Multilocus Sequence Analysis Provides Insights into Molecular Epidemiology of *Chlamydia pecorum* Infections in Australian Sheep, Cattle, and Koalas

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*Chlamydia pecorum* is a significant pathogen of domestic livestock and wildlife. We have developed a *C. pecorum*-specific multilocus sequence analysis (MLSA) scheme to examine the genetic diversity of and relationships between Australian sheep, cattle, and koala isolates. An MLSA of seven concatenated housekeeping gene fragments was performed using 35 isolates, including 18 livestock isolates (11 Australian sheep, one Australian cow, and six U.S. livestock isolates) and 17 Australian koala isolates. Phylogenetic analyses showed that the koala isolates formed a distinct clade, with limited clustering with *C. pecorum* isolates from Australian sheep. We identified 11 MLSA sequence types (STs) among Australian *C. pecorum* isolates, 10 of them novel, with koala and sheep sharing at least one identical ST (designated ST2013Aa). ST23, previously identified in global livestock isolates, was observed here in a subset of Australian bovine and sheep isolates. Most notably, ST23 was found in association with multiple disease states and hosts, providing insights into the transmission of this pathogen between livestock hosts.

The complexity of the epidemiology of this disease was further highlighted by the observation that at least two examples of sheep were infected with different *C. pecorum* STs in the eyes and gastrointestinal tract. We have demonstrated the feasibility of our MLSA scheme for understanding the host relationship that exists between Australian *C. pecorum* strains and provide the first molecular epidemiological data on infections in Australian livestock hosts.

The obligate intracellular bacterium *Chlamydia pecorum* is a globally recognized pathogen of livestock and wildlife (1, 2). The best-studied host for *C. pecorum* infections is the koala (*Phascolarctos cinereus*) (3), a native Australian arboreal marsupial. Within this host, *C. pecorum* infections are prevalent and cause acute or chronic keratoconjunctivitis, often leading to blindness (4), and genitourinary tract infections that may lead to infertility (5, 6).

*C. pecorum* also causes a range of clinically important diseases in economically significant livestock species (cattle, sheep, goats, and pigs) manifesting as encephalomyelitis (7), reduced fertility (8), vagnitis and endometritis, enteric infections, mastitis (9), pneumonia, conjunctivitis, and arthritis (stiff-lamb disease) (10, 11). *C. pecorum* infections in cattle can be subclinical and asymptomatic but nevertheless exert chronic pathological effects on animal health not seen in herds without infection (12, 13). In affected sheep flocks, *C. pecorum* infections lead to polyarthritis and conjunctivitis and spread rapidly, leading to increased morbidity and mortality (14). There are also reports implicating *C. pecorum* in ovine abortions (10). Despite the high prevalence of *C. pecorum* infections in livestock, there is a lack of information about the genetic diversity of these strains in relation to the anatomical sites that are infected and the diseases observed (15). Virtually nothing is known about the prevalence and epidemiology of infections in Australian livestock, despite the fact that cases of chlamydiosis in sheep and cattle are regularly reported in agriculturally productive areas of central New South Wales (NSW) (14, 16, 17).

The origin of *C. pecorum* infections in Australian animals is also unclear and of concern, given the potential for *C. pecorum* “spillover” or “spillback” risk between infected livestock and/or wildlife infections, respectively (18, 19). Soon after *C. pecorum* was described in the koala (20, 21), Jackson et al. (22) analyzed sequences of the *C. pecorum ompA* gene and found that (i) koala *C. pecorum ompA* sequences are highly polymorphic and (ii) some koala *C. pecorum ompA* sequences cluster more closely to sequences from Australian livestock than to those from other koalas. These earlier observations led to the suggestion that koala *C. pecorum* infections may have originated from a “recent” cross-host transmission event, possibly from livestock that were introduced to Australia following European settlement in 1788 (3).

The recent completion of the first *C. pecorum* genome sequence (23) has allowed us to revisit the relationship between *C. pecorum* infections in koalas and Australian livestock. Using novel molecular markers (tarP in addition to open reading frame 663 [ORF663], incA, and *ompA*), the findings of Marsh et al. (24) strengthened previous observations that koala *C. pecorum* strains are genetically diverse (22). In contrast to the previous study, however, the latter study showed that koala *C. pecorum* strains were phylogenetically distinct from *C. pecorum* livestock strains (24), although the study was limited by a lack of Australian livestock samples. Use of these polymorphic genes in a previous study, alone or in addition to housekeeping genes as targets of a multi-virulence locus-typing scheme (MVLST), allowed for the discrimination of *C. pecorum* strains from livestock that were isolated from diseased and healthy animals (25). Despite these reports,
significant evidence is accumulating to suggest that ompA, which encodes a highly immunogenic surface-exposed chlamydial major outer membrane protein (MOMP), is an unreliable marker for tracing the origin and relationships of other Chlamydia species (26, 27). Due to the numerous schemes presented for the typing of C. pecorum, the need for the establishment of a standardized global epidemiological tracking tool has been evident.

A number of studies have reported the use of multilocus sequence typing (MLST) (28) and multilocus sequence analysis (MLSA) to elucidate intraspecies relationships between members of the genus Chlamydia (29, 30), including analyses of strains from the same species that infect multiple animal hosts (31). These methods utilize the concatenation of four to 12 evolutionarily conserved housekeeping (HK) gene sequence fragments. HK genes are broadly distributed around the chromosome and encode conserved proteins that are not considered to be under diversifying selection (32). Phylogenies based on these loci have been useful in differentiating bacterial pathogens (33–35), including bacteria that are highly recombinogenic and undergo frequent lateral gene transfer (36, 37).

The primary objective of the current study was to develop and apply an MLSA scheme to examine the overall genetic diversity of and relationships between Australian C. pecorum isolates from different geographical origins and hosts. In doing so, we provide the first molecular epidemiological data on this important animal pathogen infecting Australian livestock.

MATERIALS AND METHODS
Chlamydial isolates. The complete list of isolates from the sequences included for analysis are described in Table S1 in the supplemental material. Five koala isolates from wild koala populations in southeast Queensland (SEQLD), Australia, were previously isolated and described by Marsh and colleagues (24). HK gene fragment sequences for three koala C. pecorum isolates from SEQLD and the sheep C. pecorum polyarthritis strain IPA were extracted from their draft genomes (A. Polkinghorne, unpublished data). HK gene fragment sequences from other U.S. sporadic bovine encephalomyelitis (SBE), arthritis, conjunctivitis, and healthy fecal C. pecorum isolates, also listed in Table S1, were obtained from the Chlamydiades MLST website (http://pubmlst.org/chlamydiades/) (38). Permission to use these sequences was granted to us by the curator, Yvonne Pannekoek, University of Amsterdam, The Netherlands.

C. pecorum-specific PCR screening of Australian livestock and wild koala samples. A total of 77 swabs were collected from 40 sheep from various flocks across central New South Wales (NSW), Australia. Swab samples were collected from the eyes, rectum, and vagina by the district veterinarians of the Livestock Health and Pest Authority (LHPA) throughout NSW, Australia, as a part of routine diagnostic testing (see Table S2 in the supplemental material). Two additional sheep with polyarthritis were sacrificed as a part of routine diagnostic testing. Fluid from diseased joints was extracted using a syringe, and swabs were dipped into the synovial fluid prior to processing. DNA extracted from a cultured C. pecorum isolate, originating from a central NSW bovine SBE case, was also used in this study. Additional swab samples were collected from wild Australian koalas from QLD, NSW, Victoria (VIC), and South Australia (SA) as part of ongoing field investigations by veterinary collaborators or for routine diagnostic testing following the presentation of sick animals to various wildlife hospitals. The testing of these swab samples has been considered by the Queensland University of Technology (QUT) Animal Ethics Committee and was approved as tissue use notification number 1100000718.

In a preliminary study to understand the relationships between these C. pecorum isolates, we selected samples from the same animal but different anatomical sites, different individuals from the same population, and/or sympatric populations for the three hosts. The abbreviated names of these C. pecorum-positive samples follow the pattern of the geographical location of the sample/animal/site of infection (e.g., Eungowa/Sheep1/Ocular = Eung/Ovi1/Eye). The samples included (i) 11 ovine C. pecorum PCR-positive samples (Eung/Ovi1/Eye, Eung/Ovi2/Eye, For/Ovi3/Eye, Dub/Ovi3/Rec, Mer/Ovi2/Int, Mer/Ovi2/Int, Nyn/Ovi1/Eye, Nyn/Ovi1/Rec, Nyn/Ovi3/Eye, Nyn/Ovi3/Int, and Nyn/Ovi4/Eye) from the collection of PCR-positive Australian livestock samples, (ii) C. pecorum PCR-positive swab samples from QUT’s extensive collection of Australian koala samples, and (iii) a cell-cultured Australian cow SBE isolate. A list of C. pecorum-positive clinical samples from Australian livestock and 17 koalas detected in this study is included in Table S1 in the supplemental material.

Animal swabs to be screened for the presence of C. pecorum DNA were processed by vortexing and centrifugation (39). For all swabs, DNA was extracted using a QIAamp DNA kit (Qiagen, Doncaster, Victoria, Australia), according to the manufacturer’s instructions. DNA purity and yield were determined using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc.). Extracted DNA was used as the template for a C. pecorum-specific quantitative real-time PCR for the detection and measurement of the infectious load, targeting a 202-bp region of the C. pecorum 16S rRNA (39). All quantitative PCRs (qPCRs) were performed as previously described (39) on a Rotor–Gene Q (Qiagen). Negative (distilled water [dH2O]) and positive (C. pecorum strain MC/Marsbar) controls were included in each amplification assay. C. pecorum genes and primers used for MLSA. The complete list of target genes and primers used in the current MLSA study is found in Table 1. Fragments of seven HK genes (en0A, oppA_3, gidA, hflX, fimC, and gatA) previously used for the typing of several species in the Chlamydiae phylum by MLST (30) were targeted for MLSA as a part of this study. Initially, we were only able to successfully amplify two HK genes (gatA and en0A) using modified pan-Chlamydiades PCR primers (30) on purified C. pecorum genomic DNA; therefore, we designed new C. pecorum-specific PCR primer pairs for the five remaining HK genes (Table 1). For the genes oppA and fimC, new primer pairs were designed using a combination of an existing modified degenerate primer (30) and a new primer. For the genes hflX, gidA, and hemN, new pairs of primers were used, which were designed based on the C. pecorum E58 bovine genome sequence (23). Using purified C. pecorum genomic DNA as a template, conventional PCR was successfully used to amplify a fragment of each of the HK genes of interest. Additionally, the primers were tested against genomic DNA samples extracted from cultured Australian avian Chlamydia psittaci and koala Chlamydia pneumoniae isolates (data not shown). These C. pecorum-specific HK primers were subsequently used for MLSA of Australian C. pecorum livestock and koala strains.

PCR and sequencing. PCRs for all target gene fragments were prepared to a total reaction mixture volume of 50 µl, including 1× AmpliTaq Gold 360 master mix (Life Technologies, Victoria, Australia), 0.3 µM each forward and reverse primer (Sigma-Aldrich, New South Wales, Australia), and 3 µl DNA template, of an average concentration of 25 ng/µl. All PCRs were performed in an S1000 thermal cycler (Bio-Rad, Singapore). Negative (dH2O) and positive (C. pecorum strain MC/Marsbar) controls were included in each amplification assay. The cycling conditions for all HK genes included an initial denaturation (10 min at 95°C) followed by 40 cycles of denaturation (30 s at 95°C), annealing (30 s at 54°C for fimC, gatA, and hemN; 30 s at 58°C for en0A, hflX, and oppA, and 30 s at 60°C for gidA), and extension (1 min at 72°C), followed by a final extension (7 min at 72°C). Upon amplification, PCR products were detected on a 2% ethidium bromide agarose gel and visualized under an UV transilluminator and purified using a High Pure PCR product purification kit (Roche, New South Wales, Australia). Each PCR product was directly sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Life Technologies, Victoria, Australia) and subsequently was purified according to the manufacturer’s instructions. Sequencing was performed at the QUT DNA sequencing facilities using the Applied Biosystems ABI3500 gene analyzer.
Sequence and phylogenetic analysis. Forward and reverse chromatograms of each sequenced gene were aligned in the Geneious Pro 6.0.4 software package, and a consensus sequence was obtained and trimmed to an appropriate size to correspond with established MLST gene sequence fragments. Concatenation of the seven HK gene fragments was performed in the same order to the established MLST scheme for Chlamydiae (30). Allele numbers for our Australian livestock and koala C. pecorum MLSA data sets were identified (see http://pubmlst.org/chlamydiales/) (38).

Sequence and phylogenetic analyses were performed using the Geneious Pro 6.0.4 software package. Sequences of individual genes and concatenated gene sequences were aligned in the Geneious Pro 6.0.4 software package. Sequences of individual genes and concatenated gene sequences were analyzed by calculating the number of synonymous (\( d_s \)) and nonsynonymous (\( d_n \)) substitutions per site and the average number of nucleotide substitutions per site between the populations (\( D_{xy} \)), with the Jukes-Cantor correction. We also calculated the number of polymorphic substitutions at sites and haplotypes, as well as testing for the minimum number of recombination events (Rm) using the Hudson and Kaplan 1985 algorithm, as implemented in DnaSP 5.0 (41). Best-fit models of nucleotide substitution for constructing phylogenies of our data sets were estimated by considering 11 substitution models using jModelTest v.2.1.1 (42). A phylogenetic tree comprising all C. pecorum strains was constructed based on concatenated MLSA sequence alignments, using the program MrBayes (43) with the HKY85G substitution model, as implemented in Geneious Pro 6.0.4. Run parameters included 10,000,000 generations, sampled every 100 generations, and with the first 1,000 trees discarded as burn-in.

Nucleotide sequence accession numbers. The HK gene sequences from Australian koala, sheep, and cow C. pecorum isolates are available in GenBank (accession numbers KC885978 to KC886180).

RESULTS

C. pecorum in Australian sheep. In order to assess the presence and prevalence of C. pecorum infections in Australian sheep populations, we screened 77 clinical swabs from a total of 40 sheep with (i) suspected chlamydiosis, (ii) no overt signs of disease, or (iii) presentation of symptoms consistent with other etiological agents from the central NSW region. This analysis revealed 13 C. pecorum-positive sheep from seven out of the nine flocks screened (see Table S2 in the supplemental material). Fifty percent of the PCR-positive swabs were from the eyes of sheep with suspected chlamydial keratoconjunctivitis. In six sheep diagnosed with keratoconjunctivitis from three different flocks (Eugowra, Forbes, and Nyngan), C. pecorum DNA was also detected at the rectal site. Screening of overtly healthy sheep revealed PCR positivity in five animals (38%), including two animals that displayed PCR positivity in ocular swabs, while the remaining three were PCR positive at the rectal sites. Due to the invasive sampling procedures, only two joint samples from sheep with suspected polyarthritis were available for screening in this study, and these were both positive for C. pecorum DNA.

Evaluation and optimization of C. pecorum-specific MLSA PCR assays. Initially, we were only able to successfully amplify two HK genes (gatA and enoA) using the modified pan-Chlamydiae PCR primers (30) on purified C. pecorum genomic DNA. To resolve these issues, multiple sequence alignments of the seven HK genes from the recently available C. pecorum E58 genome (23), and draft genomes of three koala C. pecorum isolates isolated from SEQLD and the sheep C. pecorum polyarthritis type strain IPA (Polkinghorne, unpublished), as well as other Chlamydia species, were performed, which revealed a number of single nucleotide polymorphisms that may affect the efficiency of PCR amplification using the pan-Chlamydiae degenerate primers. Due to sequence divergence within the Chlamydiaceae family, Zocovic and colleagues (44) were also able to amplify only gatA, enoA, gidA, and hflX using the pan-Chlamydiae. The newly designed C. pecorum-specific HK gene primers reported here amplified the desired sequences with high efficiency and specificity and were tested against genomic DNA samples extracted from cultured Australian C. psittaci and koala C. pneumoniae isolates (data not shown).

MLSA HK genes are subject to purifying selection. To understand the relationships and the level of genetic diversity that exists among Australian C. pecorum isolates from livestock and koalas, as well as U.S. strains, an MLSA of concatenated HK gene fragments was performed on 35 isolates, including 18 livestock isolates (11 Australian sheep, one Australian cow, and six U.S. livestock isolates) and 17 Australian koala C. pecorum isolates, detected in a variety of populations across Australia.

Evaluation of the selective pressures on the C. pecorum HK genes was performed by estimating nonsynonymous-to-synonymous-substitution (\( d_n/d_s \)) ratios, where a \( d_n/d_s \) ratio of <1 indicates negative or purifying selection (an excess of synonymous substitutions), and a

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**TABLE 1 Genes and primers used in this study**

<table>
<thead>
<tr>
<th>HK gene</th>
<th>Annotation</th>
<th>Locus tag</th>
<th>Position in the C. pecorum E58 genome*</th>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
<th>Annealing temp (°C)</th>
<th>Amplicon size (bp)</th>
<th>Size of sequence analyzed (bp)</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>opp4_3</td>
<td>Oligonucleotide-binding protein</td>
<td>G55_0967 952191–952671</td>
<td>Mlopp4A1F</td>
<td>ATGGCGAAGATCCTCGGTTG</td>
<td>58</td>
<td>605</td>
<td>483</td>
<td>30</td>
<td>This study</td>
</tr>
<tr>
<td>hflX</td>
<td>GTP binding protein</td>
<td>G55_0597 579741–580175</td>
<td>MihflX1F</td>
<td>TGAGGAGATCTCTGATCG</td>
<td>58</td>
<td>607</td>
<td>435</td>
<td>30</td>
<td>This study</td>
</tr>
<tr>
<td>gidA</td>
<td>Glucose-inhibited division protein A</td>
<td>G55_0430 429868–429935</td>
<td>MJgidA1F</td>
<td>GATGACAACAAAGAAGGCG</td>
<td>60</td>
<td>560</td>
<td>474</td>
<td>30</td>
<td>This study</td>
</tr>
<tr>
<td>enoA</td>
<td>Enolase</td>
<td>G55_0242 254779–255159</td>
<td>MJenoA1R</td>
<td>TCTTCCTCAGTATACACCGG</td>
<td>60</td>
<td>560</td>
<td>474</td>
<td>30</td>
<td>This study</td>
</tr>
<tr>
<td>hemN</td>
<td>Oxygen-independent coproporphyrinogen III oxidase</td>
<td>G55_0144 150138–150569</td>
<td>MJhemN1F</td>
<td>GATAGCAGATAGAGACCC</td>
<td>54</td>
<td>634</td>
<td>432</td>
<td>30</td>
<td>This study</td>
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<td>fumC</td>
<td>Fumarate hydratase class II</td>
<td>G55_0015 18169–17705</td>
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<td>634</td>
<td>432</td>
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<td>This study</td>
</tr>
</tbody>
</table>

* GenBank accession number.
Collectively, we have identified 11 STs in Australian sequences of the seven HK gene fragments for all 35 C. pecorum isolates using Bayesian methods (Fig. 1). Our analysis revealed that all seven loci are under purifying or negative selection, with double the number of synonymous substitutions compared to nonsynonymous substitutions, with three novel STs (A, E, and F) unique to sheep, one ST previously identified in koalas, and one ST23 previously identified in Australian livestock isolates (see Table S4 in the supplemental material). The highest number of polymorphic sites and substitutions was limited to three HK genes, gatA, gidA, and enoA. The highest number (n = 3) of nonsynonymous substitutions was observed in gatA. Overall, the number of synonymous substitutions per synonymous site (dS) occurred 3.2 times more than nonsynonymous substitutions per nonsynonymous site (dN). Analysis of the dN/dS ratios, ranging from 0 to 0.237, revealed that all seven loci are under purifying or negative selection, resulting mainly in silent substitutions. Although the level of diversity was limited, we found 26 allelic variants across the seven HK loci, with gatA exhibiting six allelic variants, making it the most diverse locus.

Diversity within livestock and koala C. pecorum populations as assessed by MLSA. MLSA of Chlamydia HK genes has previously been shown to reflect the level of genetic diversity present across the genome among representative species of Chlamydiae (30, 31, 46). To confirm whether similar observations could be made for C. pecorum, we analyzed the genetic diversity within and between C. pecorum isolates by assessing dN/dS ratios, the number of sequence types (STs), and divergence between different C. pecorum populations based on their respective hosts and/or biogeographical origins.

Koala C. pecorum isolates had the lowest dN/dS ratio among the Australian C. pecorum isolates assessed, with double the number of synonymous substitutions compared to nonsynonymous substitutions (Table 2). Seven previously undescribed and arbitrarily assigned C. pecorum STs (Aa, Ab, Ac, B, C, Ca, and D) were identified in koalas (see Table S4 in the supplemental material). The pool of samples from Australian livestock C. pecorum isolates also had low dN/dS ratios, again with synonymous substitutions in excess. Lesser ST diversity was observed among Australian livestock isolates, with three novel STs (A, E, and F) unique to sheep, one ST (Aa) shared with koalas, and one ST23 previously identified in global C. pecorum livestock isolates (see Table S4 in the supplemental material). Collectively, we have identified 11 STs in Australian C. pecorum isolates, with 10 of them being novel.

Phylogenetic relationships of C. pecorum livestock and marsupial strains assessed by MLSA. MLSA has been previously used to examine the phylogenetic relationships between closely related species of bacteria (47). In this study, the phylogenetic relationships of each strain were constructed using the aligned concatenated sequences of the seven HK gene fragments for all 35 C. pecorum isolates using Bayesian methods (Fig. 1). Our analysis revealed that C. pecorum isolates separate into three well-supported clades, with an outgroup consisting of four U.S. C. pecorum livestock isolates (Fig. 1). These four isolates had 100% sequence identity among them but differed from the rest of the koala and livestock isolates by an average of 10 nucleotides.

A second clade consisted of bovine and ovine C. pecorum isolates from the United States and Australia. Within this clade, we observed a subgroup of six Australian C. pecorum isolates (five sheep and one cow) that have 100% sequence identity to each other, as well as to the U.S. bovine E58 C. pecorum SBE type isolate. This clade also provides several potentially important epidemiological observations regarding the Australian livestock isolates, including the following: (i) isolates found causing SBE in cattle (NSW/Bov/SBE) could also be found in healthy sheep, as well as in association with conjunctivitis or polyarthritis in sheep (Dub/Ovi3/Rec, Esg/Ovi1/Eye, Mer/Ovi1/Int), (ii) the same C. pecorum STs could be found circulating within one flock (Mer/Ovi1 and Mer/Ovi2), and (iii) multiple STs could be found within one animal from various anatomical sites (Nyn/Ovi1/Eye and Rec and Esg/Ovi1/Eye and Rec).

All Australian koala C. pecorum isolates grouped together into a third distinct clade. Interestingly, three livestock isolates (U.S. cow 66P130 and Australian sheep Nyn/Ovi2/Eye and Nyn/Ovi3/Eye) also clustered with the koala isolates, sharing 99.8% and 100% sequence similarities, respectively. The identical sheep and koala STs were not sampled from animals in the same region, however. Notably, the latter ovine STs detected on the Nyngan property brought the total of unique STs detected in this single sheep flock to four. As a rule, the diversity of STs detected was more homogenous than that in the previous clade, which contained only C. pecorum isolates from livestock. Nevertheless, an analysis of the diversity of STs within this third clade of koala and livestock C. pecorum isolates showed evidence for (i) C. pecorum STs infecting different anatomical sites within the same koala, as similarly found in STs from the previous livestock clade (e.g., Gun/Koala1/Urethra and Gun/Koala1/Cloaca), (ii) the same STs infecting multiple animals within the same koala population (e.g., StB/9274 and StB/9341), (iii) the presence of multiple C. pecorum STs circulating within a single koala population (e.g., EC/Ned, EC/James, and EC/Steve), and (iv) the presence of a single C. pecorum ST in the koala populations in SA (SA/Adel/Eye), QLD (StB/9341/Eye, Bre/Kurt/UGT, MC/Marsbar, and EC/Ned/UGT), NSW (TB/Elisabeth/Eye), and VIC (Vic/JBA001/Eye).

Divergence between C. pecorum populations assessed in this study. In our assessment of the phylogenetic relationships between livestock and koala C. pecorum isolates, we observed separation into two distinct clades and a degree of association between the host and isolate. We also assessed the genetic divergence between the koala and livestock C. pecorum populations using these
seven concatenated HK gene fragment sequences. The divergence can be assessed by calculating the average number of nucleotide substitutions per site between populations ($D_{xy}$) and the number of uniform mutations within specific populations (the fixed differences) and shared polymorphisms between populations.

As observed in Table S5 in the supplemental material, $D_{xy}$ was very limited and comparable among populations, and there were no fixed differences between populations. Overall, the average number of nucleotide differences between the koala and livestock $C. pecorum$ populations was 7.25, with the highest number of differences observed between U.S. and Australian livestock isolates, which shared 10 polymorphisms. Consistent with phylogenetic observations, the smallest number of differences was noted between Australian koala and livestock, with only two shared polymorphisms.

**DISCUSSION**

$C. pecorum$ infections continue to cause significant economic losses in livestock, both in Australia and globally (14, 15). $C. pecorum$ infections are also a major contributing factor to the decline of koala populations across Australia (3, 48). Despite this, virtually nothing is known about the epidemiology and genetic diversity of these infections in both livestock and koala hosts. While molecular evidence has previously pointed to a relationship between strains infecting both hosts (24), these relationships had not been subjected to robust phylogenetic analysis using a typing scheme that was demonstrated to reflect the rates of change across the whole chromosome of *Chlamydia*.

In a pilot study to investigate the overall genetic relationships between $C. pecorum$ isolates infecting koala and livestock hosts, we developed a customized MLSA scheme, based on a previously...
published MLST scheme for member species of the *Chlamydii-
aceae* (30). Sequences of seven genetically stable HK gene frag-
ments were obtained and analyzed from a total of 35 livestock and
koala *C. pecorum* isolates. Although the level of diversity was lim-
ited among analyzed *C. pecorum* strains, we identified 10 novel
STs, six found in koala isolates only and three sequence types
observed in Australian sheep isolates. For the most part, we ob-
served a distinct phylogenetic separation of koala and livestock
isolates, with an observation of only one ST being shared between
Australian sheep and koalas.

Based on this analysis and in contrast to previous descriptions
of the genetic diversity of this species using the *ompA* gene (22), *C.
pecorum* appears to harbor limited diversity, at least when HK
genes were considered. This observation is supported by low $d_\text{sn}/d_\text{ds}$
ratios across the *C. pecorum* strains we sampled. No putative re-
combination events were detected in the seven HK gene frag-
ments, and the number of synonymous substitutions exceeded
nonsynonymous substitutions, indicating strong purifying selec-
tion. This was expected, as these genes, commonly used for MLST
in other bacteria, are widely spaced across the chromosome, are
evolutionarily conserved, experience limited or no host immune
pressures, and are representative of overall chromosomal change
(28,33). This limited diversity of the *C. pecorum* species is consist-
tent with the diversity observed in other members of the genus
*Chlamydia*, particularly that observed from MLSA and MLST
analysis of the closely related chlamydial species *C. pneumoniae*
(30,49). The number of synonymous substitutions detected in *C.
pecorum* was comparable to those observed in *Chlamydia abortus*
and *C. psittaci* (31). Our Australian *C. pecorum* strains had diver-
sity of 0.4 ST/per strain, which is consistent with observations
across the *Chlamydiae* in general (31,44).

Koala isolates displayed more diversity of STs, with six unique
STs found in this host compared to livestock. This greater ST
diversity might reflect the wider geographic range of koala popu-
lations sampled here compared to Australian livestock, which
were sampled from central NSW only. The greater ST diversity
may also reflect the recent diversification of *C. pecorum* across the
geographic range of koalas following its potential introduction
from livestock, despite HK genes being under strong purifying
selection. Different bacterial populations show different levels of
ST diversity per isolate, e.g., 0.92 ST/per strain for *Enterococcus faecalis*, 0.72 ST/per strain for *Neisseria* spp., and 0.46 ST/per
strain for *Staphylococcus aureus*, but in general, the values are on
the lower end and are comparable to each other (32).

Despite the fact that *C. pecorum* is a major pathogen of domes-
ticated animals with a worldwide distribution, still little is known
about its transmission and the factors associated with *C. pecorum*
infection in these hosts (50). With the establishment of a level of
confidence in our *C. pecorum* MLSA scheme, we then used this
scheme to provide insights into the finely detailed molecular epi-
demiology of *C. pecorum* infections in Australian livestock and
koalas. This analysis of several sheep flocks in central NSW re-
vealed at least four unique STs (see Table S4 in the supplemental
material). Among these STs, we identified Australian sheep and
cow isolates that were phylogenetically 100% identical to the pre-
viously described U.S. bovine SBE type isolate E58 (23). In Aus-
tralian livestock, this ST, previously described as ST23, could be
found in association with (i) ovine conjunctivitis, (ii) ovine poly-
arthritis, (iii) bovine encephalomyelitis, and, in the present study,
(iv) in a single case of clinically healthy ovine fecal shedding. Gas-
trointestinal strains leading to asymptomatic infection and their
fecal shedding may be common in infected Australian sheep
flocks. Fecal shedding of *C. pecorum* by carrier animal hosts has
been reported previously (8,51) and is thought to be the most
important mode of transmission (15). Other transmission routes
include sexual and vertical transmission, as *C. pecorum* iso-
lates were found in the genitourinary tracts of healthy bulls (1) and
*C. pecorum* was detected in infected calves in utero (9). Animals are
also susceptible to infection by the fecal-oral route (2). Using our
MLSA, we also observed in multiple cases that one sheep host can
harbor two distinct STs, with one found in the conjunctiva (Eug/
Ovi/Eye, Nyn/Ovi/Eye), while another novel ST was detected in the
gastrointestinal tract (Eug/Ovi/Rec, Nyn/Ovi/Rec). A variety of STs
were also present on a flock level. Three distinct STs, 23, 2013A,
and 2013Aa, detected in multiple hosts from ocular and rectal
sites, were present in a Nyngan sheep flock (see Table S2 in the
supplemental material). Infection with these *C. pecorum* STs in
the Nyngan flock could have been from multiple sources, further
supporting the possible transmission routes outlined above. The
risks of potential transmission of *C. pecorum* infections between
animals are increased with cograzing of sheep and cattle, as in this
study we observed the same *C. pecorum* ST found in both hosts.
As the cooccurrence of cattle and sheep in a geographical area is com-
mon, this should be a target area for further *C. pecorum* investiga-
tions in order to better understand the epidemiology of the *C.
pecorum* infections.

Using our *C. pecorum* MLSA scheme, we could also make similar
ly interesting observations about the epidemiology of *C. peco-
rum* infections in koalas. *C. pecorum* isolates infecting koalas
displayed more diversity than those infecting livestock, resulting in
seven novel STs being detected (see Table S4 in the supplemental
material). The distribution of STs in koala populations was similar
to that seen in our sheep epidemiological analysis, including the
observation of multiple *C. pecorum* STs within the same koala
population (EC/James, EC/Steve, and EC/Ned), the presence of a
single *C. pecorum* ST in multiple anatomical sites of the same koala
(Gun/Koala1/Urethra and Gun/Koala1/Cloaca), and the detection
of a single *C. pecorum* ST within multiple animals in the same
population (StB/9341 and StB/9274 koalas). Although koalas were
sampled from various geographical locations across Australia, we
did not observe biogeographical separation of *C. pecorum* koala
strains using MLSA. Identical STs were observed in koalas origin-
ating from different states (SA *C. pecorum* isolate SA/Adel/Eye
was 100% identical to the SEQLID isolates MC/Marsbar, Bre/Kurt/
UGT, StB/9274/RE, and StB/9341/RE, as well as the VIC and NSW
Vic/jba001/Eye and TB/Elisabeth/Eye isolates).

The majority of koalas assessed in this study displayed clinical
signs of chlamydial disease, with the exception of two koalas (StB/
9274 and StB/9341) from a geographically isolated koala popula-
tion from St. Bees Island, QLD, which were found to be *C. peco-
rum* PCR positive. ST2013Aa, observed in these samples (StB/
9274 and StB/9341) from a geographically isolated koala popula-
tion (EC/James, EC/Steve, and EC/Ned), and the detection
of a single *C. pecorum* ST within multiple animals in the same
population (StB/9341 and SB/9274 koalas). Although koalas were
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9274 and StB/9341) from a geographically isolated koala popula-
tion from St. Bees Island, QLD, which were found to be *C. peco-
rum* PCR positive. ST2013Aa, observed in these samples (StB/
9274/RE and StB/9341/RE), was the same as in the samples
derived from animals with keratoconjunctivitis and/or urogenital
infection, not allowing for the differentiation of strains based on
pathology. Interestingly, this same ST was also observed in *C.
pecorum* isolates from the two sheep samples (Nyn/Ovi2/Eye and
Nyn/Ovi3/Eye), which phylogenetically clustered in the koala
clade.

The observation of an identical *C. pecorum* ST type ST2013Aa
in two Australian sheep, alongside the clustering of a U.S. bovine

ST49 with other koala Chlamydia pecorum STs (Fig. 1), provides a potential snapshot of the risk of cross-host transmission between Australian livestock and koalas. Similar observations were reported previously in phylogenetic analyses of koala and livestock Chlamydia pecorum using the single highly polymorphic ompA gene (22, 52). In our study, koala isolates formed a single cluster within a larger livestock clade, in contrast to previous observations using novel genetic markers (24), although Australian livestock isolates were not included in the previous study. In agreement with the phylogenetic analyses mentioned above, low population divergence indices (see Table S5 in the supplemental material) also support the possibility that koala Chlamydia pecorum isolates may have diverged from livestock isolates, a potential indicator of the origin of Chlamydia pecorum infections in koalas. Low population divergence values indicate a potentially very recent evolutionary split between the koala and livestock Chlamydia pecorum populations, with no fixed differences and only shared polymorphisms. An increase in shared polymorphisms and reduced fixed differences suggest "recent" active gene flow between the local populations (53), which could explain the observed values in C. pecorum koala and livestock populations that were analyzed here. A better understanding of the origin of Chlamydia pecorum in koalas will require a larger cohort of livestock and koala samples from the same geographical area in Australia. A broader assessment of the strains reported in other Australian marsupials (54) and worldwide domesticated and wild ungulates (55, 56) would contribute to our understanding of the genetic diversity of Chlamydia pecorum and the origins of these infections. Such an analysis should also include a larger number of samples within each population to build a better picture of the intrapopulation Chlamydia pecorum genetic structures, thus improving our understanding of the epidemiology of these infections.

Collectively, our MLSA of seven HK gene fragments of Chlamydia pecorum isolates in Australian sheep and cows has provided us with the first molecular epidemiological data on infections in these hosts in Australia. An expansion of cross-sectional studies employing this Chlamydia pecorum typing scheme across entire populations of infected animals will be critical for developing effective management strategies for Australian livestock. Beyond this study, our MLSA scheme could be translated into an MLST scheme that is widely used for finely detailed epidemiological study, our MLSA scheme could be translated into an MLST scheme that is widely used for finely detailed epidemiological study, our MLSA scheme could be translated into an MLST scheme that is widely used for finely detailed epidemiological study, our MLSA scheme could be translated into an MLST scheme that is widely used for finely detailed epidemiological study, our MLSA scheme could be translated into an MLST scheme that is widely used for finely detailed epidemiological study.

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