Panton-Valentine leucocidin is the key determinant of *Staphylococcus aureus* pyomyositis in a bacterial GWAS

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Abstract: Pyomyositis is a severe bacterial infection of skeletal muscle, commonly affecting children in tropical regions, predominantly caused by *Staphylococcus aureus*. To understand the contribution of bacterial genomic factors to pyomyositis, we conducted a genome-wide association study of *S. aureus* cultured from 101 children with pyomyositis and 417 children with asymptomatic nasal carriage attending the Angkor Hospital for Children, Cambodia. We found a strong relationship between bacterial genetic variation and pyomyositis, with estimated heritability 63.8% (95% CI 49.2-78.4%). The presence of the Panton-Valentine leucocidin (PVL) locus increased the odds of pyomyositis 130-fold ($p=10^{-17.9}$). The signal of association mapped both to the PVL-coding sequence and the sequence immediately upstream. Together these regions explained over 99.9% of heritability (95% CI 93.5-100%). Our results establish staphylococcal pyomyositis, like tetanus and diphtheria, as critically dependent on a single toxin and demonstrate the potential for association studies to identify specific bacterial genes promoting severe human disease.

Introduction

Microbial genome sequencing and bacterial genome-wide association studies (GWAS) present new opportunities to discover bacterial genes involved in the pathogenesis of serious infections.[1-6] Pyomyositis is a severe infection of skeletal muscle most commonly seen in children in the tropics.[7-9] In up to 90% of cases it is caused by a single bacterial pathogen, *Staphylococcus aureus* (*S. aureus*).[7-10] Unlike infections of the skin and superficial soft tissues, the skin and subcutaneous tissues are not usually involved in pyomyositis, by contrast to intense inflammation in the infected muscles.[7,8] Pyomyositis is thought to arise from haematogenous seeding of bacteria from blood to muscle.[8] There is evidence that some *S. aureus* strains have heightened propensity to cause pyomyositis – the incidence in the USA doubled during an epidemic of community-associated methicillin resistant *S. aureus* (CA-MRSA)[11] – but molecular genetic investigation of *S. aureus* from pyomyositis has been limited.[12]

Panton-Valentine leucocidin (PVL), a well-known staphylococcal toxin causing purulent skin infections and found in epidemics caused by CA-MRSA, has been implicated in pyomyositis, pneumonia and other *S. aureus* disease manifestations, but its role in these invasive infections is disputed.[13-16] PVL is a bipartite pore-forming toxin comprising the co-expressed LukF-PV and LukS-PV proteins.[17,18] The coding sequence for PVL, *lukSF-PV*, is usually carried on bacteriophages,[13,17] which facilitate *lukSF-PV* exchange between lineages.[19] The mechanism of PVL toxicity has been shown to involve cell lysis in human myeloid cells, particularly neutrophils, by insertion into the cellular membrane,[21] leading the tissue to release inflammatory neutrophil products. [22] Neutrophil lysis is mediated by PVL binding to target complement receptors C5aR; in binding, PVL has both toxic and immunomodulatory effects, as it also inhibits C5a mediated immune activation.[23]

Although small case series testing for candidate genes have reported a high prevalence of PVL among pyomyositis-causing *S. aureus*,[11,23,24] a detailed meta-analysis found no evidence for an increased rate of musculoskeletal infection (or other invasive disease) in PVL-positive bacteria versus controls.[13] These conflicting results may reflect insufficiently powered studies, and some case series lack comparative control strains.[23] A further problem with the use of candidate gene studies in studying pathogenesis is that they may miss important variation elsewhere in the genome. One study reporting a critical role for PVL in the causation of severe pneumonia[15] was later found to have overlooked mutations in key regulatory genes, capable of producing the virulence that had been attributed to PVL by the original study.[16] Thus, while some evidence suggests an association between pyomyositis and PVL, there remains significant uncertainty regarding the bacterial genetic predisposition of *S. aureus* to pyomyositis, and whether PVL is an important virulence factor, or merely an epiphenomenon, carried by bacteria alongside unidentified genetic determinants.[25,26]

GWAS offer a means to screen entire bacterial genomes to discover genes and genetic variants associated with disease risk. They are particularly appealing because they enable the investigation of traits not readily studied in the laboratory, and do not require the nomination of specific candidate genes.[5] Proof-of-principle GWAS in bacteria have demonstrated the successful rediscovery of known antimicrobial resistance (AMR) determinants.[2,3,4] However, AMR is under extraordinarily intense selection in bacteria. More subtle traits, including host specificity[1] and the duration of pneumococcal carriage,[27] have also been demonstrated using GWAS. Promising results for GWAS in human infecting bacteria include identifying possible loci for invasive infection with *Streptococcus pyogenes*[4] and *Staphylococcus epidermidis*[28],and the identification of virulence associated genes corresponding with regional differences in disease manifestations of melioidosis.[29] Within species, lineage specific variants have been

shown to predict mortality following *S. aureus* bacteraemia.[30] These studies support the potential GWAS has to precisely pinpoint genes and genetic variants underlying the propensity to cause specific human infections, making it a promising tool to investigate the possible contribution of bacterial genomic variation to pyomyositis.

Results

To understand the bacterial genetic basis of pyomyositis, we sampled and whole-genome sequenced *S. aureus* from 101 pyomyositis infections and 417 asymptomatic nasal carriage episodes in 518 children attending Angkor Hospital for Children in Siem Reap, Cambodia between 2008 and 2012 (Supplementary File 1). As expected, we observed representatives of multiple globally common lineages in Cambodia, together with some globally less common lineages at high frequency, in particular clonal complex (CC) 121, identified by multi-locus sequence typing (MLST). There were no major changes in lineage frequency over time (Figure 1-figure supplement 1).

In our study, some *S. aureus* lineages were strongly overrepresented among cases of pyomyositis compared with asymptomatic, nasally-carried controls over the same time period. Notably, 86/101 (85%) of pyomyositis cases were caused by CC-121 bacteria, whereas no pyomyositis cases were caused by the next two most commonly carried lineages, sequence type (ST)-834 and CC-45 (Figure 1). We estimated the overall heritability of case/control status to be 63.8% (95% CI 49.2-78.4%) in the sample, reflecting the strong relationship between bacterial genetic variation and case/control status. We used *bugwas*[6] to decompose this heritability into the principal components (PCs) of bacterial genetic variation. PC 1, which distinguished CC-121 (the most common pyomyositis lineage) from ST-834 (which was only found in carriage), showed the strongest association with case /control status ($p = 10^{-29.6}$, Wald test). The strongest associations were with PC 20, which differentiated a sub-lineage of CC-121 within which no cases were seen ($p = 10^{-13.9}$), and PC 2, which distinguished CC-45 from the rest of the species ($p = 10^{-4.9}$).

We conducted a GWAS to identify bacterial genetic variants associated with pyomyositis, controlling for differences in pyomyositis prevalence between *S. aureus* lineages. We used a kmer-based approach[1] in which every variably present 31bp DNA sequence observed among the 518 genomes was tested for association with pyomyositis *versus* asymptomatic nasal carriage, controlling for population structure using GEMMA.[31] These kmers captured bacterial genetic variation including single nucleotide polymorphisms (SNPs), insertions or deletions (indels), and presence or absence of entire accessory genes. We found 10.7 million unique kmers variably present across the bacterial genomes. In total, 9,175 kmers were significantly associated with case/control status after correction for multiple testing ($10^{-6.8} \leq p \leq 10^{-21.4}$; Figure 2A). When mapped to the *de novo* assembly of a CC121 isolate from pyomyositis (PYO2134), the vast majority of these kmers (9,074/9,175; 98.9%) localised to a 45.7kb region spanning an integrated prophage with 95% nucleotide identity to φSLT (Figure 2B). Most (9,173/9,175; 99.98%) significant kmers were found at an increased frequently in pyomyositis, with odds ratios (OR) ranging from 2.7 to 139.8, indicating that the presence of each was associated with increased risk of disease. The presence of bacteriophage φSLT was thus strongly associated with pyomyositis.

We were able to localise the most statistically significant signal of association to kmers that mapped within φSLT to the *lukS-PV* and *lukF-PV* cargo genes. These genes encode the subunits of PVL, which multimerise into a pore-forming toxin capable of rapidly lysing the membranes of human neutrophils.[17,25] 1,630 kmers tagging the presence of the *lukSF-PV* coding sequences (CDS) were highly significantly associated with disease, being present in 98/101 (97%) pyomyositis cases and 84/417 (20%) carriage controls (unadjusted OR 129.5,

$p=10^{-17.9}$). Kmers tagging variation in the 389bp region immediately upstream of the CDS were also strongly associated with disease ($p=10^{-21.4}$). The most significant of these kmers were co-present with the CDS in the same cases (98/101, 97.0%), but present in fewer controls (79/417, 18.9%), producing an OR of 140.

Closer examination of this ~400bp upstream region in genomes assembled from short-read Illumina sequencing showed that assembly of the region was problematic, with breaks or gaps in the assembly (Figure 2-figure supplement 2). To improve the accuracy of this region of the assembled genomes we performed long-read Oxford Nanopore sequencing on the 37 genomes with incomplete or discontinuous assembly upstream of the PVL CDS. By integrating long-read and short-read data we were able to assemble a single contig spanning this region in all isolates (Figure 2-figure supplement 2). When these improved assemblies were introduced, the signal of association upstream of the PVL CDS was no more significant than within the CDS (Figure 2C). Therefore, the presence of genomic sequence spanning the PVL toxin-coding sequences and the upstream, presumed regulatory, region exhibited the strongest association with pyomyositis in the *S. aureus* genome. All isolates with kmers mapping to the PVL CDS had 98% or more coverage for the PVL CDS genes in *de novo* assembly (Figure 2-figure supplement 3). The signal of association in the earlier and later periods of the study were examined and found to be consistent (Figure 2-figure supplement 4).

Out of 9,175 kmers significantly associated with pyomyositis, we only found 101 kmers related to regions outside the PVL-carrying prophage (Figure 2A, Supplementary File 2). Two kmers mapping at a position near 0.2Mb in the PYO2014 reference genome showed homology to platelet adhesin *sraP* by BLAST. Thirty-five kmers mapping to a 50bp non-coding fragment at 0.6Mb and two kmers mapping to 2.8Mb showed homology to an MSSA476 intergenic sequence between adhesin-encoding *sdrC* and *sdrD* by BLAST. One kmer mapping to position 2.0Mb showed no sequence homology by BLAST. Sixty-one kmers mapping to a 61bp non-coding region at 2.7Mb showed homology to an MSSA476 intergenic sequence between acetyltransferase-encoding genes SAS2453 and SAS2454 by BLAST. In conclusion, these other signals were short, fragmentary and mostly non-coding so we did not investigate them further.

The presence of high-risk kmers mapping to the PVL region explained the vast majority of observed heritability. When the presence or absence of the most significant kmer pattern, a set of kmers with an identical pattern of presence in the population, all of which mapped to the PVL region, was included as a covariate in GEMMA, the remaining heritability not explained by other factors was estimated and found to be 0.0% (95% CI 0-2.5%). Thus, the point estimate for heritability (not explained by the inclusion of PVL-tagging kmers) is reduced by 100% (95% CI 93.5-100%), meaning we have little evidence for any remaining heritability in case/control status.

Presence or absence of the PVL region accounted for the differences in pyomyositis rates between lineages. It was common in pyomyositis-associated lineages including CC-121 and absent from carriage/non-pyomyositis-associated lineages including ST-834 and CC-45 (Figure. 1), explaining over 99.9% of observed heritability in case-control status. It was infrequent in the non-pyomyositis-associated sub-lineage of CC-121 (2/36, 5.6%), and sporadically present in pyomyositis cases in otherwise non-pyomyositis-associated, PVL-negative strains CC-1 and CC-88. Its absence from only three cases (in lineages CC-88, CC-1 and CC-121) suggested that the PVL region approached necessity for development of pyomyositis in the current setting in Cambodian children, while its presence in 20% of controls indicated that PVL-associated pyomyositis is incompletely penetrant, i.e. presence of the PVL region does not always lead to disease.

PVL genes were carried on multiple genetic backgrounds in this population, and the phage backgrounds vary by clonal complex. We examined all assemblies for sequence similarity to six known bacteriophages that carry the PVL genes,[18] as well as the 45.7kb region in PYO2134, a hypothesised integrated prophage identified in the reference genome prepared for this study, which we have called φCC121 (Figure S4). The finding of PVL genes on BLAST corresponded completely with the presence of kmers mapping across the PVL locus. We find sequences with >95% homology to four of these seven phages in the population. Regions in some assemblies showed homology for multiple phages, reflecting the similarity between φSLT, φSa2USA and φCC121 rather than the presence of multiple phages, and resolution of phages was limited by fragmented assemblies from short reads (Supplementary File 3). Phage types were restricted within most lineages, with φSLT found in ST-3206, φSa2USA in ST-1232 and φPVL in CC-1. φCC121 was the phage most often identified in the dominant pyomyositis strain CC-121, but it was absent in all but one isolate from the low risk subclade within CC-121. Strikingly, PVL negative isolates in CC-121 and ST-834 strains frequently retained sequence homologous to >95% of φSLT, suggesting that some PVL-negative CC-121 isolates lost PVL secondarily by gene deletion rather than prophage excision.

Discussion

In this study we found a strong association between pyomyositis, a highly distinctive tropical infection of skeletal muscle in children, and Panton-Valentine leukocidin, a bacterial toxin commonly carried by bacteriophages. We found that a single coding region together with the upstream sequence are all but necessary for the development of pyomyositis: its sporadic presence is associated with pyomyositis in otherwise low-frequency strains, and its absence is associated with asymptomatic carriage in a high-propensity strain. PVL appears to be carried on multiple phage backgrounds in this population, but PVL-positive lineages generally carry a single phage type, as expected given the observed strain restriction of phages in *S. aureus*.[33,34] The locally common PVL-positive CC-121 lineage contributes most strongly to the prevalence of pyomyositis in Cambodian children.

While PVL has long been thought an important *S. aureus* virulence factor,[35-37] its role in invasive disease has been controversial,[25,26] with conflicting results in case-control studies and an absence of supporting evidence on meta-analysis.[13] In previous studies the PVL positive USA300 lineage was associated with musculoskeletal infection (both pyomyositis and osteomyelitis), however in these studies almost all such infections were caused by the USA300 strain, so the role of PVL was almost completely confounded by both methicillin resistance and strain background.[11,36] In our study, this confounding influence is broken down by the movement of PVL on mobile genetic elements (MGEs). Despite the emergence of CA-MRSA in carriage in Cambodia,[38] all the pyomyositis cases were MSSA (Figure 1-figure supplement 1). By applying GWAS methods to a well-powered cohort, our study resolves the controversy around pyomyositis and PVL, demonstrating strong heritability which localises to a single region, even when the full bacterial genome is considered. Bacterial GWAS can pinpoint virulence variants when MGEs act to unravel linkage disequilibrium, if effect sizes are sufficiently strong.

There is strong biological plausibility for the association demonstrated in this study. PVL is a well characterised *S. aureus* toxin, toxic to myeloid cells, which form a first line of defence against bacterial infection,[20] and in binding to myeloid cells by a complement receptor (C5aR), exerts immunomodulatory effects.[22] The establishment of muscle abscesses is a critical step in the pathogenesis of pyomyositis, but unlike renal, hepatic and splenic abscesses, skeletal muscle abscesses are rarely observed in experimental models of bacteraemia, unless there is preceding muscle trauma.[39] *S. aureus* strains containing PVL show increased duration of

bacteraemia in a rabbit model of sepsis,[33] and result in larger muscle abscesses.[40] PVL has been found strongly bound to necrotic muscle in an individual with myositis associated with necrotizing fasciitis.[41] These observations support the hypothesis PVL may facilitate bacterial seeding to muscles via the bloodstream, and tropism for muscular infection.

These results establish that, for children in Cambodia, staphylococcal pyomyositis is a disease whose pathogenesis depends crucially on a single toxin. This property is shared by toxin-driven, vaccine-preventable diseases such as tetanus and diphtheria. Therefore, vaccines that generate neutralising anti-toxin antibodies against PVL[42] may protect human populations specifically against this common tropical disease. These results also raise the hypothesis that antibiotics which decrease toxin expression, and have been recommended in some PVL-associated infections,[43] may offer specific clinical benefit in treating pyomyositis. More generally, our study provides an example of how microbial GWAS can be used to elucidate the pathogenesis of bacterial infections and identify specific virulence genes associated with human disease.

Materials and Methods:

Patient sample collection. We retrospectively identified pyomyositis cases from the Angkor Hospital for Children in Siem Reap, Cambodia, between January 2007 and November 2011. We screened all attendances in children (≤16 years) using clinical coding (ICD-10 code M60 (myositis)) and isolation of *S. aureus* from skeletal muscle abscess pus. We reviewed clinical notes to confirm a clinical diagnosis of pyomyositis was made by the medical staff, and bacterial strains cultured by routine clinical microbiology laboratory were retrieved from the local microbiology biobank. 106 clinical episodes of pyomyositis were identified, in 101 individuals, and we included the earliest episode from each individual.

We identified *S. aureus* nasal colonisation from two cohort studies undertaken at Angkor Hospital for Children. The first were selected from a collection characterising nasal colonization in the region between September and October, 2008, which has previously been described using multi-locus sequence typing.[38] The swabs had been saved at -80°C since the study, these samples were re-examined for the presence of *S. aureus* using selective agar, confirmed using Staphaurex (Remel, Lenexa, USA) and the DNAse agar test (Oxoid, Hampshire, UK). Antimicrobial susceptibility testing was performed according to the 2014 Clinical and Laboratory Standards Institute guidelines (M100-24).[44]

We undertook a second cohort study in 2012. Inclusion criteria were children (≤16 years) attending as an outpatient at Angkor Hospital for Children with informed consent. There were no exclusion criteria. Children were swabbed between the 2-7th July 2012, using sterile cotton tipped swabs pre-moistened (with phosphate buffered saline, PBS) using 3 full rotations of the swab within the anterior portion of each nostril with one swab being used for both nostrils, the ends were broken into bottles containing sterile PBS and kept cool until plated in the laboratory (within the hour). The swabs were plated onto Mannitol Salt agar to select for *S. aureus*. The M100-24 CLSI[44] standards were followed for susceptibility testing and bacteria stored in tryptone soya broth and glycerol at -80°C.

We selected controls from carriers in these two cohorts using the excel randomization function: 222 of 519 from the 2008 cohort and 195 of 261 from the 2012 cohort.

Ethical Framework. Approval for this study was provided by the AHC institutional review board and the Oxford Tropical Ethics Committee (507-12).

Whole genome sequencing. For each bacterial culture, a single colony was sub-cultured and DNA was extracted from the sub-cultured plate using a mechanical lysis step (FastPrep; MPBiomedicals, Santa Ana, CA) followed by a commercial kit (QuickGene; Autogen Inc, Holliston, MA), and sequenced at the Wellcome Centre for Human Genetics, Oxford on the Illumina (San Diego, California, USA) HiSeq 2500 platform, with paired-end reads 150 base pairs long.

A subset of samples were sequenced using long-read sequencing technology. We selected 37 isolates with incomplete assembly upstream of the PVL locus, 22 with ambiguous base calls in the assembly, and 15 where the region was assembled over 2 contigs. DNA was extracted using Genomic Tip 100/G (Qiagen, Manchester, UK) and DNA libraries prepared using Oxford Nanopore Technologies (ONT) SQK-LSK108 library kit (ONT, Oxford, UK) according to manufacturer instructions. These were then sequenced on ONT GridION device integrated with a FLO-MIN106 flow cell (ONT, Oxford, UK). ONT base calling was performed using Guppy v.1.6.

Variant calling. For short-read sequencing we used Velvet[45] v1.0.18 to assemble reads into contigs *de novo*. Velvet Optimiser v2.1.7 was used to choose the kmer lengths on a per sequence basis. The median kmer length was 123bp (IQR 119-123). To obtain multilocus

sequence types we used BLAST[46] to find the relevant loci, and looked up the nucleotide sequences in the online database at http://saureus.mlst.net/. Strains that shared 6 of 7 MLST loci were considered to be in the same Clonal Complex.[47] Antibiotic sensitivity was predicted by interrogating the assemblies for a panel of resistance determinants as previously described.[48]

We used Stampy[49] v1.0.22 to map reads against reference genomes (USA300_FPR3757, Genbank accession number CP000255.1).[50] Repetitive regions, defined by BLAST[46] comparison of the reference genome against itself, were masked prior to variant calling. Bases were called at each position using previously described quality filters. [51-53]

After filtering ONT reads with filtlong v.0.2.0 (with settings filtlong -- min_length 1000 -- keep_percent 90 --target_bases 500000000 --trim --split 500), hybrid assembly of short (Illumina) and long (ONT) reads were made, using Unicycler v0.4.5[54] (default settings). The workflow for these assemblies is available at https://gitlab.com/ModernisingMedicalMicrobiology/MOHAWK)

Reconstructing the phylogenetic tree. We constructed a maximum likelihood phylogeny of mapped genomes for visualization using RAxML[55] assuming a general time reversible (GTR) model. To overcome a limitation in the presence of divergent sequences whereby RAxML fixes a minimum branch length that may be longer than a single substitution event, we fine-tuned the estimates of branch lengths using ClonalFrameML.[56]

Kmer counting. We used a kmer-based approach to capture non-SNP variation.[1] Using the *de novo* assembled genome, all unique 31 base haplotypes were counted using dsk[57]. If a kmer was found in the assembly it was counted present for that genome, otherwise it was treated as absent. This produced a set of 10,744,013 variably present kmers, with the presence or absence of each determined per isolate. We identified a median of 2,801,000 kmers per isolate, including variably present kmers and kmers common to all genomes (IQR 2,778,000-2,837,000).

***Calculating heritability*.** We used the Genome-wide Efficient Mixed Model Association tool (GEMMA[31]) to fit a univariate linear mixed model for association between a single phenotype (pyomyositis vs asymptomatic nasal carriage). We calculated the relatedness matrix from kmers, and used GEMMA to estimate the proportion of variance in phenotypes explained by genotypic diversity in the sample set (i.e. estimated heritability). Heritability estimates with and without a covariate (e.g. the presence of high risk kmers) are compared by testing for difference in proportions. We use the point estimate for heritability as the denominator to calculate the relative decrease proportion.

Genome wide association testing of Kmers. We performed association testing using an R package bacterialGWAS (https://github.com/jessiewu/bacterialGWAS), which implements a published method for locus testing in bacterial GWAS.[3] The association of each kmer on the phenotype was tested, controlling for the population structure and genetic background using a linear mixed model (LMM) implemented in GEMMA.[24] The parameters of the linear mixed model were estimated by maximum likelihood and likelihood ratio test was performed against the null hypothesis (that each locus has no effect) using the software GEMMA.[24] GEMMA was run using a minor allele frequency of 0 to include all SNPs. GEMMA was modified to output the ML log-likelihood under the null, and alternative and $-\log_{10} p$ values were calculated using R scripts in the bacterialGWAS package. Unadjusted odds ratios were reported because there was no residual heritability unexplained by the most significant kmers.

To address the possibility of differing effect sizes between the two control cohorts, we have repeated the analysis after splitting the study into two groups – early (2008 and earlier, n = 276,

cases n =54, controls n = 222) and late (2009 and later, n=242, cases n=47, controls n=195). We then examined the maximum likelihood estimates produced by the LMM for kmers mapping to the PVL coding sequence in each region. The 95% CI of the estimate from each sub study were overlapping (See Figure 2-figure supplement 4).

Testing for lineage effects. We tested for associations between lineage and phenotype using an R package *bugwas* (available at https://github.com/sgearle/bugwas), which implements a published method for lineage testing in bacterial GWAS.[3] We tested lineages using principal components. These were computed based on biallelic SNPs using the R function prcomp. To test the null hypothesis of no background effect of each principal component, we used a Wald test, which we compared against a χ^2 distribution with one degree of freedom to obtain a *p* value.

Kmer mapping. We used Bowtie[58] to align all 31bp kmers from short-read sequencing were to a draft reference (the *de novo* assembly of a CC-121 pyomyositis strain PYO2134). Areas of homology between the draft reference and well-annotated reference strains were identified by aligning sequences with Mauve.[59] For all 31bp kmers with significant association with case-controls status, the likely origin of the kmer was determined by nucleotide sequence BLAST[46] of the kmers against a database of all *S. aureus* sequences in Genbank.

Joint short-read and long-read analysis. 31bp kmers were counted for the 37 hybrid short-read and long-read assemblies using dsk[57]. The presence or absence of all Illumina (short-read) kmers that mapped to the two PVL toxin-coding sequences and the upstream intergenic region plus the surrounding 1kb were reassessed. For the 37 samples with hybrid assemblies, the presence/absence of these kmers was determined from the kmers counted from the hybrid assemblies. For all other samples, presence/absence was determined from the kmers counted from the short-read only assemblies. The new presence/absence patterns were tested for association with the phenotype controlling for population structure and genetic background using GEMMA[31], using the same relatedness matrix as the original short-read analysis.

Predicting presence of PVL genes and bacteriophages. We used BLAST to check for the relative coverage of the PVL CDS (From reference genome USA300_FPR3757 (CP000255.1) positions 1546170-1548350), as well as the entire sequence of 6 known PVL positive phages (φ2958(NC_011344.1), φPVL (NC_002321.1), φPVL108 (NC_008689.1), φSLT (NC_002661.2), φSa2MW (NC_003923.1), φSa2USA (CP000255.1))[18], as well as the hypothesised prophage region from PYO2134 (1571177- 1616957), which we have calledφCC121. For PVL genes, we determined relative coverage of the query sequence; over 98% coverage was used as threshold for gene presence.

Multiple testing correction. Multiple testing was accounted for by applying a Bonferroni correction;[60] the individual locus effect of a variant (kmer or PC) was considered significant if its *P* value was smaller than α/n_p, where we took $\alpha = 0.05$ to be the genome-wide false-positive rate and n_p to be the number of kmers or PCs with unique phylogenetic patterns, that is, unique partitions of individuals according to allele membership. We identified 236627 unique kmer patterns and 518 PCs, giving thresholds of 2.1×10^{-7} and 9.7×10^{-5} respectively.

Data availability. Sequence data has been submitted to Short Read Archive (Bioproject ID PRJNA418899). Clinical origins of sequenced strains are listed in supplementary information (Supplementary File 4).

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Figure 1. Phylogeny of *S. aureus* cultured from children in Cambodia shows strong strain-to-strain variation in pyomyositis prevalence. The phylogeny was estimated by maximum likelihood from SNPs mapping to the USA300 FPR3757 reference genome. Multi-locus sequence type (ST) or clonal complex (CC) groups are shown (outer grey ring). Case/control status is marked in the middle ring: pyomyositis (gold, n = 101) or nasal carriage (green, n = 417). Branches of the phylogeny that correspond to the three principal components (PCs) significantly associated with case/control status (PCs 1, 2 and 20) are marked in red, blue and yellow, respectively. Branch lengths are square root transformed to aid visualization. The presence of the kmers most strongly associated with pyomyositis is indicated by red blocks in the inner ring

Figure 1-figure supplement 1 Sampling frequencies of the major strains were stable over time. The year of sampling (2007-2008, blue shaded lines) and MRSA status (orange lines) are illustrated around the phylogeny of the bacteria sampled from pyomyositis cases (gold lines) and asymptomatic carriage controls (green lines). The three PCs most significantly associated with case/control status are also shown (PCs 1, 2 and 20 by red, blue and yellow branches respectively). Branch lengths are square root transformed to aid visualization.

Figure 2. Kmers associated with pyomyositis. **(A)** All kmers (n = 10,744,013) were mapped to the genome assembly of one CC121 pyomyositis bacterium (PYO2134). Each point represents a kmer, plotted by the mapped location and the significance of the association with disease ($-\log_{10} p$ value). Kmers are coloured by the odds ratio (OR) of kmer presence for disease risk. A Bonferroni-adjusted threshold for significance is plotted in grey **(B)** The region between 1.57-1.62 MB in greater detail. Grey arrows depict coding sequences, determined by homology to USA300 FPR3757. **(C)** Associations for kmers mapping to region 1,571 – 1,574kB is plotted. Kmer presence determined from hybrid assembly using short and long-read data for assembly. Grey arrows depict coding sequences, determined by homology to USA300 FPR3757.

Figure 2-figure supplement 1: Alignments of reference genome PYO2134 assembly (R) with 37 *de novo* assemblies of Illumina short-read sequencing (C) which feature either ambiguities (Ns) or contig boundaries in the region 389bp upstream of PVL coding sequence. Contig boundaries, when overlapping, are marked with a red diamond. Ns in the assembly are marked in dark grey. Polymorphisms are colour-coded by base.

Figure 2-figure supplement 2: Alignments of reference genome PYO2134 assembly (R) with 37 *de novo* assemblies of Illumina short-read sequencing (C) which feature either ambiguities (Ns) or contig boundaries in the region 389bp upstream of PVL coding sequence. Contig boundaries, when overlapping, are marked with a red diamond. Ns in the assembly are marked in dark grey. Polymorphisms are colour-coded by base.

Figure 2-figure supplement 3: Presence of PVL and potential PVL carrying phages across the population. Multi-locus sequence type (ST) or clonal complex (CC) groups are shown (outer grey ring). Case/control status is marked in the next outermost ring: pyomyositis (gold, n = 101) or nasal carriage (green, n = 417). Branch lengths are square root transformed to aid visualization. The presence of kmers mapping to the PVL coding sequence (dark red) or of >98% coverage of PVL coding sequence in *de novo* assembly found by BLAST (red) are marked in the next rings. Phages with >95% coverage on BLAST of the de novo assembly are marked by points (phiPVL in purple, φSaUSA2 in dark blue, φSLT in light blue and

φCC121 in orange), and bars are used to mark the phage with the greatest sequence homology.

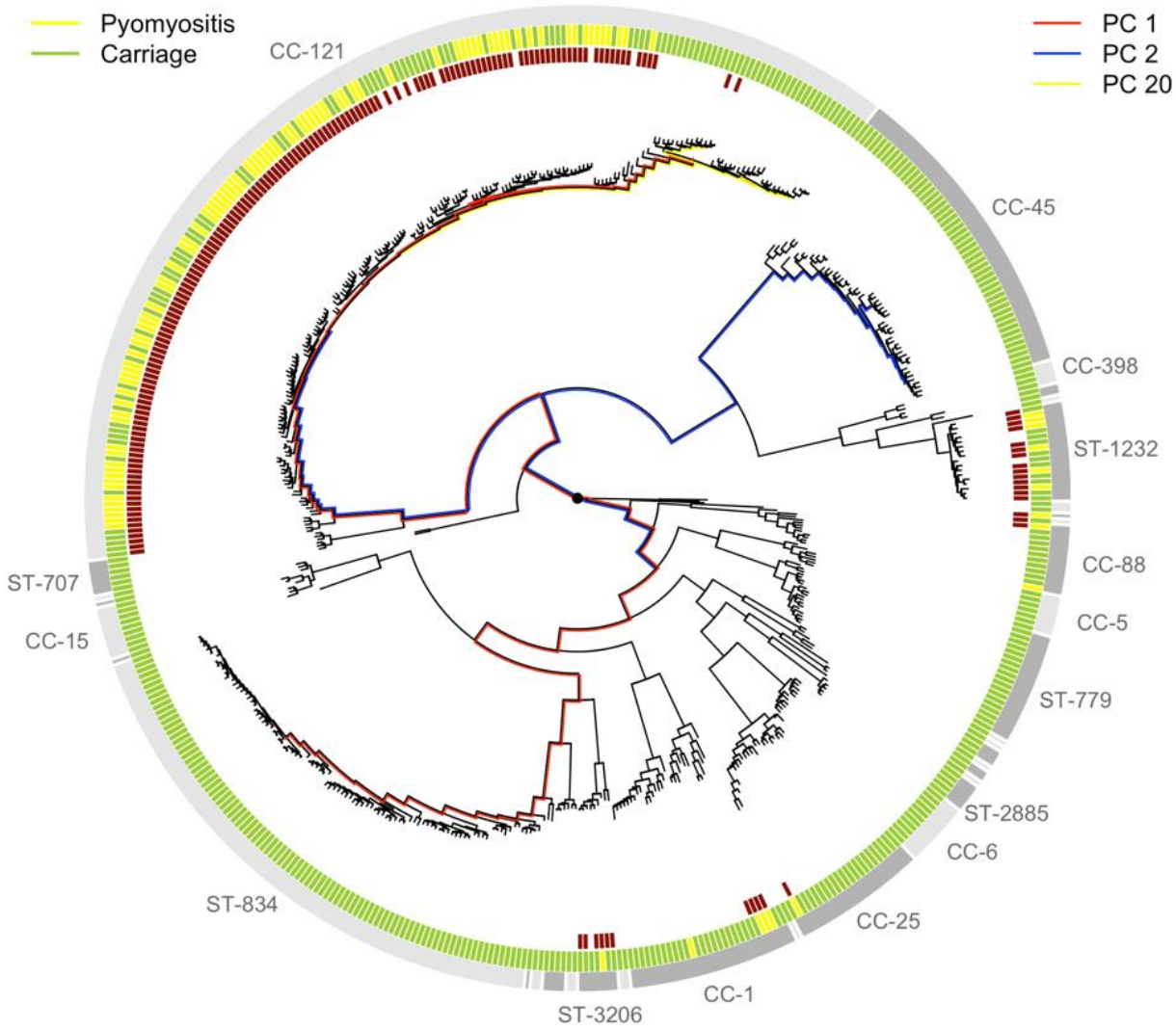
Figure 2-figure supplement 4: Significant association between case/control status and the presence of kmers mapping to PVL was consistent across early and late subsets. Results from repeat kmer GWAS using early and late sub-groups of cases and controls, as well as results from original GWAS of the entire group. We report the presence of kmers mapping across the PVL locus in each subset. Forest plot showing the effect sizes (box) with 95% CI (whiskers) determined by LMM for kmers mapping to PVL kmer in each subset, and in the complete study (diamond centred on effect size extending across 95% CI). The statistical significance of association with case/control status after controlling for population structure is shown for each subset and the complete study.

Supplementary File 1: Isolates included in this study.

Supplementary File 2: All significant kmers from short read sequencing assembly, evidence of association, frequency, location on mapping to the study reference PYO2134, best match on BLAST (blastn) to all *S. aureus* coding sequences in Genbank, and best match on BLAST (blastn) to the NCBI database.

Supplementary File 3: Presence of high risk kmers and relative coverage of PVL coding sequence and common PVL positive phages found by BLAST (blastn) of short read assemblies.

Supplementary File 4: List of all isolates, and site of isolation (carriage or invasive disease) and year of isolation. These isolate names match those used in sequence data deposition on SRA.


Pyomyositis
Carriage
PC 1
PC 2
PC 20
CC-121
CC-45
CC-398
ST-1232
CC-88
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CC-15
ST-707

Figure 1

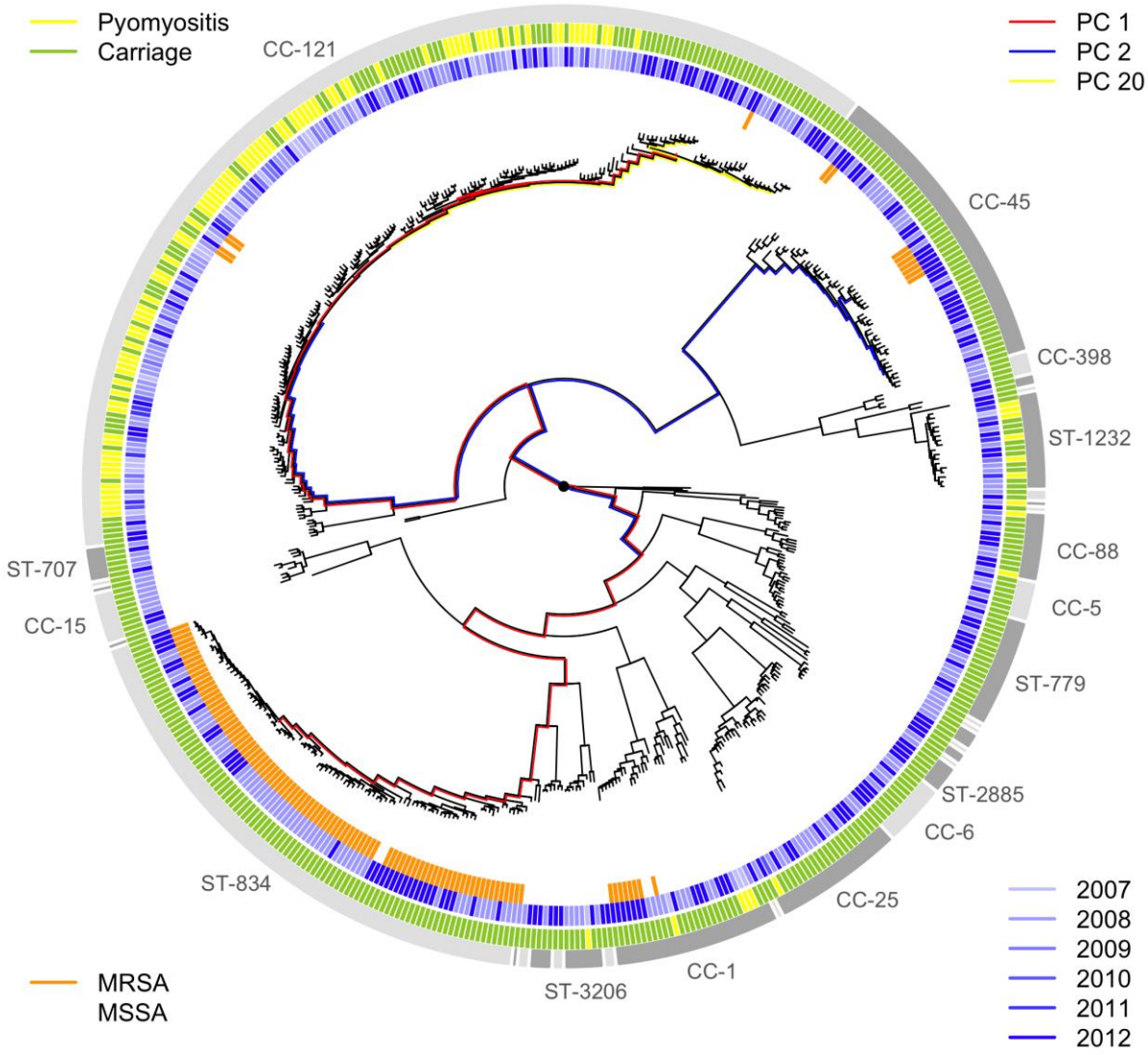
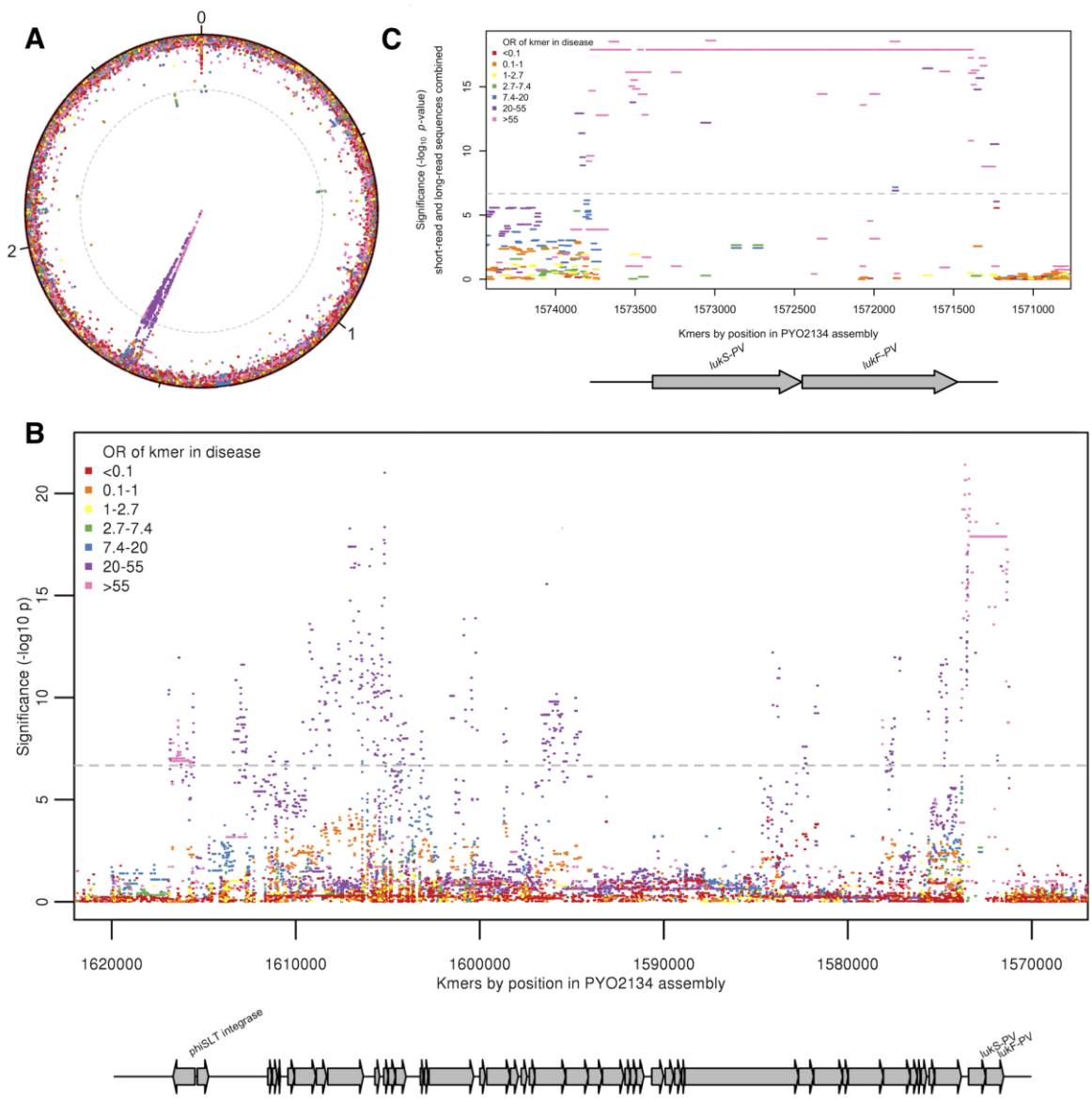


Figure 1-figure supplement 1


A
0
1
2
C
OR of kmer in disease
<0.1
0.1-1
1-2.7
2.7-7.4
7.4-20
20-55
>55
Significance (-log10 p-value)
short-read and long-read sequences combined
0
5
10
15
1574000
1573500
1573000
1572500
1572000
1571500
1571000
Kmers by position in PYO2134 assembly
lukS-PV
lukF-PV
B
OR of kmer in disease
<0.1
0.1-1
1-2.7
2.7-7.4
7.4-20
20-55
>55
Significance (-log10 p)
0
5
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20
1620000
1610000
1600000
1590000
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1570000
Kmers by position in PYO2134 assembly
phiSLT integrase
lukS-PV
lukF-PV

1

2 **Figure 2**

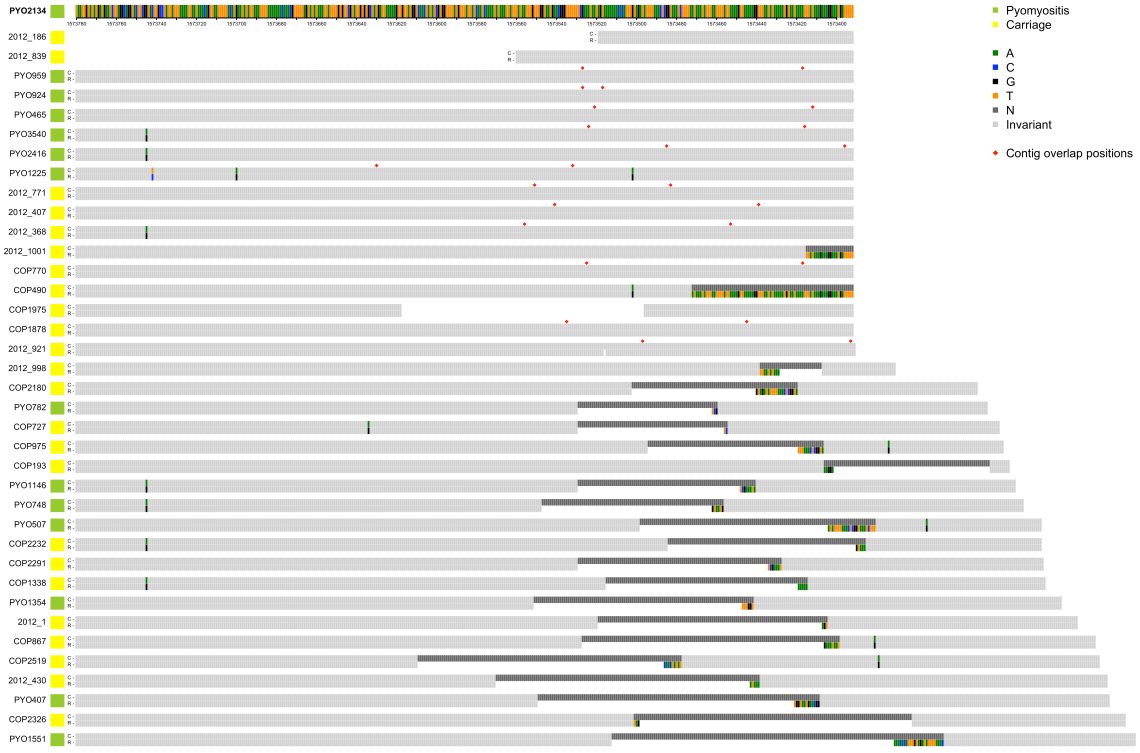
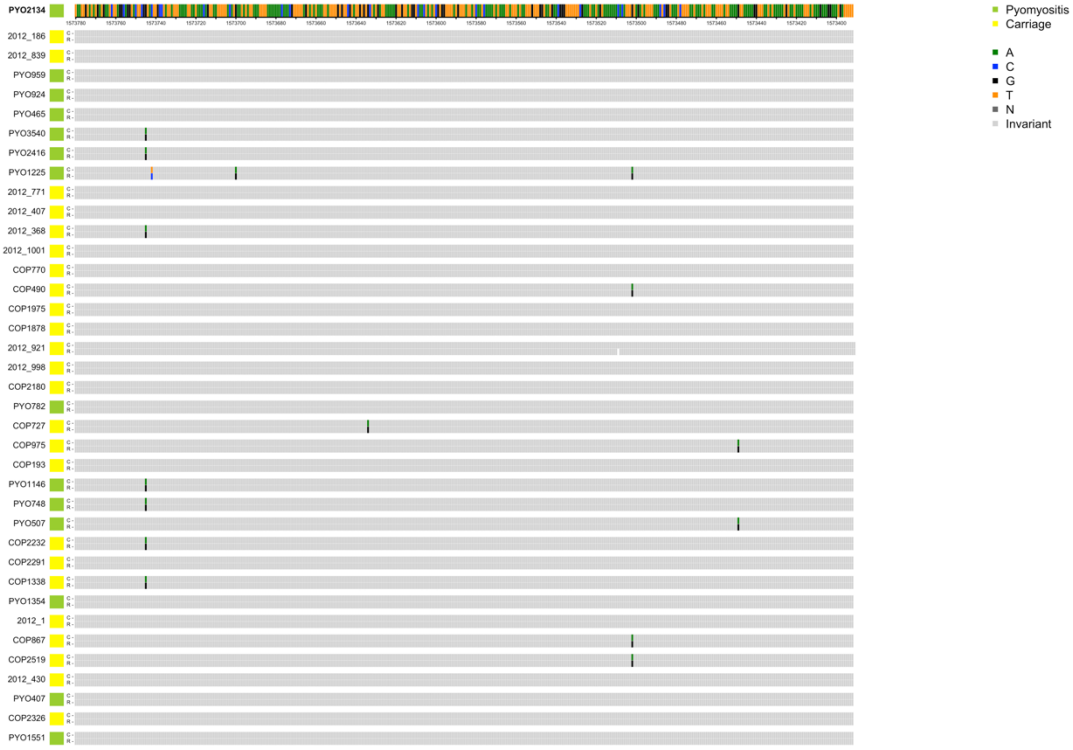


Figure 2-figure supplement 1



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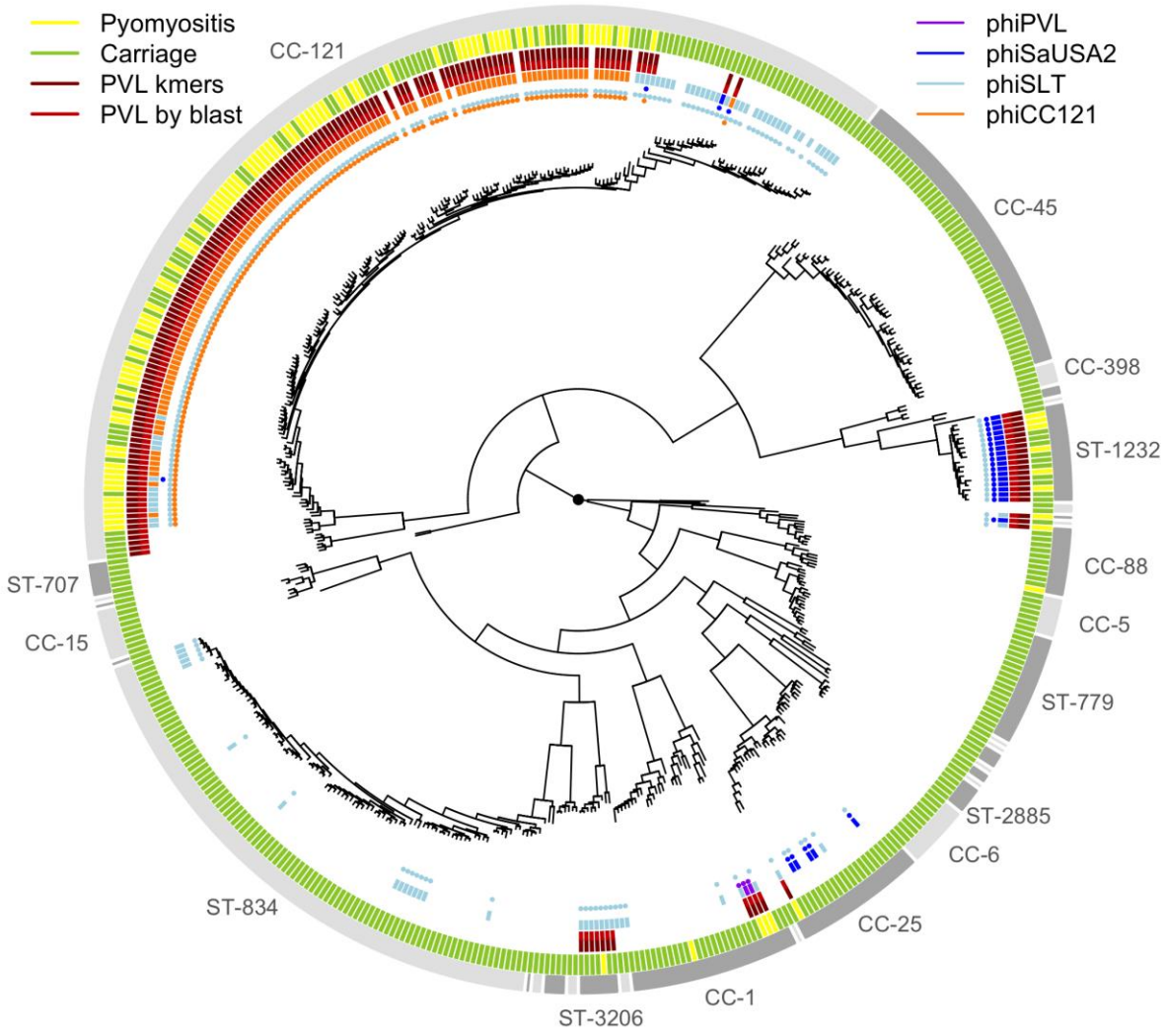

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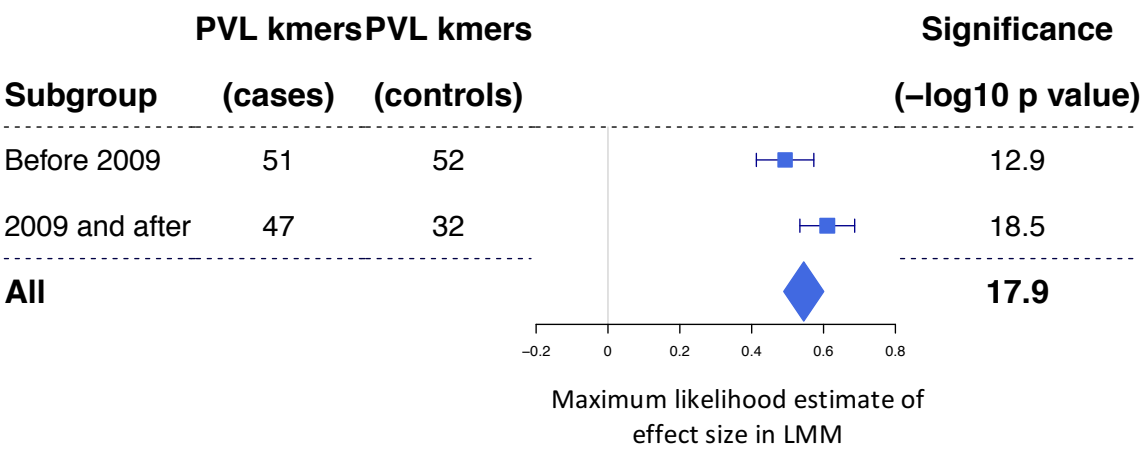

Figure 2-figure supplement 4