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Culture-Independent Genome Sequencing of Clinical Samples Reveals an Unexpected Heterogeneity of Infections by *Chlamydia pecorum*

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***Chlamydia pecorum* is an important global pathogen of livestock, and it is also a significant threat to the long-term survival of Australia's koala populations. This study employed a culture-independent DNA capture approach to sequence *C. pecorum* genomes directly from clinical swab samples collected from koalas with chlamydial disease as well as from sheep with arthritis and conjunctivitis. Investigations into single-nucleotide polymorphisms within each of the swab samples revealed that a portion of the reads in each sample belonged to separate *C. pecorum* strains, suggesting that all of the clinical samples analyzed contained mixed populations of genetically distinct *C. pecorum* isolates. This observation was independent of the anatomical site sampled and the host species. Using the genomes of strains identified in each of these samples, whole-genome phylogenetic analysis revealed that a clade containing a bovine and a koala isolate is distinct from other clades comprised of livestock or koala *C. pecorum* strains. Providing additional evidence to support exposure of koalas to Australian livestock strains, two minor strains assembled from the koala swab samples clustered with livestock strains rather than koala strains. Culture-independent probe-based genome capture and sequencing of clinical samples provides the strongest evidence yet to suggest that naturally occurring chlamydial infections are comprised of multiple genetically distinct strains.**

Chlamydia pecorum is a widespread pathogen of economically important livestock species, such as cattle and sheep. In cattle, *C. pecorum* is associated with sporadic bovine encephalomyelitis (SBE), which presents as a fever followed by limb stiffness and staggering (1). In sheep, *C. pecorum* infections commonly are linked to polyarthritis and conjunctivitis, which can spread rapidly in a flock (2, 3). While these infections are economically relevant to producers, most *C. pecorum* infections in ruminants appear to be asymptomatic or subclinical, characterized by a consistent presence in the gastrointestinal tract (4, 5). While questions remain over the impact of these infections in livestock globally, the best example of the pathogenic potential of this obligate intracellular bacterium actually is found in koalas, a native Australian marsupial that continues to experience localized extinctions. *C. pecorum* infections in koalas can cause debilitating ocular and urogenital tract diseases (6, 7). Epidemiological questions have been raised about the relationships between *C. pecorum* strains infecting domesticated animals and the koala, with a recent *C. pecorum* multilocus sequence typing (MLST) study revealing the presence of identical sequence types in samples collected from each host (8). As a follow-up to these studies, we recently sequenced the genomes of several cultured koala *C. pecorum* isolates, revealing a high degree of synteny and sequence identity (98.5 to 98.8%) with *C. pecorum* genomes from European and U.S. cattle and sheep (9).

High-throughput comparative genome sequencing of cultured isolates has fundamentally changed our understanding of the biology and genetic relationships of chlamydial species infecting a wide range of human and animal hosts (10). To date, however, a major limitation of this approach has been the requirement for extensive passaging in tissue culture to generate a sufficient concentration of chlamydial DNA for the purpose of genome sequencing (11–14). Culturing also has the potential to produce bias, with a recent study revealing that extended *in vitro* culturing can introduce mutations in the genome sequence of chlamydial isolates, presumably due to a lack of host immune pressures (15). In a major breakthrough in the *Chlamydia* research field, culture-independent sequencing methods have been developed, with one of the first approaches involving immunomagnetic separation (IMS) of chlamydial cells using antibodies specific for the chlamydial lipopolysaccharide (16). Using IMS in conjunction with multiple displacement amplification (MDA) produced whole-genome sequences for *C. trachomatis* strains from low-volume archival samples and swab samples collected from patients (17, 18).

Sequence capture by hybridization also can be used to sequence chlamydial DNA without the need for cell culturing (19). The sequence capture method involves designing customized biotin-labeled RNA probes that can hybridize to a complete target genome sequence so that magnetic beads coated with the biotin-binding protein streptavidin can be used to extract the captured sequences (20). This sequence capture method originally was designed to enrich specific regions of large eukaryotic genomes for deep sequencing of a selected subset of genes (21, 22) but has since been used for other applications, including deep sequencing of viral genomes (23). In this study, we adapted this sequence cap-

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TABLE 1 *C. pecorum* clinical samples and sequencing output

Strain	Host	Disease[a]	Amt of DNA used as template[b] (ng)	Total no. of input *C. pecorum* genome copies[c]	Total no. of reads	Mapped reads[d]		Mean read depth	Read coverage (SD)	Accession no.
						No.	%			
C. pecorum NSW/Bov/SBE	Cattle	SBE	1,011.35	4.6×10^{12}	37,110,550	36,996,629	99.69	2,632	250.81	SRR1693788
C. pecorum Gun/koa1/Ure	Koala	UTI	209.90	1.6×10^{5}	13,281,012	13,023,096	98.06	184	33.70	SRR1693763
C. pecorum SA/k2/UGT	Koala	UTI	453.92	2,253	4,623,524	3,874,162	83.79	44	18.70	SRR1693792
C. pecorum Mer/Ovi1/Jnt	Ovine	Polyarthritis	157.63	2,628	1,105,062	1,063,335	96.22	12	29.90	SRR1693791
C. pecorum Nar/S22/RE	Ovine	Conjunctivitis	173.21	10,299	4,890,048	3,576,983	73.15	11	139.47	SRR1693793
C. pecorum Nar/S22/Rec	Ovine	Conjunctivitis	84.45	2,674	27,257,774	21,705,968	79.63	21	116.61	SRR1693794
C. pecorum Nar/S42/LE	Ovine	Conjunctivitis	129.94	8,662	26,729,184	185,341	0.69	6	1.64	SRR1693795

[a] SBE, sporadic bovine encephalomyelitis; UTI, urinary tract infection.
[b] DNA concentration includes host and other resident microbiota DNA.
[c] The number of *C. pecorum* genome copies determined by qPCR that were used as input in the library preparation.
[d] Reads were mapped to the complete *C. pecorum* E58 genome.

ture technique to extract *C. pecorum* DNA sequence from clinical swabs and assess its feasibility as a novel culture-independent technique for high-throughput sequencing of *C. pecorum* strains. In doing so, we not only demonstrate that this technique is feasible for this chlamydial species but also reveal that it can be used to detect an unprecedented diversity of mixed infections in clinical samples, an observation that has ramifications for our understanding of the genetic diversity of naturally occurring infections for all chlamydial species.

MATERIALS AND METHODS

Clinical samples analyzed in this study. *C. pecorum* PCR-positive samples ($n = 6$) and a cell-cultured *C. pecorum* isolate ($n = 1$) were selected. The strain names are abbreviations referring to geographical location, the animal name, and site of infection/disease (e.g., NSW/Bov/SBE is New South Wales/bovine/sporadic bovine encephalomyelitis). The collection of previously tested *C. pecorum* PCR-positive swab samples included two urogenital tract (UGT) swabs (SA/k2/UGT and Gun/koa1/Urethra [Ure]) collected from two different koalas, one from South Australia (SA) and one from Gunnedah (Gun) (8). The remaining *C. pecorum* PCR-positive swab samples ($n = 4$) were collected from previously tested Australian sheep from the NSW regions of Narromine (Nar) and Merriwa (Mer) (8, 24), including swab samples collected from (i) the eye (*C. pecorum* Nar/S22/RE) and the rectum (*C. pecorum* Nar/S22/Rec) (8) of a single sheep (S22) presenting with conjunctivitis; (ii) the left eye of a second sheep (S42) from the same flock (*C. pecorum* Nar/S42/LE), also presenting with conjunctivitis (24); and (iii) the joint of a third sheep (Ovi1) suffering from polyarthritis (*C. pecorum* Mer/Ovi1/Jnt) (8). All collected dry swabs were kept at 4°C until processing by vortexing and centrifugation, as previously described (8). The final sample analyzed (*C. pecorum* NSW/Bov/SBE) was a cell-cultured and purified strain isolated from the brain of an NSW calf with SBE (8). Briefly, the *C. pecorum* NSW/Bov/SBE isolate was propagated in Hep-2 cells and ultrapurified using density gradient centrifugation as previously described (9).

Quantification of *C. pecorum* in swab samples. DNA was extracted from swab samples and from the *C. pecorum* NSW/Bov/SBE cell culture as previously described (8). DNA concentration for each sample was measured in duplicates on a NanoDrop instrument, and the presence of *C. pecorum* was requantified using a *C. pecorum* species-specific quantitative PCR (qPCR) assay (25). Samples with <10 copies of the *C. pecorum* 16S rRNA gene were considered negative. The total amounts of DNA and the genome copies used as the template for targeted enrichment are outlined in Table 1.

SureSelect target enrichment. The 120-mer RNA probes were designed in-house at the Institute for Genome Sciences (IGS) to span across the forward strand of the *C. pecorum* E58 genome (accession number CP002608). The custom-designed *C. pecorum* probe library was uploaded to SureDesign (https://earray.chem.agilent.com/suredesign/) and synthesized by Agilent Technologies. DNA libraries were constructed using the TrueSeq library kit, and the hybridization was done using the *C. pecorum*-specific probes and SureSelectXT reagents.

Genome sequencing. *C. pecorum* genomes were sequenced from the seven samples using Illumina HiSeq to produce paired-end 101-bp reads. Read quality was checked with FASTQC, and filtering and trimming were performed on the reads with Trimmomatic version 0.32 (26). Genomes were assembled using Spades 3.0.0 with k-mer values of 15, 21, 33, 51, and 71 (27).

Read mapping and SNP analysis. Duplicate reads were removed using Picard (https://github.com/broadinstitute/picard), and read mapping was done using the very sensitive parameters for Bowtie-2 (28) with *C. pecorum* E58 as the reference. The read mapping was visualized using the BLAST ring image generator (BRIG) program (29). Single-nucleotide polymorphisms (SNPs) were detected using Freebayes with min-alternate-fraction (minimum allele frequency) set to 0.05 and ploidy set to 10 (30).

Detection of mixed infection in samples. A custom Python script called groupFlows.py was used to assess the distribution of variants in the reads for the identification of separate strains in each swab sample (https://github.com/mjsull/HaploFlow). Briefly, for each site with two genomic variants (A_n and B_n) identified by Freebayes, groupFlows.py iterated through the reads that aligned to that position using the Python module, pysam, and sorted reads based on which variant is present in the reads. Once reads had been assigned to either variant, groupFlows.py determined how many of the assigned reads also were assigned to each variant at the site immediately upstream (A_{n-1} and B_{n-1}). If either upstream variant shared two or more reads with the downstream variant, a link was created, with up to four links possibly being created (A_{n-1}-A_n, A_{n-1}-B_n, B_{n-1}-B_n, and B_{n-1}-A_n). A cutoff of two reads allowed us to reduce the amount of links being created incorrectly due to read error. In most cases, this resulted in variant A at site *n* linked to a single variant at site *n*-1 and variant B at site *n* linked to the alternate variant (i.e., if variant A_{n-1} linked to variant B_n, then B_{n-1} linked to A_n). If the distance between adjacent variant sites was too long to be spanned by paired-end reads, then it was not possible to form links. In regions where there is a large amount of sequencing or mapping errors, low-complexity regions where non-*C. pecorum* DNA is captured, and regions where additional subpopulations at low levels are observable, three or four links are found. When this occurs, the variants are marked as unlinkable. This process then was repeated for all variants in the genome, resulting in chains of linked variants separating into two distinct groups (e.g., A_{n-4}-B_{n-3}-A_{n-2}-A_{n-1}-B_n and B_{n-4}-A_{n-3}-B_{n-2}-B_{n-1}-A_n) separated by unlinkable regions or gaps that cannot be spanned by reads. These chains then were assigned to strains by looking at the prevalence of variants in the chain that have not undergone a coverage

drop significant enough to make the coverage of two strains indiscrete (greater than half the median coverage of the genome). If there is contradictory information between reporting sites, groups are not assigned to strains (as coverage in the strains was completely distinct, all groups were assigned). If the distance from the first variant in the chain to the last variant in the chain was less than 100 bp and unlinkable regions were found on either side of the chain, the area was marked as "noisy" and the variants weren't assigned to strains. Once variants had been assigned to either the dominant or minor strain, all reads associated with those variants also were assigned and a BAM file of both the dominant and minor strain was created. SAMtools (31) then was used to call the consensus with bases with a PHRED quality score of less than 30 and identified noisy regions being masked. Finally, we iterated through each base in the original BAM file, and if there was no significant coverage drop (coverage of more than half the median coverage) and a 98% or greater consensus between reads mapping to that site, the base was called for the dominant strain. This allowed us to call bases for the dominant strain in regions with no interstrain variation.

Phylogenetic analyses. A whole-genome alignment of the six dominant *C. pecorum* draft genomes (excluding *C. pecorum* Nar/S42/LE), two minor *C. pecorum* strains, and the eight publicly available *C. pecorum* genomes (E58, PV3056, W73, P787, VR629, IPTaLE, DBDeUG, and MC/Marsbar) (32, 33) was performed using Mugsy with default settings (34). Syntenic alignments across all 16 genomes were concatenated using a custom Perl script to construct a core alignment where poorly aligned regions were filtered from the alignment using GBLOCKS version 0.91b with the minimum length of a block set to 5, and no gap positions were allowed. A phylogenetic tree was constructed using the core genome alignment of the 16 *C. pecorum* genomes with PhyML 3.1 using the generalized time reversible (GTR) model, and bootstrap values were calculated using 1,000 replicates.

Short read sequence accession numbers. The sequence data were submitted to the National Center for Biotechnology Information (NCBI) short read archive (SRA) database, and the accession numbers are listed in Table 1.

RESULTS

Probe-based sequence capture enables sequencing of *C. pecorum* strains directly from clinical samples. SureSelectXT sequence capture was performed using *C. pecorum*-specific probes on extracted DNA from six clinical swab samples and a single cell-cultured sample. To determine the efficacy of the SureSelectXT sequence capture method to enrich for *C. pecorum* DNA in samples without prior genome purification, we compared the total number of *C. pecorum* genomes copies to the number of reads that mapped to the complete *C. pecorum* E58 genome and the mean read depth (summarized in Table 1). Cultured *C. pecorum* NSW/Bov/SBE resulted in the highest percentage of mapped reads (99.69%) as well as the highest mean read depth (2,632×). The most likely factor contributing to the high yield of the *C. pecorum* reads in this sample was the high genome copy number of 14.6×10^{12} as a result of extensive passaging through cell culture.

For the swab samples, *C. pecorum* Gun/koa1/Ure had the highest genome copy number (1.6×10^5 copies), and 98.06% of the reads mapped to *C. pecorum* E58 with a mean read depth of 184×. The *C. pecorum* SA/k2/UGT, *C. pecorum* Mer/Ovi1/Jnt, *C. pecorum* Nar/S22/RE, and *C. pecorum* Nar/S22/Rec samples each had a comparatively lower number of genome copies (2,253 to 10,299 copies), but the sequencing still yielded >71% of reads that mapped to *C. pecorum* E58; however, the mean read depth varied greatly among these samples. *C. pecorum* SA/k2/UGT had a mean read depth of 44×, while *C. pecorum* strains Mer/Ovi1/Jnt, Nar/S22/RE, and Nar/S22/Rec had a mean read depth of 12×, 11×, and 21×, respectively. However, the standard deviations of read depth for Mer/Ovi1/Jnt, Nar/S22/RE, and Nar/S22/Rec are higher than the mean read depth, suggesting there is atypical distribution of read depth in these samples. Read quality checking revealed that these three samples have a large number of duplicated reads (>90%) occurring at a specific location, which can be seen in Fig. 1 as coverage spikes. The coverage spikes can be observed in the other samples but the reads are more evenly distributed, resulting in better read depth across the rest of the genome. The coverage spikes correlated with the presence of genes containing tandem repeats (data not shown). Lastly, while the *C. pecorum* Nar/S42/LE sample had a genome copy number of 8,662 copies, only 0.69% of the reads mapped to *C. pecorum* E58; however, a mean read depth of 6× was achieved.

The read mapping revealed that 100% coverage of the reference genome was achieved for the four *C. pecorum* samples collected from sheep (*C. pecorum* Mer/Ovi1/Jnt, *C. pecorum* Nar/S22/RE, *C. pecorum* Nar/S22/Rec, and *C. pecorum* Nar/s42/LE) and the cattle sample, *C. pecorum* NSW/Bov/SBE (Fig. 1). For both koala *C. pecorum* strains, SA/k2/UGT and Gun/koa1/Ure, approximately 95% of the reference genome was covered. Gaps in the coverage were found in a hypervariable region called the plasticity zone (PZ) and in the polymorphic membrane protein (PMP) gene cluster. The cultured *C. pecorum* NSW/Bov/SBE genome was assembled into two contigs, while *C. pecorum* Gun/koa1/Ure, Nar/S22/Rec, SA/K2/UGT, and Mer/Ovi1/Jnt were assembled into 8 contigs, 15 contigs, 17 contigs, and 27 contigs, respectively. Lastly, the assemblies for *C. pecorum* Nar/S22/RE and Nar/S42/LE were poorer, and both assembled into >554 contigs.

Culture-independent sequencing of *C. pecorum* revealed mixed infections. An SNP analysis revealed that four of the seven samples contained heterogeneous sites in the read depth data, indicating the presence of multiple *C. pecorum* strains. This was determined by the identification of a set of variants that occur only in a subset of reads: 8.0% of reads in *C. pecorum* SA/k2/UGT, 13.4% of reads in *C. pecorum* Gun/koa1/Ure, and 23.4% of reads in *C. pecorum* Mer/Ovi1/Jnt (Table 2 and Fig. 2). Although a high level of heterogeneity was detected in *C. pecorum* Nar/S22/Rec, suggesting the potential presence of >2 separate *C. pecorum* strains, the read depth of 21× was too low to accurately predict the prevalence of different strains in this sample. The low read coverage in *C. pecorum* Nar/S22/RE and *C. pecorum* Nar/S42/LE swab samples meant that we also were unable to reliably identify variant reads. We did not detect any variants in the *C. pecorum* NSW/Bov/SBE cultured isolate, suggesting it is a clonal isolate. Table 2 summarizes the number of SNPs identified in the dominant and minor strains of all samples compared to the reference *C. pecorum* E58 genome. The dominant strain is identified by the Greek letter alpha at the end of the strain name (e.g., *C. pecorum* Mer/Ovi1/Jnt-α), while the minor strain is designated by the letter beta at the end of the strain name (e.g., *C. pecorum* Mer/Ovi1/Jnt-β). The cultured *C. pecorum* NSW/Bov/SBE and *C. pecorum* E58 genomes from the brains of cattle with SBE in Australia and the United States, respectively, differ by 61 SNPs. Likewise, both dominant ovine strains (*C. pecorum* Mer/Ovi1/Jnt-α and Nar/S22/RE-α) differed from *C. pecorum* E58 by 59 SNPs. Higher numbers of SNPs were detected in the ovine minor strains (*C. pecorum* Mer/Ovi1/Jnt-β and Nar/S22/RE-β), ranging from 449 to 589 SNPs. The dominant koala strains, *C. pecorum* SA/k2/UGT-α and *C. pecorum* Gun/koa1/Ure-α, differ from *C. pecorum* E58 by 4,775

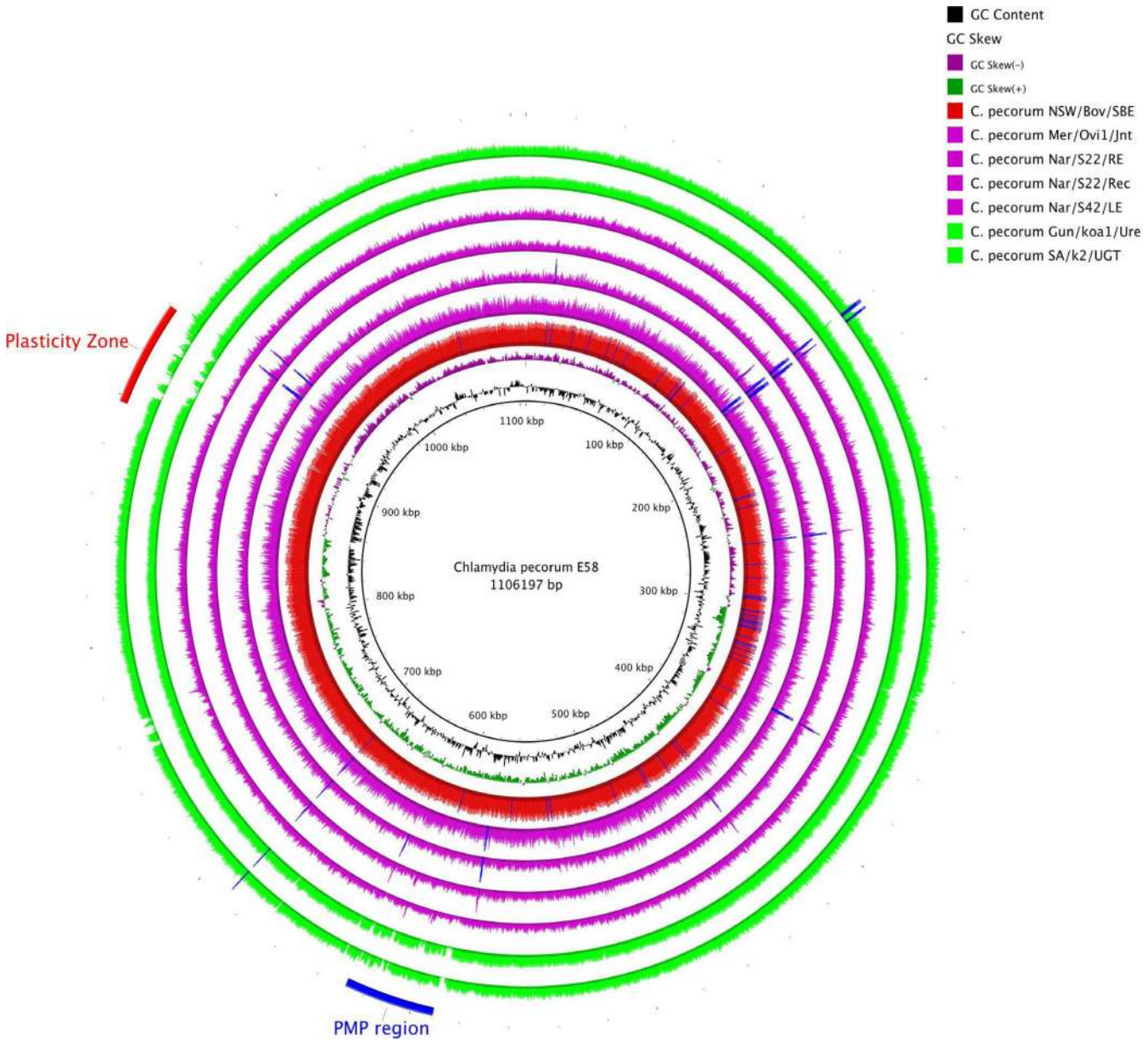

FIG 1 Coverage of *C. pecorum* E58 reference genome. BRIG image shows *C. pecorum* E58 as the reference and the rings (from inner to outer) for GC content, GC skew, and filtered reads mapped against the reference with Bowtie2. The bovine strain *C. pecorum* NSW/Bov/SBE is shown as the red ring. The ovine strains *C. pecorum* Mer/Ovi1/Jnt, *C. pecorum* Nar/S22/RE, *C. pecorum* Nar/S22/Rec, and *C. pecorum* Nar/S42/LE are shown as purple rings. The green rings represent the koala strains *C. pecorum* SA/k2/UGT and *C. pecorum* Gun/Koa1/Ure. Blue spikes represent read-enriched regions that cause large coverage spikes. Only coverage across the reference genome is shown, and read depth is not to scale.

and 6,191 SNPs, respectively. However, there are fewer predicted SNPs (267 SNPs for *C. pecorum* Gun/koa1/Ure-β and 1,391 SNPs for *C. pecorum* SA/k2/UGT-β) in the minor koala *C. pecorum* strains. Figure 3 shows all of the heatmap comparisons of the number of variant differences between each of the *C. pecorum* strains identified from the swab samples, including dominant and minor strains. The number of different variant sites between each of the minor strains is 571 to 1,488 SNPs, suggesting that it is unlikely that the presence of these minor strains was caused by cross-contamination of the samples. The high number of different variant sites between the dominant strains and the minor strains from the same swab samples is further evidence for the presence of multiple genetically distinct *C. pecorum* strains coinfecting the same anatomical sites in both koalas and sheep.

Reassembly of the dominant and minor *C. pecorum* strain reads. In order to understand the phylogenetic relationships between the dominant and minor strains, the raw reads needed to be separated and the consensus sequences called individually. For the most part, an analysis of genetic variants in the sequence reads for each swab sample revealed the presence of two discrete populations of variants that could be separated easily into dominant and minor strains (Fig. 2). There were two exceptions to this observa-

TABLE 2 Prevalence of the dominant strain and the total number of SNPs in dominant and minor strains using *C. pecorum* E58 as a reference

Strain	No. of SNPs in:		Prevalence rate (%) of dominant strain (based on assigned reads)
	Dominant strain	Minor strain	
C. pecorum NSW/Bov/SBE	61	NA[a]	100.0
C. pecorum Gun/koa1/Ure	4775	267	86.6
C. pecorum SA/k2/UGT	6191	1391	92.0
C. pecorum Mer/Ovi1/Jnt	59	449	76.6
C. pecorum Nar/S22/RE	59	589	Unknown[b]

[a] NA, not applicable.
[b] Read coverage was too low to accurately determine the prevalence of the dominant and minor strains.

tion, however, including regions that are (i) low-complexity regions where non-*C. pecorum* DNA potentially has been captured by probes, (ii) reads belonging to additional putative subpopulations, and (iii) regions with low read coverage. Strain-specific coverage drops occurred when one strain in the sample varied too much from the sequence capture probes at the nucleotide level, resulting in less sequence from that area of the strain being captured. As the minor strain may have high similarity in these regions, we predicted that the dominant strain often would be less prevalent at these locations.

Minor *C. pecorum* strains were successfully separated from the dominant *C. pecorum* Gun/koa1/Ure-α and SA/k2/UGT-α strains in the koala UGT swab samples. The minor strain, *C. pecorum* Gun/koa1/Ure-β, was assembled into 648 contigs with a total combined sequence of 915,868 bp. The other minor strain, *C. pecorum* SA/k2/UGT-β, was assembled into 2,070 contigs, with a combined length of 519,725 bp. The assemblies of Gun/koa1/Ure-β and SA/k2/UGT-β are less than the 1.1-Mbp chromosome size of *C. pecorum* E58, suggesting that only partial genomes were acquired for these minor strains. The successful identification of the minor strains for both koala swab samples was possible because of the high starting DNA concentration and the high number of sequence reads generated. In contrast, the low coverage of chlamydial reads in both of the Nar/S22 swab samples made it impossible to separate a minor strain. There also was difficulty with identifying a minor strain in the *C. pecorum* Mer/Ovi1/Jnt sample because of the relatively small amount of sequence reads produced for this sample.

Phylogenetic analysis revealed genetically distinct *C. pecorum* strains coinfecting the same anatomical site. A core genome alignment of 279,714 bp was built using all sequenced *C. pecorum* genomes, including the two minor strains, *C. pecorum* Gun/koa1/Ure-β and SA/k2/UGT-β. Since only partial genomes could be acquired for the minor strains, the resulting core alignment only accounts for 25% of the *C. pecorum* chromosome. A maximum likelihood tree showed that all of the koala and livestock *C. pecorum* strains form separate, well-supported clades, with the exception of the koala *C. pecorum* SA/k2/UGT and cattle *C. pecorum* PV3056 genomes, which cluster together in a distant clade (Fig. 4). This suggests that the population structure of koala *C. pecorum* strains is more complex than what was initially established in other phylogenetic analyses, which showed all koala *C. pecorum* strains forming a single lineage (8, 9). Remarkably, both koala minor strains (*C. pecorum* SA/k2/UGT-β and *C. pecorum* Gun/koa1/Ure-β) belong to the clade comprised of livestock *C. pecorum* E58 and other Australian *C. pecorum* livestock strains, which are different from that of the associated dominant strains, indicating an unexpected degree of diversity in *C. pecorum* strains infecting the same anatomical sites of animals. In order to evaluate the topology of this tree, a maximum likelihood tree constructed using the partial gene fragments of seven *C. pecorum* housekeeping genes targeted in our previous *C. pecorum* MLST study (8) were extracted from the genome sequences analyzed in this study and previously (9, 33), revealing topology similar to that observed from our whole-genome phylogeny (see Fig. S1 in the supplemental material).

DISCUSSION

High-throughput sequencing of *C. pecorum* genomes is limited by cell culturing, which is time- and labor-intensive and not always successful. In addition, it has been shown that extended *in vitro* culturing of other chlamydial species may alter the genotype of these isolates (15). Due to these issues, culture-independent methods have been devised to sequence *Chlamydia* genomes directly from clinical swabs (17, 18). In this study, a probe hybridization sequence capture method was used to extract *C. pecorum* DNA from swab samples collected from koalas and Australian sheep and a cell-cultured Australian bovine isolate (20). The sequence capture resulted in sequence data for the five livestock *C. pecorum* genomes that enabled complete coverage across the reference genome of *C. pecorum* E58. Complete coverage was almost achieved for the two koala *C. pecorum* genomes (SA/k2/UGT and Gun/koa1/Ure); however, the coverage decreased at the PZ and PMP region. The PZ encodes several virulence factors that have been linked to host-pathogen interactions and appear to undergo rapid evolution (35). The PMPs are a family of autotransporter surface proteins that are thought to be important for adhesion to host cells and, like the PZ, are a major source of diversity in *Chlamydia* genomes (36). Between koala and livestock *C. pecorum* strains, there is a significant degree of nucleotide variability in both PZ (678 SNPs over 42 kb) and the PMP gene cluster (894 SNPs over 36 kb) (9). Since the probes were designed using the sequence of the cattle *C. pecorum* E58 strain, the decrease in coverage at the PZ and PMP cluster likely is caused by reduced binding affinity of the probes. Interestingly, this was not an issue for another traditionally hypervariable *C. pecorum* gene, *ompA* (37), encoding the chlamydial major outer membrane (data not shown), as full-length sequences could be retrieved for each of the completely assembled genomes. One possible explanation for the success in sequencing this variable gene, however, was that the *ompA* sequence from all of the livestock strains sequenced was indeed identical to the E58 *ompA* sequence, while the other two *ompA* genotypes, detected in each of the dominant koala *C. pecorum* genome sequences, contained 84 to 133 different SNPs compared to the E58 sequence.

Nevertheless, we anticipate that this limitation can be overcome in future iterations of the capture probe systems by designing additional probes that capture the sequence diversity present in previously sequenced koala *C. pecorum* genomes (9). The percentage of mapped reads is proportional to the total number of genome copies, which also has been observed when this culture-independent sequencing method was applied to *Chlamydia trachomatis* (19). In this study, it was observed that a genome copy number greater than 100,000 results in >98% of reads mapping to

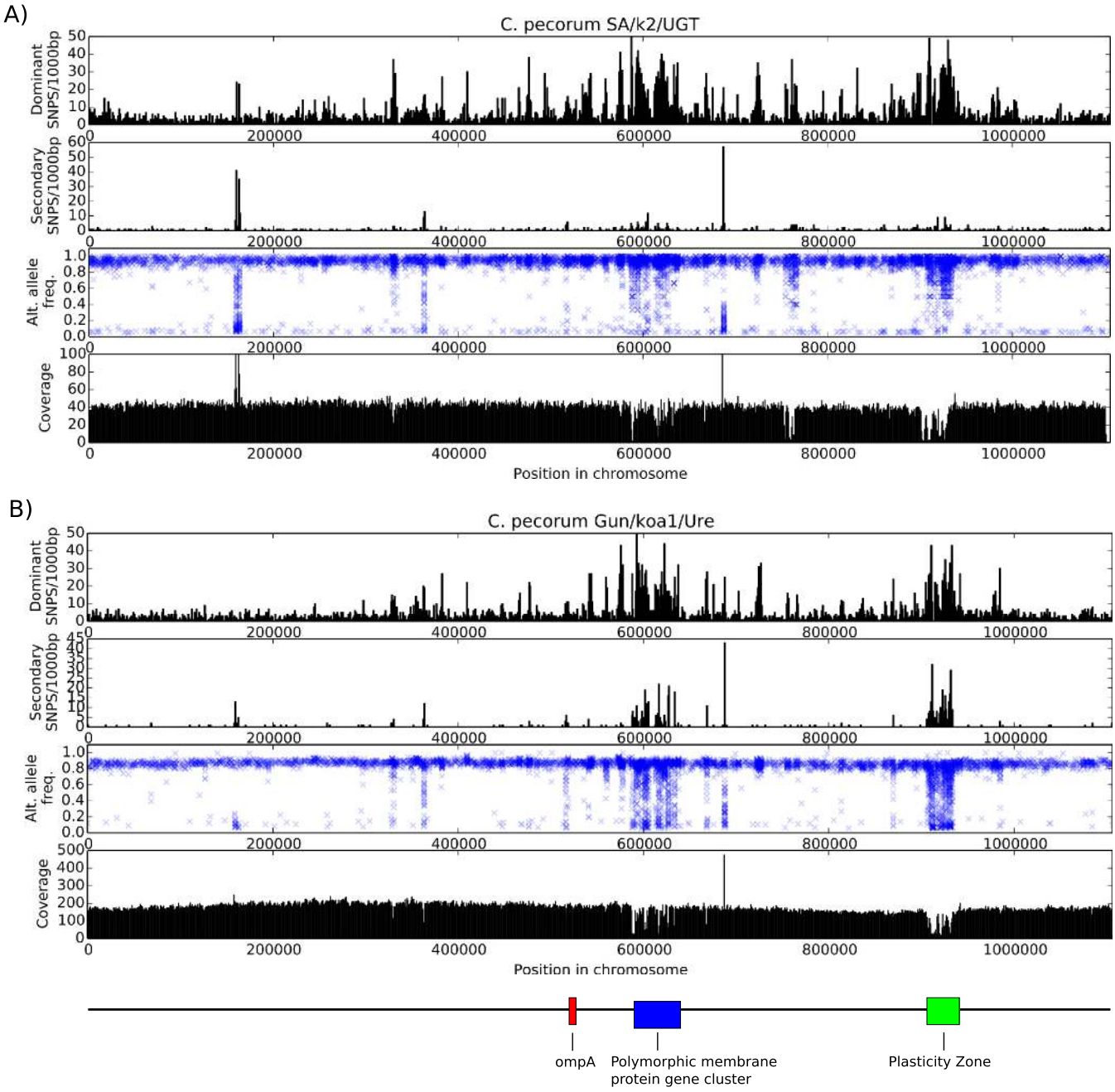

FIG 2 SNP frequency and coverage for *C. pecorum* SA/k2/UTG (A) and *C. pecorum* Gun/koa1/Ure (B). The first graph shows frequency per 1,000 bp of SNPs with an allele frequency of ≥0.5. The second graph shows frequency per 1,000 bp of SNPs with an allele frequency of <0.5. The third graph plots a cross for each SNP variant in the genome. The *x* axis represents the position in the genome, and the *y* axis indicates the frequency with which the SNP appears in the raw reads. The bottom graph shows average read coverage along the genome.

C. pecorum E58 and a mean read depth of >100×. However, samples with a genome copy number as low as 2,253 still can result in >71% of the reads mapping to the reference and a mean read depth of at least 10× even if high numbers of reads are the result of read duplication.

Using this culture-independent technique, we demonstrated compelling evidence to suggest that, at least in the case of *C. pecorum*, naturally occurring chlamydial infections are comprised of multiple infections by genetically distinct strains. Mixed infections previously have been detected in other bacterial species, such as *Clostridium difficile*, where a dominant strain and at least one minor strain were identified in 15 clinical samples (38). Limited evidence of mixed infections also exists for *C. trachomatis*, where DNA was extracted using IMS-MDA (17). One of the *C. trachomatis* samples that were extracted with IMS-MDA showed a high level of heterogeneity in the read sequences, with a major variant appearing in ~85% of the reads and a minor variant appearing in ~15%, which is indicative of a mixed infection by two *C. tracho-*

Strain	*C. pecorum* NSW/Bov/SBE	*C. pecorum* Gun/koa1/Ure-α	*C. pecorum* Gun/koa1/Ure-β	*C. pecorum* SA/k2/UGT-α	*C. pecorum* SA/k2/UGT-β	*C. pecorum* Mer/Ovi1/Jnt-α	*C. pecorum* Mer/Ovi1/Jnt-β	*C. pecorum* Nar/S22/RE-α	*C. pecorum* Nar/S22/RE-β
C. pecorum NSW/Bov/SBE		4794	222	6203	1396	57	439	32	594
C. pecorum Gun/koa1/Ure-α	4794		5010	6069	4769	4242	4014	3831	3573
C. pecorum Gun/koa1/Ure-β	222	5010		6187	1416	220	571	174	710
C. pecorum SA/k2/UGT-α	6203	6069	6187		5694	5481	5514	4985	5061
C. pecorum SA/k2/UGT-β	1396	4769	1416	5694		1238	1488	1066	1447
C. pecorum Mer/Ovi1/Jnt-α	57	4242	220	5481	1238		468	58	527
C. pecorum Mer/Ovi1/Jnt-β	439	4014	571	5514	1488	468		360	738
C. pecorum Nar/S22/RE-α	32	3831	174	4985	1066	58	360		604
C. pecorum Nar/S22/RE-β	594	3573	710	5061	1447	527	738	604	

FIG 3 Heatmap of the number of nucleotide variant differences between each *C. pecorum* strain identified in the swab samples.

matis strains (17). However, there is no data on the clinical ramifications of these mixed infections.

In the case of *C. pecorum* specifically, a previous MLST study on livestock *C. pecorum* strains showed that a single animal could be infected by multiple strains at different anatomical sites, with speculation only on shedding multiple strains from the same site (8, 39). However, this is the first study to reveal the presence of multiple *C. pecorum* strains at the same anatomical site in both koalas and sheep. *C. pecorum* MLST was performed with bidirectional dideoxy Sanger sequencing of target amplicons resulting in a pair of single chromatograms, making it unlikely to detect the signals from minor genotypes. In this study, using culture-independent and deep whole-genome sequencing, we were able to capture and confirm the presence of minor strains. The DNA for at least two separate and genetically distinct *C. pecorum* strains were captured via probe hybridization for each of the clinical swab samples; however, no additional strains were detected in the cell-cultured *C. pecorum* NSW/Bov/SBE sample. The most likely explanation for this is that the *C. pecorum* NSW/Bov/SBE isolate cultured was the dominant strain found in the clinical sample at the time of culturing, and its growth was favored to the exclusion of others through subsequent passaging. Alternatively, since this was a brain isolate, it also might reflect the success of this strain in invading the central nervous system over other strains infecting the animal at the same time, a possibility given our recent observation of genetically distinct strains present in the gastrointestinal tract versus central nervous system tissues of calves with SBE (39).

The observation of mixed infections in the *C. pecorum*-positive swab samples analyzed provides further significant insight into the complex genetic relationships that exist between *C. pecorum* strains detected in koalas and Australian sheep. While this analysis revealed one more example (Gun/koa1/Ure-α) of a *C. pecorum* strain that is phylogenetically related to other koala strains (Fig. 4), phylogenetic analysis of SA/k2/UGT-α, SA/k2/UGT-β, and Gun/koa1/Ure-β provided examples of koala strains more similar to Australian and European livestock *C. pecorum* strains than to other koala strains (Fig. 4). Our group previously provided evidence to suggest that genetically similar, if not identical, strains of *C. pecorum* can be found in Australian sheep, cattle, and koalas (6, 8, 39). The whole-genome phylogenies constructed for the strains sequenced from northern New South Wales (Gun/koa1/Ure) and South Australian koalas (SA/k2/UTG) appear to confirm this finding. Although the small pool of samples analyzed obviously limits firm conclusions, it is tantalizing to speculate on what this means for our understanding of the potential origin of koala *C. pecorum* infections. In terms of the *C. pecorum* strains present in the major koala clade, it is possible that these strains are the result of millions of years of evolution or by cross-host transmission from Australian cattle and sheep in the past following European colonization. Furthermore, the presence of minor koala *C. pecorum* strains in clades dominated by Australian livestock supports ongoing exposure and potential cross-transmission of livestock strains into koalas from sheep and cattle. Development of a molecular clock for *C. pecorum* and extensive sampling of Australian sheep and cattle and koalas from sympatric regions is warranted to confirm these hypotheses and to gain insight into the origin of this pathogen in Australian native animals.

This study also led to the development of a new approach for detecting mixed infections from bacterial whole-genome sequencing data based on detecting variants within the raw reads (groupFlows.py). This was possible due to the presence of only two observable strains with distinct levels of coverage in the sample and a significant amount of variation between strains across the entire genome. If these two conditions are met, this application could be applied to nonrepetitive regions in other mixed-strain samples.

In conclusion, this study highlights the efficiency of the probe-based sequence capture method for rapidly acquiring genome sequences of *C. pecorum* strains directly from clinical swabs. In doing so, we have provided compelling evidence to show that we can reliably detect the genomes of at least two genetically distinct *C. pecorum* strains infecting an animal at the same anatomical site. The presence of multiple strains in a single host suggests that the chlamydial infection process is more complicated than initially expected and has ramifications for our broader understanding of chlamydial infections in a variety of hosts. The mixed population of *C. pecorum* strains will need to be investigated further to determine if they affect pathogenesis. In order to unravel these potentially complex mixtures of strains, we also developed a method for separating reads belonging to different *C. pecorum* strains, which will be critical for future studies relying on this sequence capture method. While these experiments were largely successful and provided powerful new data on the genetic diversity of *C. pecorum* infections, we were able to extract only minor strains from two samples, indicating that a significant amount of starting DNA is required for the minor strain to be assembled with acceptable coverage. It is more than likely that additional strains also are present, but higher sequence coverage would be required to identify them. Further optimization of the sequence capture method and increasing the depth of the sequencing runs will confirm if the

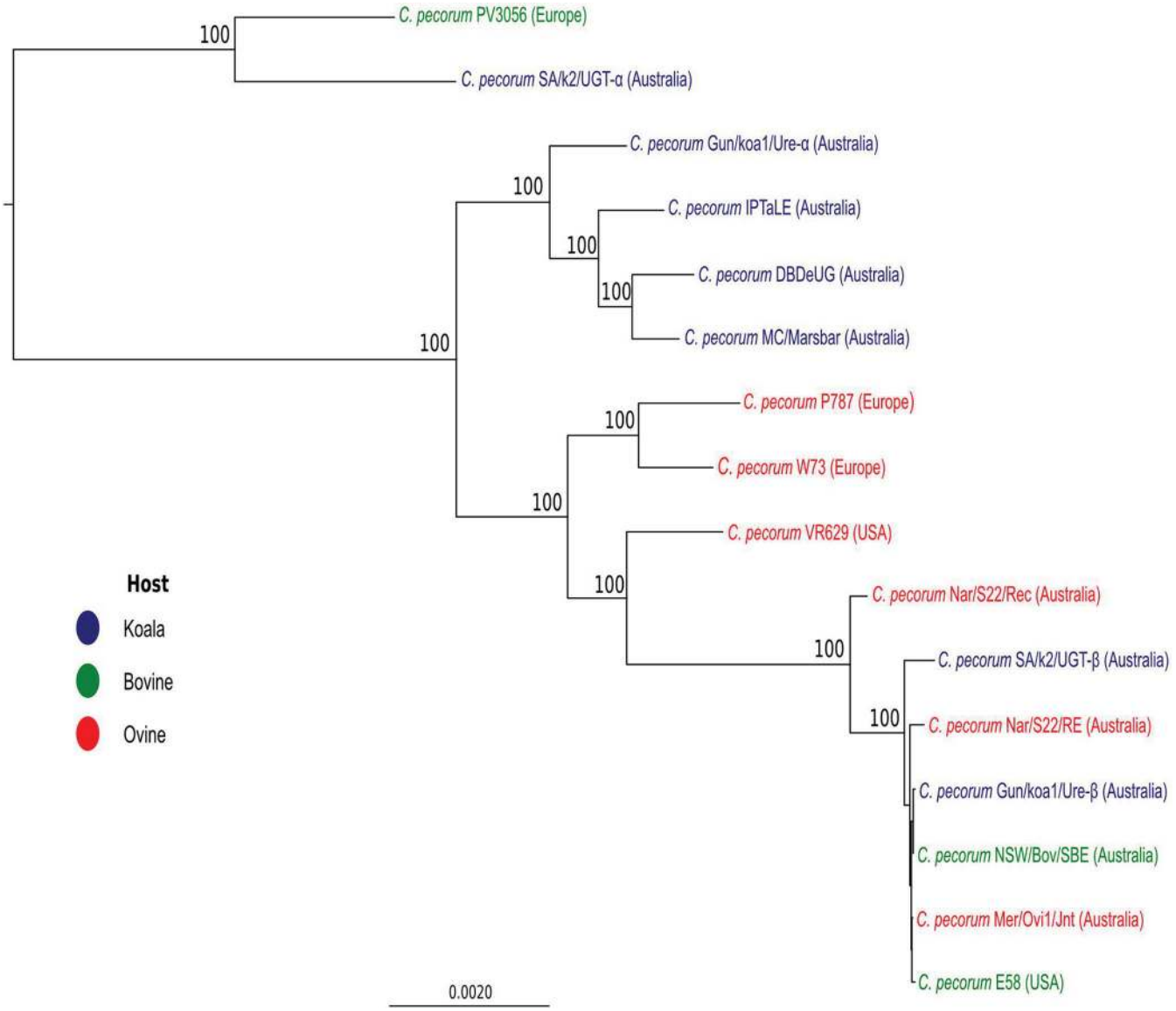

FIG 4 Maximum-likelihood tree based on 279,714-bp genome alignment. The phylogenetic tree was constructed using PhyML with the GTR substitution model. Bootstrap values are shown as percentages of 1,000 replicates. Bootstrap values lower than 85% are not shown.

diversity of *C. pecorum* strains in a single swab sample extends beyond two strains.

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