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Genetic differences in *Chlamydia pecorum* between neighbouring sub-populations of koalas (*Phascolarctos cinereus*)

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ABSTRACT

Chlamydiosis, caused by *Chlamydia pecorum*, is regarded as an important threat to koala populations. Across the koala's geographical range, disease severity associated with *C. pecorum* infection varies, with pathogen diversity and strain pathogenicity being likely important factors. To examine *C. pecorum* diversity on a sub-population level a Multi-Locus Sequence Typing (MLST) scheme, containing the housekeeping genes; *gat*A, *opp*A_3, *hfl*X, *gid*A, *eno*A, *hem*N and *fum*C, was used to type strains from two sub-populations of koalas from the Liverpool Plains, NSW, Australia, with different disease expressions. Typing of samples from 2015 to 2017, revealed a significant association between sequence type ST 69 and clinical disease and a significant difference in sequence type frequencies between sub-populations. Sequence type ST 69 has previously been identified in both sub-clinical and clinically diseased koalas indicating that these markers alone are not illustrative of pathogenicity. However, recent emergence of this sequence type in a naïve population may explain the differing disease expressions. Sequence types ST 73 and ST 69 have been described in koalas across a broad geographic range, indicating multiple introduction events and/or a limited veracity of the MLST loci to explore fine scale epidemiological investigations, particularly those examining the interface between pathogenic strain and disease outcome.

1. Introduction

Chlamydiosis is the leading cause of keratoconjunctivitis (Brown and Grice, 1984; Cockram and Jackson, 1981), urogenital disease and infertility in the koala (Brown and Grice, 1984; McColl et al., 1984), with *Chlamydia pecorum* being the main causative chlamydial species (Jackson et al., 1999). It is transmitted predominantly venereally but can also be transmitted vertically from mother to young (Jackson et al., 1999; Nyari et al., 2017; Russell et al., 2018).

Although this pathogen is sometimes regarded as an important driver of population declines (Griffith et al., 2013; Rhodes et al., 2011) there is little direct evidence demonstrating this, and the impact of chlamydiosis across most koala populations in Australia remains poorly understood (McCallum et al., 2017). Among infected koalas subclinical chlamydial disease is common (Jackson et al., 1999; Nyari et al., 2017) and koalas with clinical signs display varying degrees of severity (Wan et al., 2011; Weigler et al., 1988). Differences in disease expression are observable across koala populations throughout Australia (Kollipara et al., 2013; Legione et al., 2016; Patterson et al., 2015; Polkinghorne et al., 2013) with the factors driving these variabilities being multi-factorial and complex. Such factors include the immunological profile of individuals (Mathew et al., 2014), co-infection with other pathogens such as koala retrovirus (Waugh et al., 2017) and differences in *C. pecorum* strains. Genetic variation has been reported amongst strains

Abbreviations: NSW, New South Wales; MLST, multi-locus sequence typing; ST, sequence type; PCR, polymerase chain reaction

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isolated from different regions (Jackson et al., 1997; Jelocnik et al., 2013; Kollipara et al., 2013; Legione et al., 2016; Marsh et al., 2011) with it being suggested that *C. pecorum* strains may likely differ in virulence (Higgins et al., 2012).

Improved understanding of pathogen diversity and significance within populations is needed to refine the knowledge on the epidemiology of *C. pecorum* infections and evaluate the risks of pathogen transfer when managing translocations and constructing wildlife corridors between koala subpopulations.

Meta-analysis of *C. pecorum* diversity in koalas is challenging because of the variation in methods and gene targets used in different studies. Single-locus typing of the *ompA* gene coding for the major outer membrane protein has been commonly applied (Jackson et al., 1997; Kollipara et al., 2013). Yet the accuracy of using this gene alone to infer relatedness and biogeographic spread of strains is considered suboptimal compared to its use in combination with other loci (Marsh et al., 2011). Whole genome sequencing (WGS) has been used to characterise varying regions within the *C. pecorum* genome (Bachmann et al., 2014). However, the high cost of applying this method makes it impractical when dealing with large sample sizes. Thus, there is a need for a reliable typing method with sufficient resolution to characterize strains and to be applicable across various typing studies. Addressing the limitations of single locus typing and whole genome analysis, Multi-Locus Sequence Typing (MLST) has been applied as a useful typing tool with sufficient sensitivity to elucidate the diversity of strains (Maiden, 2006). MLST types strains based on housekeeping genes that have been demonstrated to be useful markers due to their highly conserved nature (Eisenberg and Levanon, 2013; Feil, 2004; Maiden, 2006; Martin et al., 1998). In previous typing studies of *C. trachomatis, C. pneumoniae* and *C. pecorum,* MLST has suggested the potential for sequence types (ST) to be associated with certain clinical outcomes (Jelocnik et al., 2014; Pannekoek et al., 2008). Current typing studies of *C. pecorum* within Australia have centred on highly conserved housekeeping genes applied to limited sample sizes of koalas (Jelocnik et al., 2013, 2014). The application of MLST to a greater sample size of koala isolates is required to better understand both intra- and inter-population strain diversity (Jelocnik et al., 2013).

The Gunnedah Shire (30° 59′ S, 150° 16′ E) is situated within the Liverpool Plains, northern NSW, Australia (Fig. 1) and is home to a large number of free-ranging koala sub-populations in fragmented habitats (Crowther et al., 2014; Lunney et al., 2012). The emergence of chlamydiosis in the Gunnedah regional koala population, with distinct sub-population variation in disease expression over a 10-year period (M. Krockenberger, 2017, personal observation) offered an ideal clinical scenario to test the value of MLST for fine scale epidemiology of disease emergence of koala chlamydiosis. In addition, it also allowed investigation of the association of MLST strain variation in *C. pecorum* with disease outcomes in two apparently discontinuous koala sub-populations.

2. Materials and methods

2.1. Ethics statement

All handling and sampling of koalas was conducted by experienced veterinarians under the University of Sydney Animal Ethics Approval Number 2016/955 and NPWS Scientific License SL101687.

2.2. Animals included from the Gunnedah Shire and disease severity

Samples and data were from 140 koalas captured in 2015 (n = 24), 2016 (n = 73) and 2017 (n = 43) with 68 specimens from the southeast sub-population and 72 from the northwest sub-population (Table 2) (Fig. 1). Three clinical swabs were collected per koala: two ocular swabs from the conjunctiva of the left and right eye and one urogenital (UGT) tract swab. Koalas were captured and sedated (alfaxalone [Alfaxan, Jurox] approximately 2 mg/kg intramuscularly) to facilitate sample collection. Ocular and urogenital scores were assigned numbers between 0–9 (Griffith, 2010), where 0 represented an absence of visible disease and 9 severe observable disease. Samples were identified as 'subclinical' for grading score 0 if they were PCR positive for *C. pecorum*, 'mild clinical disease' for scores 0.5–3 and 'severe clinical disease' for scores between 4–9 (Supplementary Table 1). Koalas were identified by unique ear tags and microchip transponder numbers. Recaptures allowed some koalas to be sampled multiple times for the presence of *C. pecorum* and for disease progression but MLST of *Chlamydia* from many samples was unsuccessful due to non-amplification where insufficient amounts of chlamydial DNA were present, resulting in some animals being unable to be typed and all recaptured koalas only being typed once. Animals were assigned the disease state at the capture time point at which they were typed.

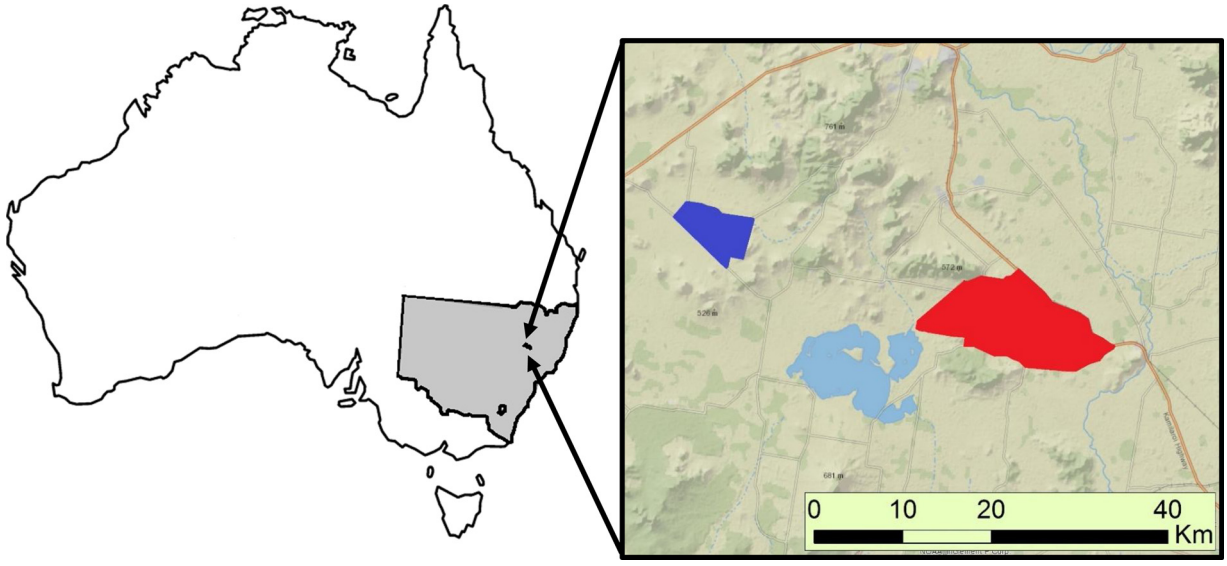

Fig. 1. Regional map of Australia showing the study sites sampled in the Liverpool Plains, NSW, Australia. The area from which samples were obtained from each subpopulation is shown on the map with the area in blue representing the northwest site and red the southeast region. A distance of approximately 40 km separates the two subpopulations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.3. DNA extraction and MLST sequence typing

DNA was extracted from swabs using the Kingfisher Duo Magjet Genomic DNA Kit #K2721 (Thermo Scientific). Ocular swabs (left and right) were pooled for each animal. A blank (sterile swab) was included to control for any contamination upon extraction. Real-time PCR used primers for *Chlamydia (*23S gene) (F: 5′-CTGAAACCAGTAGCTTATAA GCGGT-3′, R: 5-'ACCTCGCCGTTTAACTTAACTCC-3′) (Ehricht et al., 2006) and *C. pecorum (ompB* gene) (F: 5′−CCTTGTGAAGCGGAATT TGT-3′, R: 5′-CATCTTTCGCCTTGTCCTAAA-3′) (Griffith, 2010) and *C. pneumoniae* (*ompB* gene) (F: 5′-TCCGTTCCAGAATACGCTAC-3′, R: 5′-CATCACTTGTAGGGGTTGTTTC-3′) (Govendir et al., 2012; Griffith, 2010). The koala *β-actin* gene (F: 5′-TGCATCTAGCTCCTCTCTGG-3′, R: 5′-GCATCGGAACCTCTCGT-3′) (Markey et al., 2007), was used as a quality control for the amount of sample DNA extracted. PCR reactions of 20 µl compromised 225 nM of each primer, 10 µl of SYBR Green Supermix (SsoAdvanced™ Universal SYBR® Green Supermix – BIO RAD), 7.1 µl of dH_2O and 2 µl of DNA. Samples were run undiluted and diluted (1:10) to account for any inhibitors that might impede amplification, as well as to act as a replicate. A positive (pure cultured *C. pecorum* DNA extract at 1.8×10^3 EB/µl) and a negative control (dH_2O) were included in each PCR run. Melt curve temperatures of 82.5 °C for the 23S gene, 81 °C for *C. pecorum ompB* gene and 80.5 °C for *C. pneumoniae ompB* gene were considered indicative of specific amplification. Inhibitors were considered present when the C_T values for the diluted samples were lower than the C_T values for undiluted samples.

New, specific primers based on the previously published *Chlamydiales* MLST scheme containing the following 7 MLST loci; *gat*A, *opp*A_3, *hfl*X, *gid*A, *eno*A, *hem*N and *fum*C, were designed and optimised using the *C. pecorum* E58, complete genome (CP002608.1) to allow for the same cycling conditions (Jelocnik et al., 2013; Pannekoek et al., 2008) (Table 1). PCR reactions of 50 µl comprised 1 ng/µl of each primer, 0.4 mM dNTPs, 3 mM $MgCl_2$, 0.05 u/µl of Taq polymerase (Cat. No. 21105, Bioline Pty Ltd), 5 µl of buffer 10x, 24.5 µl of H_2O and 2 µl of DNA. A 1:10 DNA dilution was used if inhibitors were detected on initial chlamydial qPCR. A positive (cultured *C. pecorum* DNA extract at 1.8×10^3 EB/µl conc) and negative control (dH_2O) was included in each PCR run. PCR cycling conditions were the same for each MLST locus: initial denaturation (3 min at 95 °C), then 40 cycles of denaturation (30 s at 94 °C), annealing (45 s at 58 °C), and extension (2 min at 72 °C), and then final extension (7 min at 72 °C). PCR products were separated on a 1% agarose gel at 80 V for 40 min and visualized under UV trans illumination. Amplicons of the appropriate size were sent for sequencing and purification by Macrogen Inc., Korea. When faint or double bands were present they were excised and purified using the Promega - Wizard SV Gel & PCR Clean-up system and then re-amplified for the respective targets before preparation for sequencing. Sequences were trimmed using the Sequencher DNA Sequence Analysis Software Tool version 5.4.6, to the appropriate MLST amplicon size (bp) (Table 1). Using the Chlamydiales MLST database (https://pubmlst.org/chlamydiales/) (Jolley and Maiden, 2010), allele IDs for each housekeeping gene and sequence type numbers were obtained. Where novel allele IDs were identified, relevant targets were re-amplified and sequenced, and the data submitted to the Chlamydiales MLST database.

2.4. Statistical analysis

Only specimens with sequences for the full set of all 7 MLST loci were used in the statistical analyses. An odds-ratio test with 95% confidence intervals, was conducted to assess the odds of disease outcome [subclinical and clinical] to site [southeast and northwest]. Chi-squared analyses with Yate's corrections for small sample sizes were performed to assess the relationship between sequence type [ST 73 and ST 69] to disease outcome [subclinical and clinical], to site [southeast and northwest] and to year [2015, 2016, 2017]. For disease outcome clinical disease included both mild and severe disease together. The association between sequence type and year was only able to be tested within the northwest sub-population as ST 69 was not described within the southeast sub-population. Recaptured animals included in the analysis were only typed once by MLST and therefore were treated as single captures in the analysis.

2.5. MLST sequence typing of outgroup samples

Koalas previously tested as positive for *C. pecorum,* from Port Macquarie Koala Hospital (n = 4), Friends of the Koala Lismore (n = 1), Adelaide Koala & Wildlife Hospital (n = 3), RSPCA Wildlife Hospital Queensland (n = 3), Port Stephens (n = 1), Victoria (n = 1) and the Southern Highlands region (n = 7) were also analysed by MLST as outgroups to compare the diversity of *C. pecorum* strains within the Gunnedah shire to those from koala populations from other locations across Australia.

2.6. Phylogenetic sequence analysis obtained from the specimens from the Gunnedah Shire to outgroup sequence types

Specimens with sequences from less than 7 MLST loci (n = 12) were not included in the phylogenetic analysis. Sequences were concatenated for the 7 loci, aligned and the best fit nucleotide substitution models were identified using MEGA7 (Kumar et al., 2016). A Maximum Likelihood tree was then constructed with 1000 bootstrap probabilities using MEGA7. Bootstrap values greater than 70 were considered an

Table 1
Primers, sequences and their product sizes for the 7 housekeeping loci: *gat*A, *opp*A_3, *hfl*X, *gid*A, *eno*A, *hem*N, *fum*C, analysed by MLST within this study.[a]

MLST gene locus	Primer name	Primer sequence (this study)	Amplicon size (bp)	Previous MLST primers amplicon size (bp)	Amplicon size analysed by MLST (bp) (Jelocnik et al., 2013; Pannekoek et al., 2008)
*gat*A	gatA_F	TGAGAGCTTCTCTCGTCAGGA	737	512 (Pannekoek et al., 2008)	424
	gatA_R	AGGGCCAATCTGATCCAATGA			
*opp*A_3	oppA_F	ACCGGTATACGTTCCCTCTG	830	605 (Pannekoek et al., 2008)	483
	oppA_R	GCATCGGGGATAAAGTGTATGG			
*hfl*X	hflx_F	CGACATTTTTAAATGAAGGGAAGC	820	607 (Jelocnik et al., 2013)	435
	hflx_R	GGAAGCGCATCGACTTTGTT			
*gid*A	gidA_F	TGACCACATAAAAATAGCCCTTCT	824	560 (Jelocnik et al., 2013)	474
	gidA_R	CGGGCTCAAGTGGATAAGCA			
*eno*A	enoA_F	TCTGCTACATGGCTATAGCGTC	807	431(Pannekoek et al., 2008)	381
	enoA_R	GCCTCAGAAGTTTCTGTGAGTG			
*hem*N	hemN_F	AGTTCCCCGTTTCTCGAATCC	799	634 (Jelocnik et al., 2013)	432
	hemN_R	TCCTTGGAGAGCTCTTTGCTT			
*fum*C	fumC_F	TCCTTGGAGAGCTCTTTGCTT	787	572 (Pannekoek et al., 2008)	465
	fumC_R	CGCAAACTCTGCGTTCTCAAA			

[a] The primers in this study amplified longer fragments of the 7 MLST loci. To obtain the allele numbers, the fragments were trimmed to the same amplicon size as previously analysed by MLST (Jelocnik et al., 2013, 2014).

acceptable level of support for a clade (Hillis and Bull, 1993). Sequence types previously described in MLST studies of koalas, sheep and cattle within Australia (ST 23, ST 62, ST 63, ST 70, ST 71, ST 72, ST 73, ST 74, ST 75, ST 76, ST 77, ST 78, ST 79, ST 80, ST 81, ST 82, ST 83) (Jelocnik et al., 2013, 2014) were included within the tree to contextualize the sequence types from the present study, including novel sequence types with other Australian *C. pecorum* sequence types (Supplementary Table 2).

2.7. Accession numbers

Accession numbers for the housekeeping gene sequences for all sequence types identified in this study are available in GenBank (MH266781 – MH267194).

3. Results

3.1. Local epidemiology and sequence type diversity

Between 2015 and 2017, specimens from 140 koalas were collected and screened for the presence of *Chlamydia* with 90/140 (64%) being positive for *C. pecorum* at the urogenital site (Table 2). Of the 34/140 (24%) koalas positive for *C. pecorum* at the ocular site, 31/140 (22%) were also positive at the urogenital site. The proportion of samples from the southeast sub-population with *C. pecorum* at the ocular 20/68 (29%) and urogenital sites 58/68 (85%) from 2015 to 2017 was comparatively higher than the northwest sub-population at 14/72 (19%) and 32/72 (44%) (Table 2).

All seven MLST loci were successfully amplified from 47/93 koala specimens from the Gunnedah shire. In the remaining 46 specimens also identified as positive for *C. pecorum,* the amplification success across the seven loci was variable, and these specimens were not able to be included in further analyses. Of the 47 specimens with concatenated MLST loci, 11/47 specimens were from the ocular site and 36/47 from the urogenital site (Table 2). Subclinical koalas accounted for 7/47 specimens while 40/47 with clinical disease ranging between mild to severe (Supplementary Table 1). From the current study, 3 sequence types were identified across the two koala sub-populations (ST 73, ST 198, ST 69), with ST 198 being novel (Supplementary Table 1). Sequence types ST 73 and ST 198 were identified within the southeast sub-population and sequence types ST 73 and ST 69 within the northwest sub-population with the differences in sequence type frequencies being significant (χ^2 = 26. 706, df = 1, p = < 0.001).Within the northwest sub-population, the association between year versus sequence type was significant where the proportion of samples that were ST 69 had significantly increased from 2015 to 2017 (2015, 1/2; 2016; 4/4; 2017; 8/9; χ^2 = 10.233, df = 2, p = 0.001; Table 2). In one koala, the STs differed between the urogenital site (ST 73), and the ocular site (ST 198). Sequence type ST 73 was described in all disease states; subclinical to mild and severe clinical disease while ST 69 was only described in koalas with mild to severe disease (Table 2). From 2015 to 2017, there was a significant association between ST 69 and clinical disease (χ^2 = 4.187, df = 1, p = 0.041). This association was further supported by the odds-ratio, with the odds being 9.47 indicating that koalas from the southeast site were 9.47 times more likely to display subclinical disease compared to koalas from the northwest site.

An odds-ratio above one indicated a positive association between being subclinically diseased and living in the southeast population. The 95% confidence intervals [1.0987, 81.684] did not include one indicating that this association was significant.

3.2. Phylogenetics and diversity of sequence types

From the outgroup koala isolates, MLST was obtained from koalas in Port Macquarie (n = 1), the Southern Highlands (n = 5) and Victoria (n = 1) (Supplementary Table 1). A total of 4 STs were identified (ST 69, ST 199, ST 200, ST 202) with ST 199, ST 200 and ST 202 being novel. Sequence type ST 69 was described in the Port Macquarie isolate, ST 199 and ST 202 in koalas from the Southern Highlands region and ST 200 in a koala from Victoria.

The similarity and relatedness between the koala STs identified from within the Gunnedah shire to STs identified from the outgroup koalas from this study and those previously described in koala, cattle and sheep, is reflected through a Maximum Likelihood tree using the Hasegawa Kishino model (Fig. 2). The topology of the tree shows that *C. pecorum* STs segregate into two major clades. Interestingly, not all STs identified from the same geographical regions clustered together. Sequence types ST 73 and ST 69 did not cluster with any isolates identified in ruminants, but only clustered with other sequence types previously described from koalas (ST 75, ST 77, ST 76). Three of the sequence types identified in koalas (ST 198, ST 199 & ST 70) grouped into one clade, which contain all the ruminant isolates.

Across the seven MLST loci alleles *gat*A, *eno*A and *fum*C were the only variable markers able to distinguish between the three sequence types identified in the Gunnedah Shire as all the other allele IDs for the remaining MLST genes (*opp*A_3, *hfl*X, *gid*A, *eno*A, *hem*N and *fum*C) were identical. The *gat*A allele types 30 and 27 were distinguishable based on one single nucleotide polymorphism (SNP) within the sequences and were the only markers found to be different between ST 73 and ST 69. This allowed for the prediction of whether STs for those samples with sequences for only 5–6 of the MLST loci (n = 5) would be ST 73 or ST 69 based on whether the *gat*A allele ID was 30 or 27 (Supplementary Table 1). Allele ID's *gat*A 31, *eno*A 66, *fum*C 20 were only present in ST 198, which was only identified in one koala. In a comparison of the allele IDs for the sequence types identified within the Gunnedah shire to the outgroup sequence types (ST 199, ST 200, ST 202) only allele IDs *gid*A, *eno*A and *fum*C were found to be different. At the *gid*A allele, allele ID 31 was identified in ST 199 and ST 202, *fum*C allele ID 48 in ST 202 and *eno*A allele ID 67 in ST 200. The allele IDs *eno*A_66, *eno*A_67 and *fum*C_48, were newly identified in the present study.

Table 2
Sample size of specimens collected at each site for each year, including recaptured koalas, the number of swabs positive for *C. pecorum* at each ocular and urogenital site, and the number of samples with full MLST.

Year	Site	Sample size	No. of *C. pecorum* positive swabs at ocular and urogenital sites		No. of koala specimens with full MLST		Proportion ST 69 [a]
			'Ocular'	'Urogenital'	'Ocular'	'Urogenital'	
2015	South-East	12	3/12	9/12	0/5	2/9	0/2
	North-West	12	5/12	7/12	2/3	2/7	1/6*
2016	South-East	39	11/39	33/39	6/11	13/33	0/19
	North-West	34	4/34	9/34	0/4	4/9	4/4
2017	South-East	17	6/17	16/17	2/6	6/16	0/8
	North-West	26	5/26	16/26	1/4	9/16	10/11**

[a] *two ST 73 based on < 7 loci; **one ST 73 based on < 7 loci.

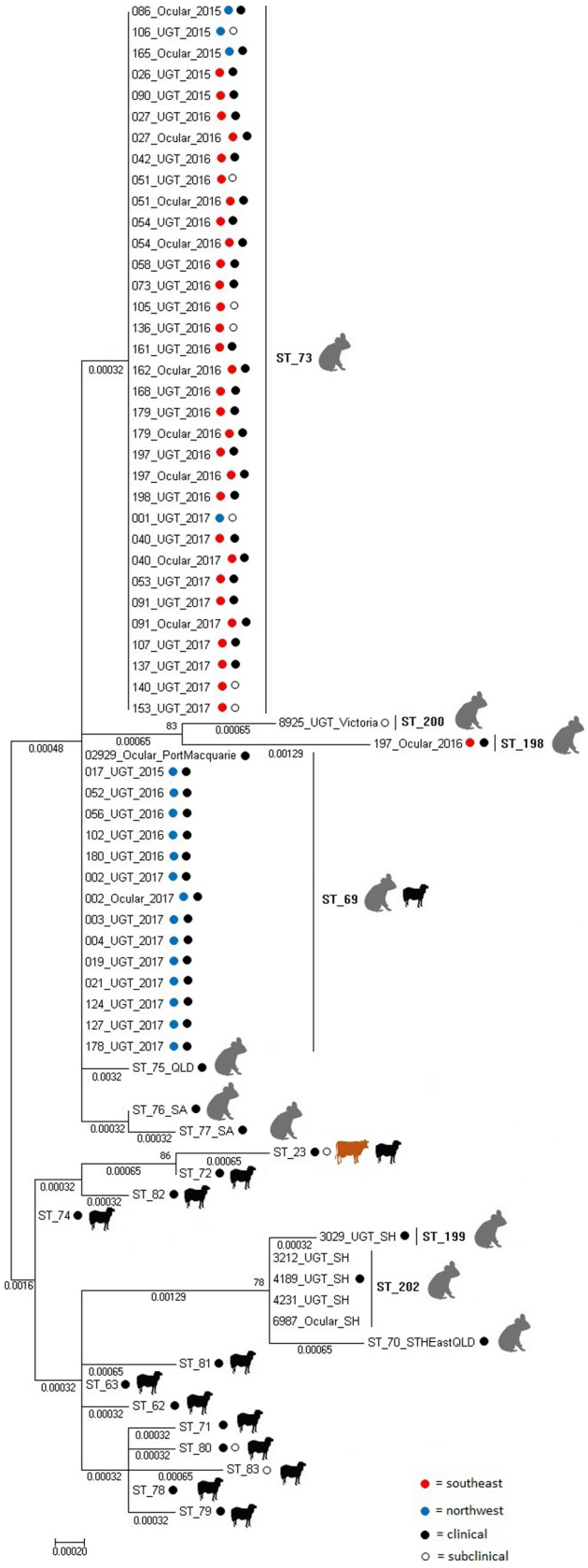

Fig. 2. Maximum Likelihood phylogenetic tree with 1000 bootstrap probabilities, of the concatenated MLST loci for all koala specimens (n = 54) typed in this study. Sequence types identified in this study (ST 73, ST 69, ST 202, ST 198, ST 199, ST 200) and sequence types (ST 23, ST 62, ST 63, ST 71, ST 72, ST 75, ST 76, ST 77, ST 78, ST 79, ST 80, ST 81, ST 82, ST 83) (Jelocnik et al., 2013, 2014) identified from previous MLST studies of Australian koalas, sheep and cattle, are included. The location of koala and cattle samples from each subpopulation; southeast and northwest, is indicated by the coloured circles next to each sample ID, as well as the year (2015–2017) in which these specimens were collected and the clinical presentation of animals with these sequence types. Images of koalas, sheep and cattle are included next to each sequence type to show in which hosts these strains have been identified. Only bootstrap values over 70 are shown. Branch lengths are representative of the level of genetic change between sequence types indicated by the nucleotide frequencies.

4. Discussion

Two sub-populations of koalas in the Gunnedah shire in the Liverpool Plains which differed in disease prevalence and severity, had different associated chlamydial sequence type patterns. In this region, ST 69 was described in only one of the two koala sub-populations (the northwest sub-population), in association with clinical disease at the urogenital and ocular regions. In contrast, ST 73 was found across both sub-populations, where it was associated with a higher apparent prevalence and lower severity of disease including subclinical disease. The significant differences in the frequencies of sequence types ST 73 and ST 69 over time within the northwest sub-population suggests multiple introductions of *C. pecorum*, illustrating the value of employing molecular typing tools to understand pathogen diversity and transfer within populations.

4.1. Association between sequence type and disease outcome

The greater proportion of clinical disease observed since 2015 attributable to ST 69, could be a result of the introduction of a novel strain into a naïve population causing a high prevalence of acute, severe disease. As ST 73 has been described within the Liverpool Plains since 2015, albeit at low levels of clinical disease expression, it is probable that it has been present within both sub-populations for a relatively long time, consistent with its association with chronic, low-grade to subclinical disease. The significant increase in the proportion of ST 69 over the years 2015–2017 and its association with clinical disease is thus most suggestive of a recent introduction of this strain into the northwest sub-population. However, the alternative plausible hypothesis is that ST 69 has been present in both sub-populations in the past but has been eliminated or disappeared by chance from the southeast sub-population. Segregation of ST 69 in only one sub-population suggests that while the populations may have previously been contiguous, they are likely segregated now. Genetic analysis of subpopulations in this region may be useful to determine possible segregation.

The association of ST 69 with only clinically diseased koalas from the present study is congruent with the findings of this sequence type in sheep with clinical signs (Jelocnik et al., 2014) but not consistent with having been identified in both subclinical and clinically diseased koalas in other populations (Jelocnik et al., 2013, 2014). Without a recent introduction to explain the apparently greater virulence of ST 69 in this population, an inherent difference between the virulence of ST 69 strains from the current study and those found to be less associated with disease elsewhere should be explored through additional analysis of loci with an associated virulence role.

4.2. Geographical distribution and phylogenetics of MLST sequence types

The use of MLST has previously demonstrated that *C. pecorum* strains have a wide distribution in koalas, sheep and cattle throughout

the koalas habitat range (Jelocnik et al., 2014). As demonstrated in *C. trachomatis* (Peuchant et al., 2012), MLST aids in increasing the understanding of the distribution of existing strains and introduction of new strains into adjacent populations. The observations concerning sequences types ST 73 and ST 69 in this study are consistent with the presence of these sequence types in other koala populations (Jelocnik et al., 2013, 2014). Sequence type ST 73 and ST 69 are broadly dispersed across considerably distant populations in Australia, having been described in koalas in South East Queensland, Central to Eastern NSW, Victoria (Jelocnik et al., 2013, 2014) and recently from this study in Port Macquarie, suggesting that multiple introductions of these strains have likely occurred across the koalas range. It appears possible that ST 69 has been similarly introduced to the northwest sub-population. It is conceivable that the strain could have been introduced by ruminants with mixed ruminant enterprises being present as the main farming practices within the Liverpool Plains up until the 1970′s (Rob Frend, 2018, personal communication) or alternatively through connectivity with neighbouring koala populations where ST 69 may be continuously distributed.

Phylogenetic analysis revealed that sequence types identified within koalas from the Gunnedah Shire (ST 73, ST 69, ST 198) did not all associate with each other (Fig. 2). Sequence type ST 73 was more closely related with ST 69, ST 75, ST 76, ST 77, with strains of ST 75 being identified in Queensland, ST 76 in South Australia and ST 77 in eastern NSW (Jelocnik et al., 2013). Examining the phylogenetic relatedness of *C. pecorum* strains across the koalas range was not an aim of the present study, and would require a more in depth phylogenetic comparative analysis to discern the relatedness between STs from koala populations from differing geographical regions.

The evolutionary origin and history of *C. pecorum* in koalas still remains somewhat controversial with evidence suggesting a spill-over of *C. pecorum* from ruminants to koalas (Jackson et al., 1997; Polkinghorne et al., 2013). This analysis was unable to establish a firm link between the pathogen in koalas and ruminants as there was limited grouping of strains in the koala to those in sheep and cattle, with most koala sequence types clustering together (Fig. 2). Although spill-over events of *C. pecorum* to koalas have previously been associated with cattle (Jackson et al., 1997; Polkinghorne et al., 2013), repeated findings of identical STs within both sheep and koalas (Jelocnik et al., 2014), highlights sheep as possible sources for spill-over events.

4.3. Improving on the utility of the MLST scheme

While MLST has proven to be useful in showing the distribution, transmission and diversity of *C. pecorum* strains between koala sub-populations, it was limited in its ability to investigate the pathogenicity of strains on the fine scale needed for the present study. The four most variable loci identified in this study; *gat*A, *eno*A, *fum*C and *gid*A, were the most informative in distinguishing between strains with the remaining 3 loci; *opp*A_3, *hfl*X and *hem*N, having shown no variability between all the samples. Limited differences between strains is expected, as the loci in the MLST scheme are known to be highly conserved, based on their function and because they are under strong purifying selection (Jelocnik et al., 2013), evident by the low nucleotide changes indicated on the branch lengths of the phylogenetic tree (Fig. 2). Despite this, differences between strains infecting koalas were found confirming the hypothesis that disease severity differences between two sub-populations are associated with strain variation. These differences provide the foundation to investigate less conserved markers or apply whole genome sequencing to better characterise identical MLST chlamydial strains within koalas with differing disease outcomes, and further differentiate the same strains identified across physically distant populations. Such genes could represent loci with putative virulence roles, such as the combination of *ompA,* ORF663 and *incA* (Marsh et al., 2011). Analysis of these genes has identified a large variety of *C. pecorum* genotypes in koalas with varying disease outcomes (Jackson et al., 1999; Marsh et al., 2011), which might better reflect the pathogenicity of strains, helping further link specific strains to disease outcomes. There is also the possibility of sampling bias, with MLST not representing all STs that could be present across the two sub-populations and across koalas with variable disease outcomes. Samples with greater chlamydial DNA load were more successfully amplified across all 7 MLST loci. The limited success in typing samples with low chlamydial loads could result in an under representation of the diversity of the *C. pecorum* strains within the region and across subclinical and clinical animals.

5. Conclusion

The inclusion of two sub-populations that display different disease expression, while sharing similar habitats, provided a model to explore the diversity of *C. pecorum* and its role in disease severity at a population level. Significant differences in the frequency of sequence types ST 73 and ST 69 between the two sub-populations, has demonstrated that differences in *C. pecorum* strains can and do exist between neighbouring populations from within the same region, suggesting multiple disease emergence events. This highlights the need to consider pathogen diversity of *C. pecorum* to identify risks of pathogen transmission and transfer across metapopulations. The addition of virulence-associated loci to the current MLST scheme may help in identifying an association between strain type and disease outcome. In conjunction, additional data from the host such as co-infection with KoRV-B and immunological profiles of important cytokines involved in immune defence and clearance of *Chlamydia* should be included in future analyses to further explain variabilities in, and better quantify the relative contribution of strain to, disease outcome. This is especially true in the situation where the association between ST 69 and clinical disease in the present study is not congruent with the behaviour of the sequence type in other koala populations across Australia and the broad geographic distribution of current MLST types.

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Conflicts of interest

The author acknowledges no conflicts of interest.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.020.

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