 2003) for each sequence alignment, and Bayesian phylogenetic reconstructions were produced from alignments using MrBayes (Ronquist and Huelsenbeck, 2003) on the CIPRES Science Gateway (Miller, 2010), with an MCMC length of 1,100,000, burn-in of 10,000, and subsampling every 200 iterations.

3. Results

3.1 Detection of *Ehrlichia* and *Anaplasma* in *Amblyomma triguttatum* subsp.

A total of 609 questing *Am. triguttatum* subsp. nymphs and adults collected across the four study sites (Table 1; Fig. 1), were screened for the presence of *Anaplasma* and *Ehrlichia* DNA, using a variety of PCR assays targeting the 16S, *groEL*, *gltA*, and *map1* genes (Table S1). All gene sequences generated in the present study were deposited in GenBank under accessions KY425419 - KY425526.

Ehrlichia DNA was detected in questing *Am. t. triguttatum* from Yanchep NP, Bungendore Park, and Innes NP, but not in *Am. t. ornatissimum* from Barrow Island. In Yanchep NP *Ehrlichia* DNA was detected in 18 (6.4%) *Am. t. triguttatum* individuals, including one male (0.4%), three females (1.1%), and 14 nymphs (5%). Eight (17.4%) *Am. t. triguttatum* nymphs from Bungendore Park contained *Ehrlichia* DNA; no males or females were infected. In the Innes NP population nine (4.2%) individuals contained *Ehrlichia* DNA including one male (0.5%), four females (0.9%), and four nymphs (0.9%).

Anaplasma DNA was detected in only two of the study sites: Yanchep NP (in *Am. t. triguttatum*) and Barrow Island (in *Am. t. ornatissimum*). In Yanchep NP *Anaplasma* DNA was identified in 12 (4.3%) individuals, including 11 (3.9%) nymphs and one female (0.35%), while on Barrow Island *Anaplasma* DNA was only detected in a single *Am. t. ornatissimum* female (1.4%). Co-infections with both *Anaplasma* and *Ehrlichia* were not detected in ticks from any of the study sites. No extraction reagent control was positive for *Anaplasma* or *Ehrlichia* DNA.

3.2 Molecular characterisation of '*Candidatus* Ehrlichia occidentalis'

In total, 1,370 bp 16S, 1,067 bp *groEL*, 1,048 bp *gltA*, and 655 bp *map1 Ehrlichia* gene sequences were generated from the 34 *Ehrlichia*-infected *Am. t. triguttatum* from Yanchep NP, Bungendore Park, and Innes NP. Two distinct *Ehrlichia* 16S sequences were generated that differed by only two SNPs at nucleotide positions 209 and 1,243. The *Ehrlichia* 16S sequences (1,370 bp) were most similar to *Ehrlichia* sp. Panola Mountain (Mtn.) (Genbak: DQ324367) (98.1%) from Georgia, USA (Loftis et al., 2006), and to *E. ruminantium* (Genbank: CR925677)

(98.1%), and were less than 97.7% similar to other *Ehrlichia* species. At the *gltA* locus (1,048 bp), all sequences generated were identical, and were most similar to *Ehrlichia* sp. Panola Mtn. (GenBank: DQ363995) (83%), and then to *E. ruminantium* (Genbank: DQ513396) (82.3%), and less than 80.6% similar to *gltA* sequences from any other *Ehrlichia* species. No *groEL* gene sequences were available from the Panola Mtn. *Ehrlichia* sp. and the *Ehrlichia groEL* gene sequences (1,067 bp) generated in the present study, were most similar to *E. ewingii* (Genbank: AF195273) (90.9%), and then to *E. ruminantium* strain Gardel *groEL* (Genbank: CR925677) (90.1%). All *Ehrlichia groEL* sequences generated in the present study were identical from each sample. At the *Ehrlichia map1* locus, two variants were sequenced that differed only by a single SNP at nucleotide position 313. As with the 16S and *gltA* loci, *Ehrlichia map1* gene sequences from *Am. t. triguttatum* were most similar to *Ehrlichia* sp. Panola Mtn. (Genbank: EU272347)(70.2%) and then to *E. ruminantium* (Genbank: AF125274) (69.9%).

At the 16S, *groEL,* and *gltA* loci, the interspecific distances between the *Ehrlichia* sp. from *Am. t. triguttatum* and all other *Ehrlichia* species (98.3-97.2%, 90.9-88.4%, and 83-79.6%, respectively) are within the accepted ranges that genetically differentiate all other *Ehrlichia* species at these loci (98.5-97%, 94.5-86.5%, and 86.9-78.3%, respectively), indicating that sufficient genetic dissimilarity exists to delimit the *Ehrlichia* from *Am. t. triguttatum* as a distinct *Ehrlichia* species. This distinction is also supported by phylogenetic reconstructions at all loci studied (Fig. 2).

Bayesian phylogenetic reconstructions based on 16S, *gltA,* and *map1* sequences all consistently grouped the *Ehrlichia* from *Am. t. triguttatum* with *Ehrlichia* sp. Panola Mtn. and *E. ruminantium,* to the exclusion of all other *Ehrlichia* species with very high statistical support (Fig. 2). Unlike the other loci, phylogenetic reconstructions based on *groEL* sequences did not cluster the *Ehrlichia* from *Am. t. triguttatum* with *E. ruminantium,* but formed its own monophyletic clade positioned between *E. ruminantium* and *E. ewingii* (Fig. 2). However, this phylogenetic clustering had only moderate statistical support (0.54), and it is likely that the addition of *Ehrlichia* sp. Panola Mtn. *groEL* sequences may alter this topology. Unfortunately,

the *groEL* gene from the Panola Mtn. *Ehrlichia* sp. has not been sequenced (Loftis et al., 2006; Yabsley et al., 2008).

Analysis at all loci studied revealed consistently, and with high statistical support, that the novel *Ehrlichia* sp. from *Am. t. triguttatum* was district from any other described *Ehrlichia* species, and that sufficient genetic variation occurred at these loci to delimit it as a new species. Based on the genetic information presented here, we propose to designate this species '*Ca*. Ehrlichia occidentalis', denoting its discovery in WA. For clarity, we herein refer to this species by its proposed name.

3.3 Molecular characterisation of *Anaplasma bovis* genotype Y11

Almost complete 16S (1,307 bp) and *groEL* (1,256 bp) gene sequences were generated from the 12 *Anaplasma*-infected *Am. t. triguttatum* from Yanchep and from one *Anaplasma*-infected *Am. t. ornatissimum* from Barrow Island. Three unique *Anaplasma* 16S sequences were generated from *Am. triguttatum* subsp. samples, differing by only one SNP and one indel at nucleotide positions 1,259 and 1,257, respectively. Phylogenetic reconstructions based on 1,265 bp 16S sequences clustered the *Anaplasma* sequences from *Am. triguttatum* subsp. samples within the monophyletic *A. bovis* clade with high statistical support (Fig 3). However, within this clade, the *A. bovis* sequences from *Am. triguttatum* subsp. were clearly distinct from previously described isolates, and to differentiate it from other isolates we have defined it as *A. bovis* genotype Y11 (Fig. 3). Almost all *A. bovis* 16S sequences worldwide share at least 99.3% similarity; however, the *A. bovis* genotype Y11 sequences analysed here were highly divergent from other *A. bovis* strains, sharing only 97.3-99.5% similarity. *Anaplasma bovis* genotype Y11 sequences shared a most recent common ancestor with *A. bovis* isolates (Genbank: KM114611-3) from wild crab-eating macaques (*Macaca fascicularis*) from Malaysia (Fig. 3) (Tay et al., 2015), which are also the geographically closest *A. bovis* isolates to Australia.

Phylogenetic analysis was also performed on 1,184 bp *groEL* sequences which showed that *A. bovis* genotype Y11 was most similar (85%) to other *A. bovis* isolates, and less than

78.5% similar to any other *Anaplasma* species (Fig. 3). *Anaplasma bovis* genotype Y11 *groEL* sequences also clustered with *A. bovis* isolates from northern China and the Amur region of Russia (Rar et al., 2013) with high statistical support (Fig. 3). However, the genetic diversity of the *A. bovis groEL* gene has not been greatly explored worldwide, and only three full-length sequences were available for comparison in the present study, likely heavily biasing the phylogenetic analysis.

4. Discussion

Worldwide, *Anaplasma* and *Ehrlichia* species are associated with a wide range of mammalian hosts and ixodid vectors, and novel genetic variants and new species are frequently described (Cabezas-Cruz et al., 2016; Guo et al., 2016; Kawahara et al., 2004; Rar et al., 2015). Here we describe the first two *Anaplasma* and *Ehrlichia* species that we believe are likely endemic to the Australian continent; *A. bovis* genotype Y11 and '*Ca*. E. occidentalis'.

Phylogenetic analyses of 16S, *groEL*, *gltA*, and *map1* gene sequences unambiguously demonstrate that '*Ca*. E. occidentalis' is unique and distinct from any other described *Ehrlichia* species, but shares common ancestry with *E. ruminantium* from sub-Saharan Africa and *Ehrlichia* sp. Panola Mtn. from the eastern USA. Both *E. ruminantium* and the Panola Mtn. *Ehrlichia* sp. are animal pathogens, that have been demonstrated to have zoonotic potential (Allsopp et al., 2005; Reeves et al., 2008). In Australia, there is currently no reliable evidence of autochthonous *Ehrlichia* sp. infections in domestic animals or people; however, very few studies have been performed (Mason et al., 2001; Mayne, 2015). The clinical impact and zoonotic potential of '*Ca*. E. occidentalis' remains unknown and needs to be explored in more detail given the current public health concerns regarding tick-transmitted diseases in Australia (Beaman, 2016; Collignon et al., 2016).

Anaplasma bovis is a well-documented bovine pathogen in Africa and Asia, and has been reported recently for the first time in Europe (Spain) (Palomar et al., 2015; Rar and Golovljova, 2011). *Anaplasma bovis* has been detected in a wide variety of wildlife species

including deer (Kang et al., 2011; Kawahara et al., 2006; Palomar et al., 2015), goats (Ge et al., 2016), raccoons (Sashika et al., 2011), cotton-tailed rabbits (Goethert and Telford, 2003), wildcats (Tateno et al., 2013), and crab-eating macaques (Tay et al., 2015); however, to date, there is no evidence that *A. bovis* is zoonotic. Subclinical and asymptomatic infections are common, particularly in wildlife that have co-evolved with *A. bovis* (Kang et al., 2011; Kawahara et al., 2006; Tay et al., 2015). *Anaplasma bovis* has also been detected in a wide variety of tick species worldwide, including *A. variegatum* (Dumler et al., 2001), *Dermacentor andersoni* (Dergousoff and Chilton, 2011), *H. longicornis* (Kawahara et al., 2006; Kim et al., 2006), *H. concinna* (Rar et al., 2010; Rar et al., 2013; Shpynov et al., 2006), and *I. scapularis* (Goethert and Telford, 2003), but their vectorial capacity and role in the epidemiology of infections of many of these tick species is unknown.

Phylogenetic analysis presented here shows that extensive genetic divergence exists between *A. bovis* genotype Y11 and other *A. bovis* isolates from around the world. We hypothesise that, unlike other *Anaplasma* species that were recently introduced to Australia, *A. bovis* genotype Y11 has occurred naturally in Australia for a significant period of time, and has co-evolved with endemic ixodids, such as *Am. t. triguttatum* and *Am. t. ornatissimum*, and presumably endemic marsupial or eutherian reservoir hosts. Further research is required to confirm this proposition.

Phylogenetic reconstruction of 16S gene sequences shows that *A. bovis* genotype Y11 shared a most recent common ancestor (96.1% identity) with *A. bovis* from crab-eating macaques (*Macaca fascicularis*) from Malaysia, whose natural distribution extends as far south as the Indonesian islands of Java and Bali, approximately 1,200 km northeast of the Australian mainland. The genetic relatedness of these genotypes is interesting and the relative geographic proximity of these isolates suggests that *A. bovis* may have migrated from southeast Asia into Australia via natural dispersal events. Some species of fruit-eating bats have been documented flying between Indonesia and New Guinea, and New Guinea and Australia, and such migratory animals may have aided in the dispersal of infectious organisms across sea barriers (Breed et

al., 2010). *Anaplasma bovis* has also been identified in ticks on migratory birds in Europe, which are thought to act as carriers of *A. bovis* over large geographic distances (Palomar et al., 2015). Assessment of the presence and genetic relatedness of *A. bovis* from islands in close proximity to Australia, such as Timor and New Guinea, would help to confirm this hypothesis. Alternatively, *A. bovis* may have entered the Australian continent with the dispersal and subsequent radiation of rodents from Asia, 5-10 million years ago (Breed, 2007).

The data presented here suggests that *Am. t. triguttatum* and *Am. t. ornatissimum* are natural hosts and potential vectors of *A. bovis* genotype Y11 and '*Ca*. E. occidentalis'. The transovarial transmission of *Anaplasma* and *Ehrlichia* species in ixodids is thought not to occur, or to occur only sporadically (Baldridge et al., 2009; Long et al., 2003; Stich et al., 1989). Rather, the maintenance of these bacteria in ixodids primarily occurs through horizontal transmission between mammalian hosts and ticks during blood feeding. Furthermore, the presence of *A. bovis* genotype Y11 and '*Ca*. E. occidentalis' in unfed questing *Am. triguttatum* subsp. ticks, demonstrates the transstadial persistence of these bacteria within the ticks' tissues through life stage moults. The transstadial persistence of *Anaplasma* and *Ehrlichia* species between life stages is a critical mechanism that facilitates transmission of the bacteria to new mammalian hosts during the next life stages blood meal (Parola and Raoult, 2001). The data presented here therefore suggests that, like their close relatives overseas, *A. bovis* genotype Y11 and '*Ca*. E. occidentalis' circulate in natural cycles of mammal-tick-mammal transmission involving *Am. t. triguttatum* and *Am. t. ornatissimum* as a vectors; however, transmission experiments are needed to validate this hypothesis.

Amblyomma triguttatum subsp. are widely distributed in Australia, naturally occurring throughout northern New South Wales, Queensland, the Northern Territory, and WA (Roberts, 1962). In WA, *Am. t. triguttatum* is exclusively found in the southwest (including study sites Yanchep NP and Bungendore Park), and *Am. t. ornatissimum* is found predominantly in the Northern tropics of the state (including Barrow Island); however, several recordings have been made of this subspecies in the southwest (Roberts, 1962). The population of *Am. t. triguttatum*

studied here from Innes NP on the southern tip of the Yorke Peninsula (SA) (Fig. 1), is thought to have established in the area in only the last 30 years, aided by human and domestic animal movement (McDiarmid et al., 2000).

Amblyomma triguttatum subsp. have a relatively wide host distribution and will readily parasitise a range of wildlife species, as well as companion animals, livestock and humans. In some areas, including southwest WA, *Am. t. triguttatum* is the most common tick that bites humans (Gofton et al., 2015a). The natural wildlife hosts of *Am. triguttatum* subsp. adults and nymphs are well documented, and include a range of native macropods such as *Macropus fuliginosus*, *M. robustus*, *M. rufus* as well as smaller marsupials such as *Myrmecobium fasciatus* (Barker and Walker, 2014; Roberts, 1970). It is therefore likely that one or more of these marsupial species may act as reservoirs for *A. bovis* genotype Y11 and '*Ca.* Ehrlichia occidentalis'.

In the present study '*Ca*. E. occidentalis' was detected in only three of the study sites: Yanchep NP, Bungendore Park, and Innes NP, but not in Barrow Island. *A. bovis* was only detected in Barrow Island and Yanchep NP but not in the other two study sites. These observed differences in the distribution of *Anaplasma* and *Ehrlichia* may be related to the different mammalian fauna that occurs across the four study sites and may act as reservoirs for these bacteria. For example *Macropus fuliginosus* occurs across all three sites in which '*Ca*. E. occidentalis' was identified, but does not occur on Barrow Island. Future studies investigating the presence and distribution of *Anaplasma* and *Ehrlichia* within Australian wildlife species are needed to improve our understanding of the epidemiology of these bacteria.

In the present study, 37 *Am. t. triguttatum* nymphs (78.7% of *Ehrlichia* or *Anaplasma*-infected individuals) were infected with *A. bovis* genotype Y11 or '*Ca.* E. occidentalis'; therefore, the hosts of larval *Am. t. triguttatum* appear to be significant reservoirs of these bacteria. However, the hosts of larval *Am. triguttatum* subsp. have not been explored to a great

extent, largely because the larval stages for all *Am. triguttatum* subsp. have not been described (Barker and Walker, 2014; Roberts, 1962; Roberts, 1970). Molecular techniques such as cytochrome c oxidase I (COI) gene barcoding (Lv et al., 2014), could aid in the identification of larval *Am. triguttatum* subsp. and their hosts in future studies.

5. Conclusions

The pathogenic and zoonotic potential of *Anaplasma* and *Ehrlichia* species worldwide is well recognised, and in endemic areas concerted efforts are made to monitor, manage, and mitigate the impact of these emerging tick-borne infections (Colwell et al., 2011; Parola and Raoult, 2001). *Anaplasma bovis* genotype Y11 and '*Ca.* E. occidentalis' add to the growing number of novel tick-associated bacteria in Australia, following the recent description of two novel '*Ca.* Neoehrlichia' species and a novel *Borrelia* species (Gofton et al., 2016; Loh et al., 2016) from native Australian ixodids. The identification of these tick-associated organisms in Australia is of potential public health importance, since a large number of Australians are exposed each year to tick bites through occupation or recreation, and there is current hightened public concern regarding tick-transmitted disease in Australia (Beaman, 2016; Collignon et al., 2016). In Australia, a concerted scientific contribution within a One Health research framework is required to determine the ecology of these novel tick-borne bacteria, and assess any potential risks these novel organisms pose to human and animal health.

Competing interest

The authors declare that they have no competing interests.

Funding

This study was supported by the Australian Research Council (LP13010050), in conjunction with Bayer Healthcare and Bayer Australia Ltd. The funders had no role in the study design, data collection and analysis, decision to publish or preparation of the manuscript.

Ethics approval and consent to participate

This research complies with the *Australian Code for the Responsible Conduct of Research, 2007*. No specific ethics approval was required for this research.

Authors' contributions

AWG, UR, and PI conceived the study and reviewed the manuscript. AWG collected and identified specimens, performed laboratory and computational analyses, and drafted the manuscript. TLG, SP, and HPW collected and identified specimens and reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and material

All DNA sequences generated as part of this study have been deposited in GenBank under accessions KY425419 - KY425526. The datasets supporting the conclusions of this article are included with the article and its supplementary materials.

Acknowledgments

The authors wish to acknowledge Frances Brigg and the Western Australian State Agriculture Biotechnology Centre (Murdoch University) for Sanger sequencing. The authors also thank Kimberly Onton from the Department of Parks and Wildlife at the Barrow Island Nature Reserve, who kindly volunteered her time to collect samples for this study, Jacqui Morgan for assisting with sample collection, and Matthew Gill for organizing the transport of South Australian tick samples.

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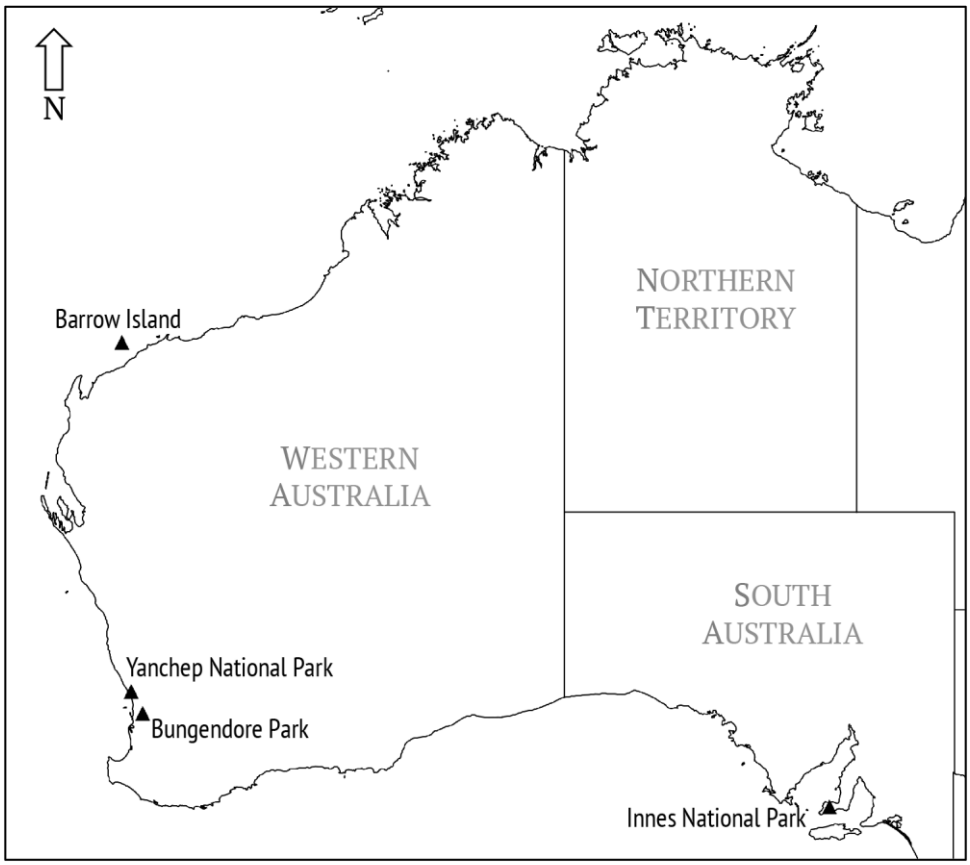
Figure legends

Figure 1. Map of Australia showing locations of study sites.

Figure 2. Bayesian phylogenetic reconstructions of *'Ca*. Ehrlichia occidentalis' based on 16S rRNA (1,281 bp), *groEL* (1,067 bp), *gltA* (1,033 bp), and *map1* (655 bp) gene sequences. Bold typeface indicates sequences from this study. All posterior probabilities are > 0.95, except where indicated. Genbank accessions are shown in parenthesis. Scale bars indicate the

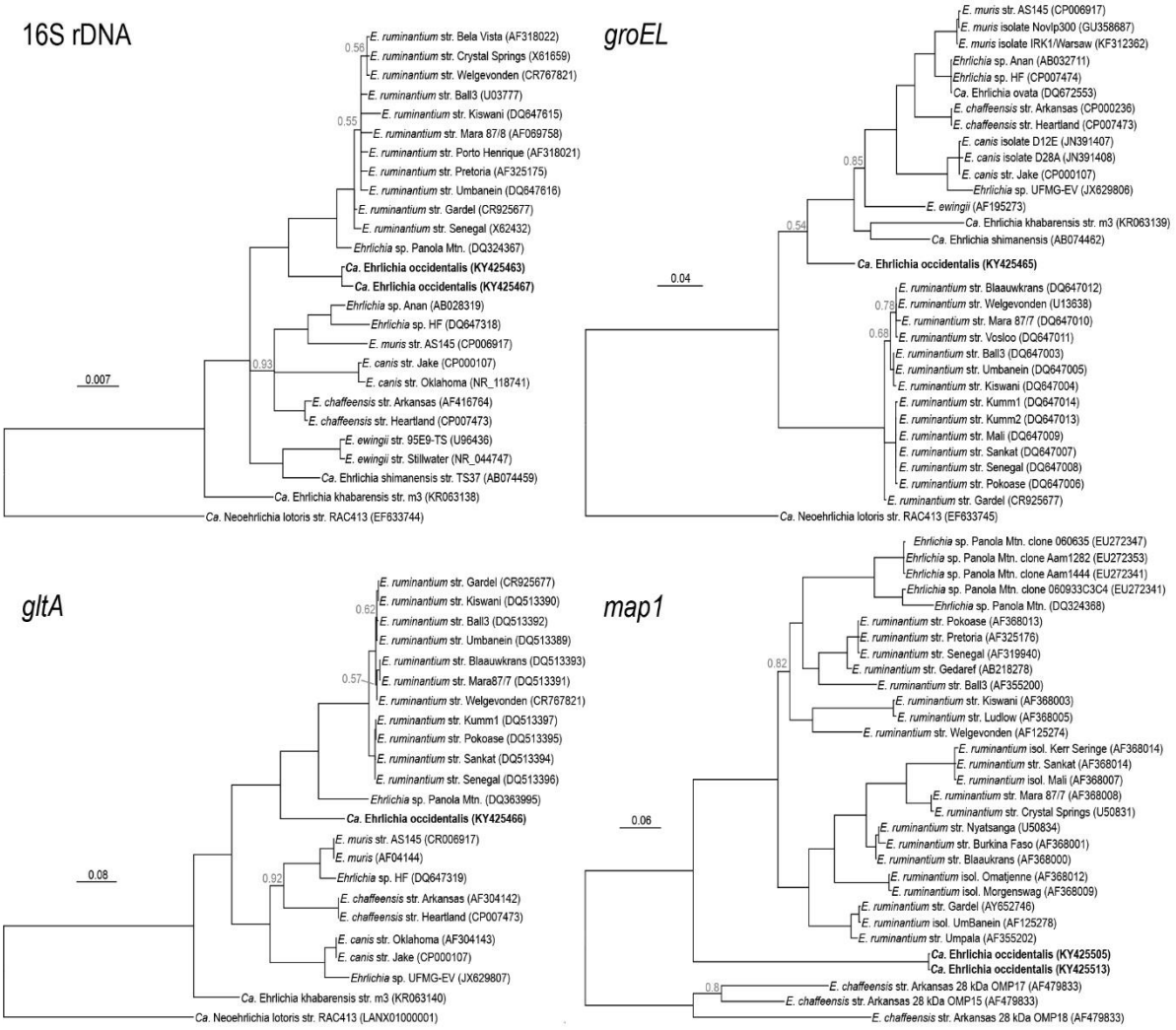
number of substitutions per nucleotide position. 16S, *groEL*, and *gltA* and *map1* phylogenies were produced using K2P, HKY85, and GTR substitution models, respectively.

Figure 3. Bayesian phylogenetic reconstructions of *Anaplasma bovis* genotype Y11 based on 16S rRNA (1,265 bp) and *groEL* (1,184 bp) gene sequences. Bold typeface indicates sequences from this study. All posterior probabilities are > 0.95, except where indicated. GenBank accessions are shown in parenthesis. Scale bars indicate the number of substitutions per nucleotide position. 16S and *groEL* phylogenies were produced using K2P and TN93 substitution models, respectively.



Figr-1

Figr-2



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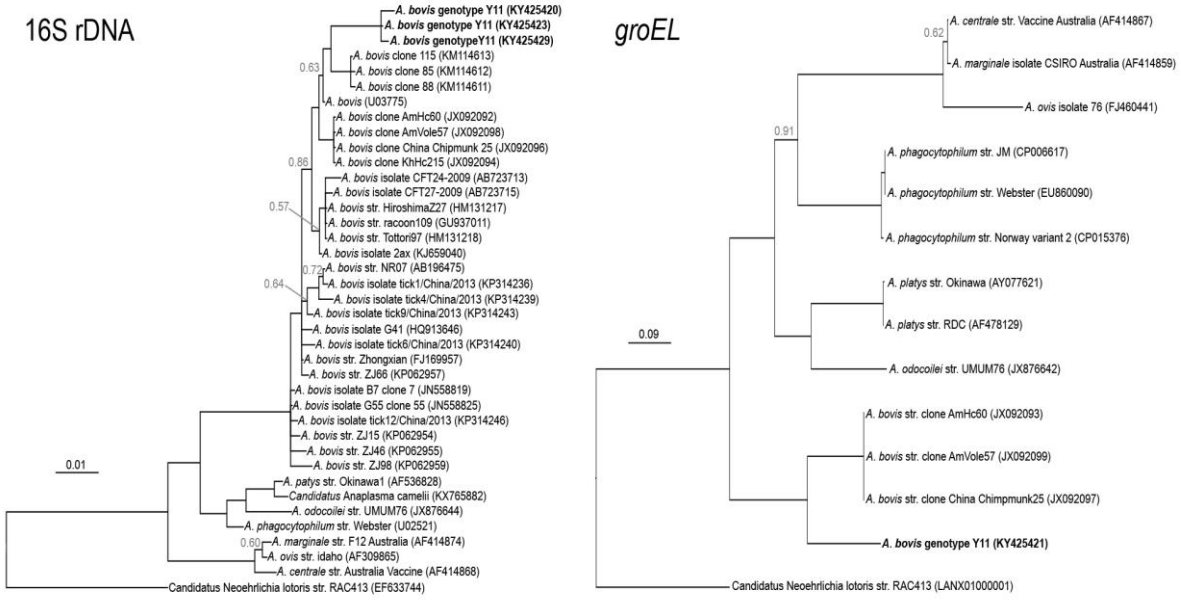

Table 1 Summary of *Amblyomma triguttatum* subsp. life stages collected and the number of positive samples at each site.

Location	Tick species	Nymphs	Females	Males	Total	*Ehrlichia* positive	*Anaplasma* positive
Yanchep National Park	*Amblyomma t. triguttatum*	237	15	28	280	18 (6.4%)	12 (4.3%)
Bungendore Regional Park	*A. t. triguttatum*	41	3	2	46	7 (15.2%)	0
Yorke Peninsula	*A. t. triguttatum*	83	68	63	214	9 (4.2%)	0
Barrow Island	*A. t. ornatissimum*	0	42	27	69	0	1 (1.4%)