

Identifying possibly novel sources of antimicrobial resistance in uncultivated bacterial and archeal metagenome-assembled genomes

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Abstract

Antimicrobial resistance (AMR) is a global threat to human health. Efforts to prevent the spread of AMR rely on surveillance of possible AMR determinants. Using phylogenetics to highlight potential sources of AMR could guide researchers in choosing organisms for phenotypic resistance testing. A systematic way of classifying AMR gene variants is important in comparing these phylogenetic relationships. In this study, the phylogenetic neighborhoods of several named AMR genes are characterized by their diversity, spread, and potential for discovering possibly novel AMR variants. Canonical sequence data from the Comprehensive Antibiotic Resistance Database (CARD) was used to query CARD prevalence data, NCBI sequence data, and draft quality metagenome assembled genomes (MAGs) from various uncultivated bacterial and archeal sources (UBAs). Genes which were potentially associated with mobile colistin resistance (MCR) were found in the UBA sources. New Delhi beta-lactamases (NDM), *Klebsiella pneumoniae* carbapenemases (KPC), and OXA beta-lactamases were not found to be represented in the UBAs, [TODO: “gradient of the AMR trees”] [TODO: conclusion: lots of novelty that cannot be accommodated by existing nomenclature schemes?]

Introduction

Antimicrobials, substances which can kill or inhibit the growth of microbes [1], are key effectors in the ecology of microorganisms. The evolution of resistance to these substances, known as antimicrobial resistance (AMR), is an important and ancient adaptive process [2]. In the last century we have adopted and used antimicrobials to great effect in clinical medicine and agriculture. However, the use and abuse of antimicrobials has led to increasing levels of observed AMR [3]. This poses a growing global health risk by undermining our ability to treat infectious diseases and perform surgeries [4]. Infections by resistant microbes have greater mortality and morbidity [4] with the European Centre for Disease Prevention and Control estimating that 25,000 people per year die due to AMR [5].

AMR can be due to intrinsic resistance within the organism or the acquisition of resistance via lateral gene transfer [2]. These mechanisms allow for the constant change of the resistome, making determining the scope of AMR within both pathogenic and non-pathogenic bacteria difficult [3]. To mitigate the spread of AMR the World Health Organization (WHO) created an action plan which emphasizes the need to strengthen our understanding and surveillance of AMR. An important aspect of this research is to examine the spread of AMR between environmental and clinical samples (<https://www.who.int/antimicrobial-resistance/global-action-plan/en/>). One way to try to understand the evolution and spread of AMR is to perform large-scale evolutionary analyses with samples from lots of different environments/contexts.

Some of the most critical pathogens are the multi-drug resistant “ESKAPE” pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. [6]. These are the leading cause of hospital-acquired infections and are largely only treatable with carbapenem or polymyxin antibiotics [5]. Carbapenems are broad spectrum beta-lactam antibiotics typically used to treat life threatening, high risk, and multi-drug resistant bacterial infections. beta-lactamase producing bacteria have difficulty degrading carbapenems when combined with beta-lactamase inhibitors making them effective treatments against pathogens with multi-drug resistance [5]. However, there has been a recent emergence of resistance to these antimicrobials, presenting a new threat to public health. One way microbes can develop carbapenem resistance is through the acquisition of carbapenamase genes. [5]. Three main carbapenamase genes of concern are *Klebsiella pneumoniae* carbapenamase (KPC), New Delhi metallo-beta-lactamase (NDM) and OXA beta-lactamases [5].

Carbapenamases are classified as one of four classes (A-D) based on their molecular structure [7]. Class A, C, and D both use a serine residue to hydrolyze beta-lactam antibiotics [8]. KPC genes are a

part of class A. Many variants of KPC are spreading and being discovered, however carbapenamases within this class are more rare than the other three classes [5]. Class D contains the OXA family of beta-lactamases. Unlike KPC and NDM, this family is characterized by phenotype rather than genotype. This family is characterized by specific hydrolases that can hydrolyze oxacillin and cloxacillin, with the enzyme being poorly inhibited by clavulanic acid

(<https://card.mcmaster.ca/ontology/36026>). This results in a low amount of sequence homology within the family. A subset of this family, OXA-48 possesses carbapenem hydrolyzing activity [9]. The OXA-48 subfamily consists of five variants: OXA-48, OXA-162, OXA-163, OXA-181, OXA-204, and OXA-232 [10]. Class B beta-lactamases are metallo-enzymes that use a zinc active site to hydrolyze beta-lactam antibiotics. The NDM gene is classified as a class B beta-lactamase [5]. When pathogens become resistant to carbapenems, the last line of defense is the polymyxin antibiotic colistin.

Polymyxins act via disrupting membrane permeability [11]. Colistin is limited to a last line defense against carbapenem resistant infections due to its neurotoxic and nephrotoxic effects [11]. Despite its already limited use, mobilized colistin resistance (MCR) have been discovered [12]. Due to the emergence of resistance to many last-line antibiotics, it is important to characterize the total diversity and spread of these key resistance determinants. However, to perform these types of analyses we need two resources: a clear consolidated definition of what constitutes an AMR-related gene and a large amount of sequencing microbial genomes.

Naming and defining AMR genes is difficult due to the legacy of research pre-DNA sequencing technology and the large number of different researchers and stakeholders involved in AMR. AMR determinants are typically classified into AMR families, some of which are based on phenotypic properties, and some based on sequence variation. The nomenclature is usually an acronym representing the mechanism of resistance, along with a numerical value to distinguish variants. Each sequence, determined to be novel by some arbitrary criteria, is assigned a new number. Sequences are further sub-categorized when the sequence similarity is high, and as much as a single amino acid difference has given rise to a newly named determinant. This system for classification has the potential to be misleading when conducting AMR research. AMR families could appear to have a large amount of diversity, when in reality, sequences are closely related, and only a small number are actually distinct. Additionally, sequences which are not homologues, could potentially be classified in the same family, simply based on their function. This is a problem when attempting to characterize the AMR determinants by sequence similarity. [TODO: analogy to multi-locus sequence typing?]

High-quality manually curated AMR databases such as the Comprehensive Antibiotic Resistance Database (CARD) [13] provide a unified resource for the definition, nomenclature and classification of AMR genes. CARD organises this information through the antibiotic resistance ontology (ARO), a controlled vocabulary with defined relationships. There are two data-sets within CARD, canonical and prevalence. Canonical is a conservative set of AMR genes and mutations that have been experimentally verified as being associated with resistance in a peer-reviewed publication. This is therefore slightly biased towards the organisms that have been most heavily studied. On the other hand, prevalence contains AMR sequences that have been detected by searching a broader set of WHO priority organism genomes from repositories such as National Center for Biotechnology Information (NCBI) for sequences similar to canonical sequences. This *in silico* search is performed using CARD's BLAST-based Resistance Gene Identifier (RGI) tool and greatly increases the sequence diversity in CARD [13].

However, this is still limited to a subset of all genomes currently available in central repositories like NCBI. If we want to thoroughly understand the evolution and spread of AMR genes we need to analyse as many genomes as possible. Unfortunately, the genomes in databases are largely sequenced from microbes that can be easily cultured. As only a subset of microbial diversity can be cultured, this means many of our existing genomes aren't necessarily representative of the environment from which they were sampled. Recently, techniques have been developed that allow the recovery of genomes from metagenomic data e.g. [14]. As metagenomic sequencing, the direct

sequencing of all DNA in a sample, doesn't require culturing these metagenome-assembled genomes (MAGs) represent a huge source of novel microbial diversity. Parks et. al. [16], generated new 7,903 bacterial and archaeal (Uncultured Bacteria and Archaea; UBA) MAGs representing >30% increase in the sampled phylogenetic diversity of the bacteria and archaea.

Identifying and phylogenetically analysing key carbapenemase and colistin resistance genes in this dataset, CARD, CARD-prevalence and NCBI genomes, could greatly improve AMR surveillance of these genes. We would characterise previously unseen diversity in genomes not yet analyzed for AMR, and provide insights into the diversity of AMR across non-clinical samples. This could inform our understanding of the transmission of these mobile critical AMR genes and help refine their current nomenclature. Therefore, in this work we present a comprehensive phylogenetic survey and analysis of KPC, NDM, OXA-48, and MCR across all currently sequenced genomes and large sets of previously uncharacterised metagenome-assembled genomes.

Methods

A data-set of 7903 draft quality MAGs which were recovered from the Sequence Read Archive by Parks @wrBRBdFb were used for this analysis. These genomes were chosen specifically because they were likely to be from lineages which were under-sampled. Environmental and non-human gastrointestinal samples were the main focus of these samples.

RGI version 5.0 with CARD database version 3.02 [3](#) was run on the contigs of the 7903 MAGS with the inclusion of loose, perfect, and strict hits.

CARD prevalence data version 3.0.4 ("based on sequence data acquired from NCBI on 28-Feb-2019, analyzed using RGI 4.2.2 (DIAMOND homolog detection) and CARD 3.0.1") was used to query the UBA data. [TODO: Other versions of software]

32 canonical MCR sequence variants, 14 canonical NDM sequence variants, 6 canonical OXA-48-like sequence variants, and 18 canonical KPC sequence variants (see Table S1) as indicated by CARD for the , were obtained from the CARD database.

In the case of OXA beta-lactamase, only OXA-48-like genes were used for analysis. The OXA family is characterized by phenotype rather than genotype, and results in a low amount of sequence homology within the family. The phenotype of OXA-48 results from carbapenem hydrolyzing activity [9](#). This subfamily of OXA contains homologous sequences suitable for this study. Incorporating other subfamilies of OXA proved to be too cumbersome.

Additional putative AMR gene family sequences were accumulated by querying the CARD prevalence data. The prevalence data was translated to a BLAST database. The CARD canonical sequences from each family were used to perform a multiple query BLASTP against the prevalence blast database with a e-value threshold and query coverages shown in Table [1](#)

Many of the sequences are nearly identical, thus they were further processed by clustering with CD-HIT at a minimum sequence identities indicated also in Table [1](#)

The CARD canonical sequences were also used to perform a multiple query BLASTP against the NCBI non-redundant database with a e-value threshold, and query coverage indicated in Table [1](#)

For simplicity in identifying the taxonomic history of the non-redundant hits, MULTISPECIES sequences were removed from the analyses. [TODO: In discussing taxonomy this may be a problem, for example, Acinetobacter is not showing up in the phylogeny and may be relevant] There are many

highly sampled taxa and genes in the non-redundant database. To balance the distribution, and reduce the size of the non-redundant sequence set, CD-HIT was used to cluster the data as per table 1.

Sequences possibly containing AMR gene prediction data for each gene family was produced by filtering RGI output based on its association with the search strings for each determinant in Table 1. The filtered data were translated to a blast database. The CARD canonical sequences were used to perform a multiple query BLASTP against this UBA blast database with a e-value threshold and query coverage in Table 1.

Table 1: [TODO: Make table more readable/better labels etc] e-value, query coverage, and cluster percentage used for each AMR family experiment for the prevalence, ncbi non-redundant, and UBA databases.

A. MCR phosphoethanolamine transferase		B. KPC beta-lactamase		C. NDM beta-lactamase		D. OXA beta-lactamase	
Tree	LG+I+G4	Tree	LG+I+G4	Tree	LG+I+G4	Tree	LG+I+G4
e-value_p	1e-160	e-value_p	1e-100	e-value_p	1e-160	e-value_p	1e-100
e-value_n	1e-160	e-value_n	1e-40	e-value_n	1e-160	e-value_n	1e-100
e-value_u	1e-160	e-value_u	1e-10	e-value_u	1e-10	e-value_u	1e-10
coverage_p	98	coverage_p	99	coverage_p	98	coverage_p	90
coverage_n	98	coverage_n	99	coverage_n	98	coverage_n	99
coverage_u	98	coverage_u	60	coverage_u	60	coverage_u	95
clustering_p	100	clustering_p	99	clustering_p	100	clustering_p	N/A
clustering_n	100	clustering_n	70	clustering_n	100	clustering_n	N/A
clustering_u	100	clustering_u	100	clustering_u	100	clustering_u	N/A

Redundant results for the prevalence, NCBI, and UBA queries were filtered from these BLASTP results by retrieving only the longest sequence for each uniquely labeled result.

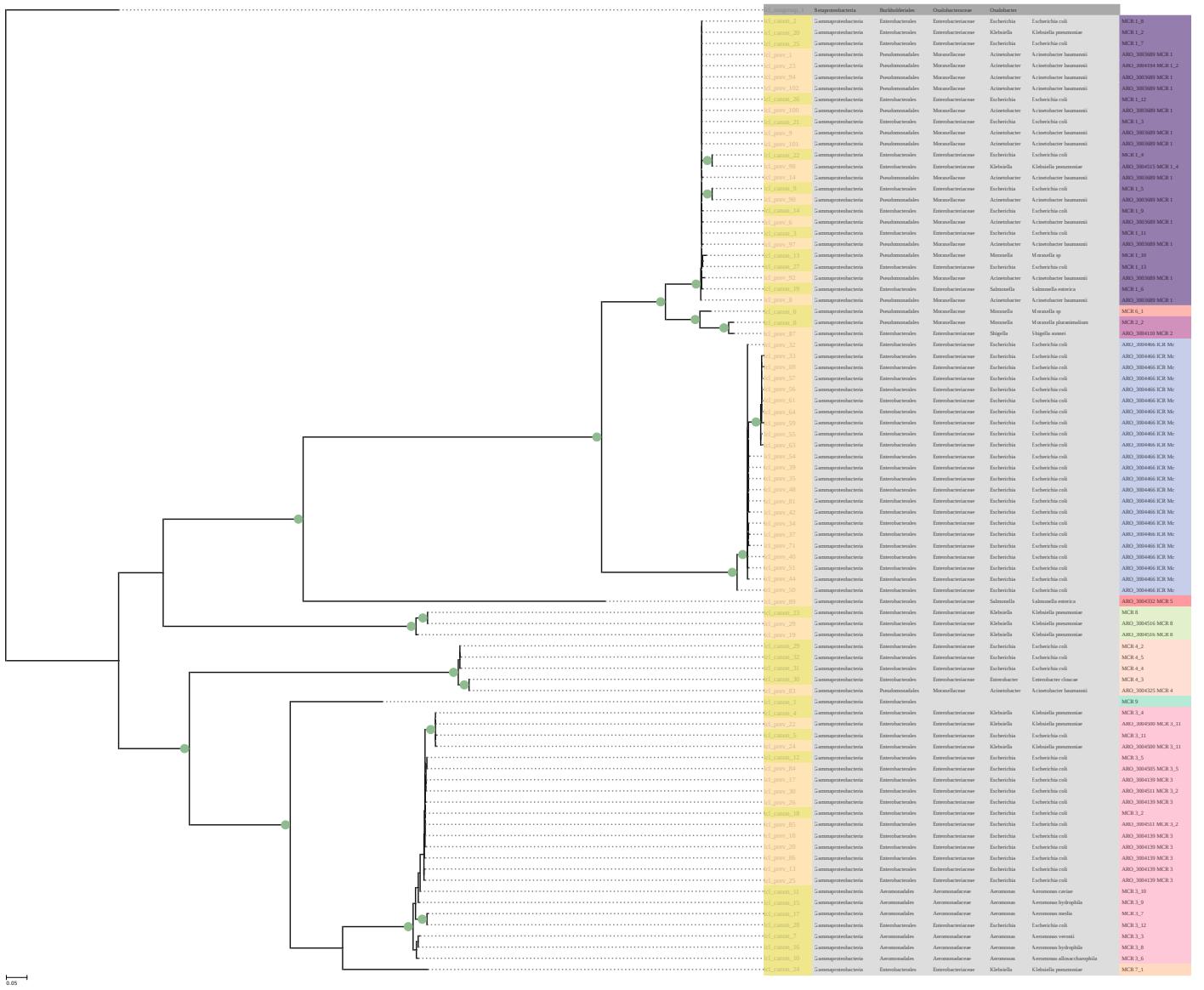
To compare the phylogenetic relationship of only the putative sequences, the sequences from the CARD prevalence data were concatenated to one multi-FASTA format file with the canonical sequences. These the concatenated amino acid sequences were aligned with MAFFT-LINSI. The aligned sequences were trimmed by trimal using the automated1 option. IQ-TREE was then used to build a bootstrapped tree with -bb 1000 with the G+I+G4 model of substitution.

For the overall comparison of sequences, the filtered sequences from NCBI non-redundant data, CARD prevalence data, UBA data were all concatenated to one multi-FASTA format file with the canonical sequences. These the concatenated amino acid sequences were aligned with MAFFT-LINSI. The aligned sequences were trimmed by trimal using the automated1 option. IQ-TREE was then used to build a bootstrapped tree with -bb 1000 with the G+I+G4 model of substitution. All leaves of the trees were labeled with as much taxonomic information as possible for each rank, based on information from metadata [TODO: supplemental].

Results

Phylogenetic analysis of MCR sequences

The phylogenetic relationships of the CARD canonical sequences, and the CARD prevalence sequences involving the MCR family were investigated to show the phylogenetic relationship of only the putative MCR sequences without the noise of additional sequences. A total of 87 genes, an out-group, the 32 canonical sequences, and the 54 prevalence sequences (clustered as per Table 1), were selected for analyses. The tree in Figure 1 shows several distinct clades. Each MCR variant forms a clade. MCR-1, MCR-2, and MCR-6 form a clade, appearing to more closely related to one another than with the other MCR family members. This clade is also closely related to the ICR-Mc clade. ICR-Mc 17 is another phosphoethanolamine transferase which confers colistin resistance.



missing, where unrepresented clades of MCR could exist.

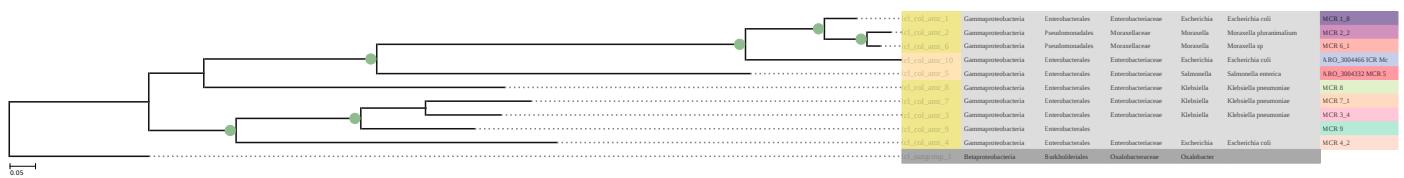


Figure 2: Phylogenetic relationship of 9 MCR family sequences, and an outgroup from Betaproteobacteria.

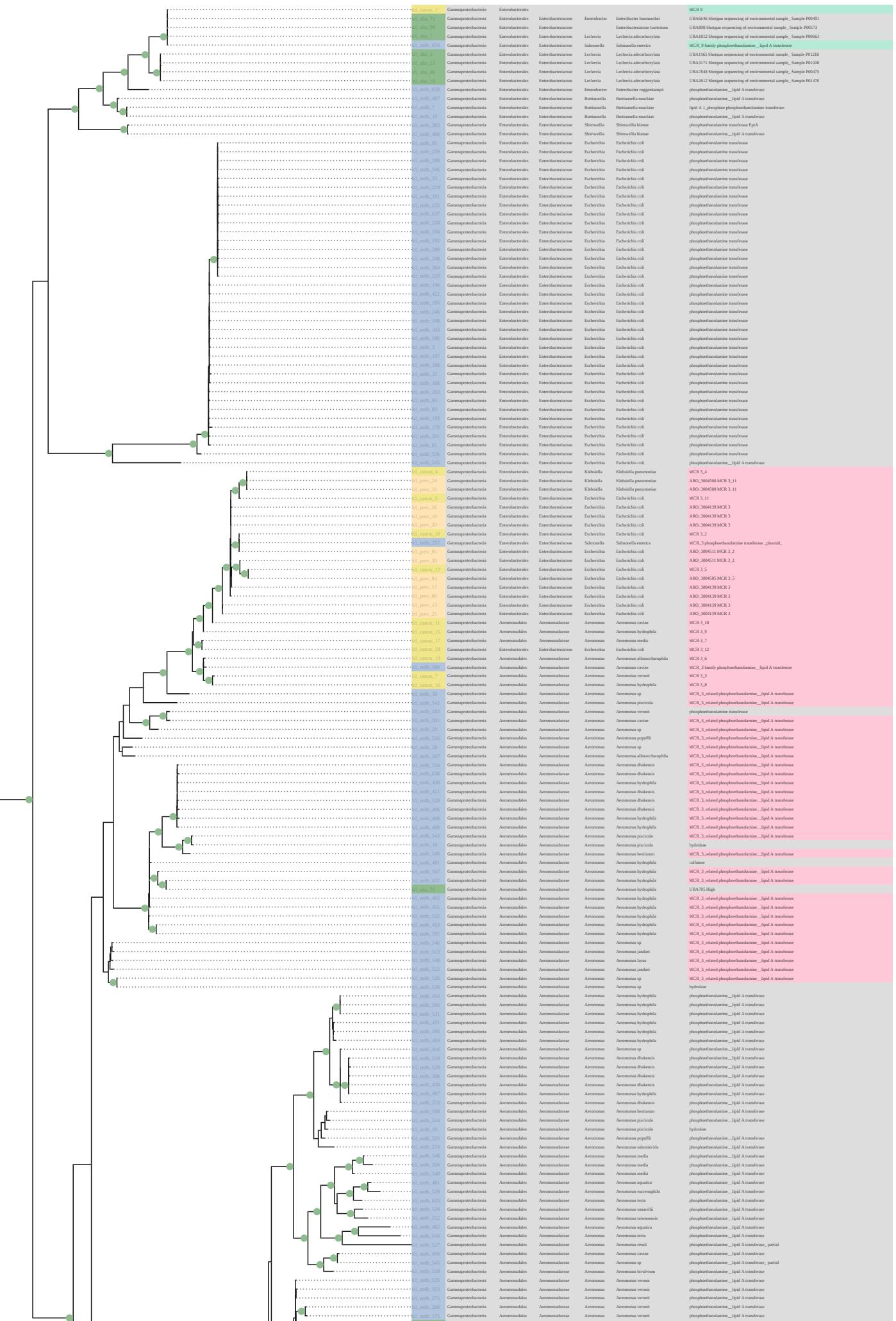
In an attempt to discover these potential clades between these named MCR families, sequences from the NCBI non-redundant data were added to the analysis to be compared with the canonical and prevalence sequences. This resulted in a total of 409 sequences for subsequent analysis, all labeled as phosphoethanolamine lipid A transferase genes, where 104 hits were labeled as MCR family genes.

In addition, the 7903 draft quality MAGs were queried for AMR genes with RGI. RGI produced 1457246 results AMR determinants under the loose cutoff from the UBA data, 7171 for the strict cutoff, and 310 for the loose cutoff. It was hoped that phylogenetic analysis could find AMR determinants would be found in under the loose criterion that may have been missed by RGI-CARD. The UBA BLAST results were included in the phylogeny in Figure 8 for the analysis. The remainder of the analysis deals with relationships which are deemed to be interesting based on the locations of the UBAs between MCR family clades.

Between the clade containing MCR 3, and the most recent common ancestor of MCR 3 and MCR 7 clades (figure 3), there is a clade of sequences from NCBI which have been reported as MCR 3 [TODO: look at linked literature]. Present within this clade is a single UBA result, UBA705, which the Parks data 18 reports as a Comparative metagenome analyses of anode-associated microbial communities developed in rice paddy field-soil microbial fuel cells, is reported to be *Aeromonas hydrophilia*. This present within the clade alone with several other canonical and non-redundant Aeromonadalacea. *Aeromonas hydrophilia* is a species which has been found to have an MCR-3 gene. Even though qualities vary (Table ??), the sequences branch in the expected location.

Between the clade containing MCR 7, and the most recent common ancestor of MCR 3 and MCR 7 clades, a clade of phosphoethanolamine lipid A transferase clade appears. This clade consists of the genus of gram negative bacteria, *Aeromonas*, 19 which is sometimes involved in human infection. This clade also includes an *Aeromonas veroni* hit from from epidermal mucus of *Anguilla anguilla* in the UBA data. Between MCR 9 and the most recent common ancestor of MCR 3 and 9, a clade of *Aeromonas* associated phosphoethanolamine lipid A transferases appear.

The MCR 9 containing clade contains 8 UBAs and several NCBI non-redundant hits not reported as MCR family genes. 5 Loose hits for MCR in a *Leclercia adecarboxyla* branch within this clade. These *Leclercia* branch below the common ancestor of MCR-9 and MCR-3 which are well supported. The *Leclercia* UBAs were all sampled from New York City MTA subway samples Metagenome. Acinetobacter is another opportunistic pathogen 20 which is becoming resistant to many antimicrobials.



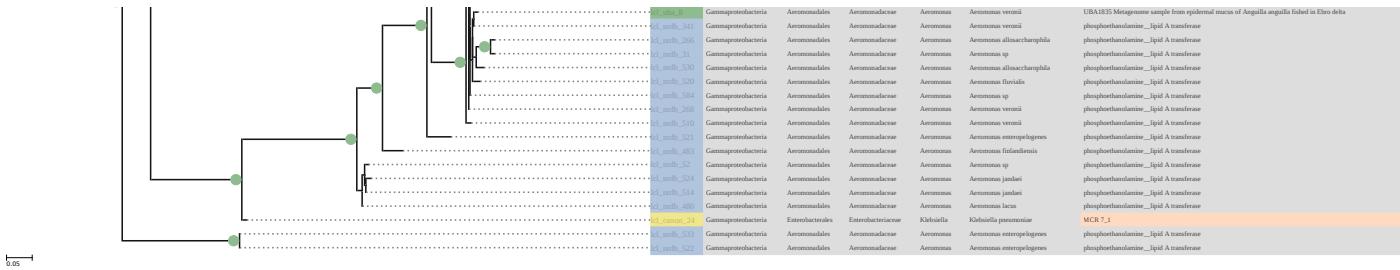


Figure 3: Clade containing putative MCR3 and MCR 9 clades pruned from Phylogenetic relationship of 32 canonical (labels prefixed with lcl_canon_ in yellow), and 54 prevalence (labels prefixed with lcl_prev_ in tan) MCR family sequences, 595 NCBI non-redundant sequences (labels prefixed with lcl_prev_ in blue), and 91 UBA sourced sequences (labels prefixed with lcl_prev_ in green). Each MCR variant is coloured based on its primary numerical value. If the sequence is not reported to be MCR family it is coloured in grey.

Between MCR 5 and ICR-Mc (figure {#fig:mcr-5-icr}), there appear clades of diversity in the genus *Psychrobacter*. Branching between these two clades, as a descendant to this ancestor, is a clade of *Psychrobacter* species bacteria. This clade includes several hits from the non-redundant database, and two hits from the UBA data. According to the Parks [18](#) data, the identity of these samples are, UBA3068, A *Psychrobacter* sp., sampled from Oil polluted marine microbial communities from Coal and Oil in Point Santa Barbara, California, USA and, UBA4193, a *Psychrobacter* sp., sampled from the New York City MTA subway samples. The quality information for these sequences, shown in Table ??, shows that UBA3068 is near complete, while UBA4193 is only partial. It is encouraging to see that even with the quality difference, these two sequences branch in the expected clade. *Psychrobacter* [21](#) is a Genus is widespread and includes many cold adapted bacteria, it is an opportunistic pathogen, and has been found sometimes be a cause of infections in humans, animals, and fish. Many new species of *Psychrobacter* have been discovered in cold climates [22](#). Some of the species have been shown to be resistant to colistin, like *Psychrobacter vallis* ps. nov. and *Psychrobacter aquaticus* ps. nov. [23](#) and is sister to [TODO: display new tree such that *Acinetobacter* is shown] Another clade between these two variants contains phosphoethanolamine transferases from the genus *Stenotrophomonas*, including *Stenotrophomonas maltophilia* and *Stenotrophomonas acidaminiphila* which multidrug-resistant opportunistic pathogen [24](#). Several UBA hits for *Stenotrophomonas maltophilia* show up as shotgun sequencing of environmental samples. [TODO: quality information]

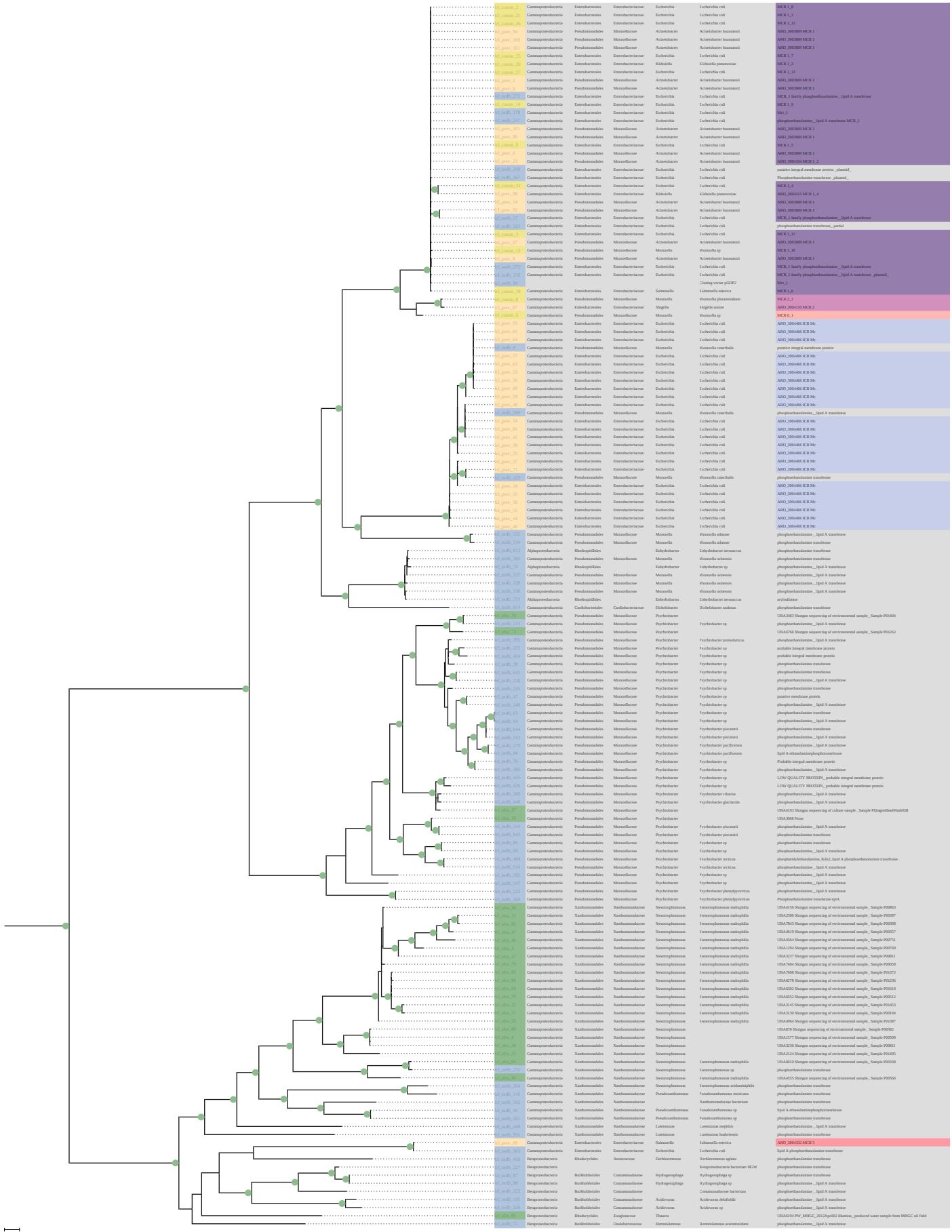
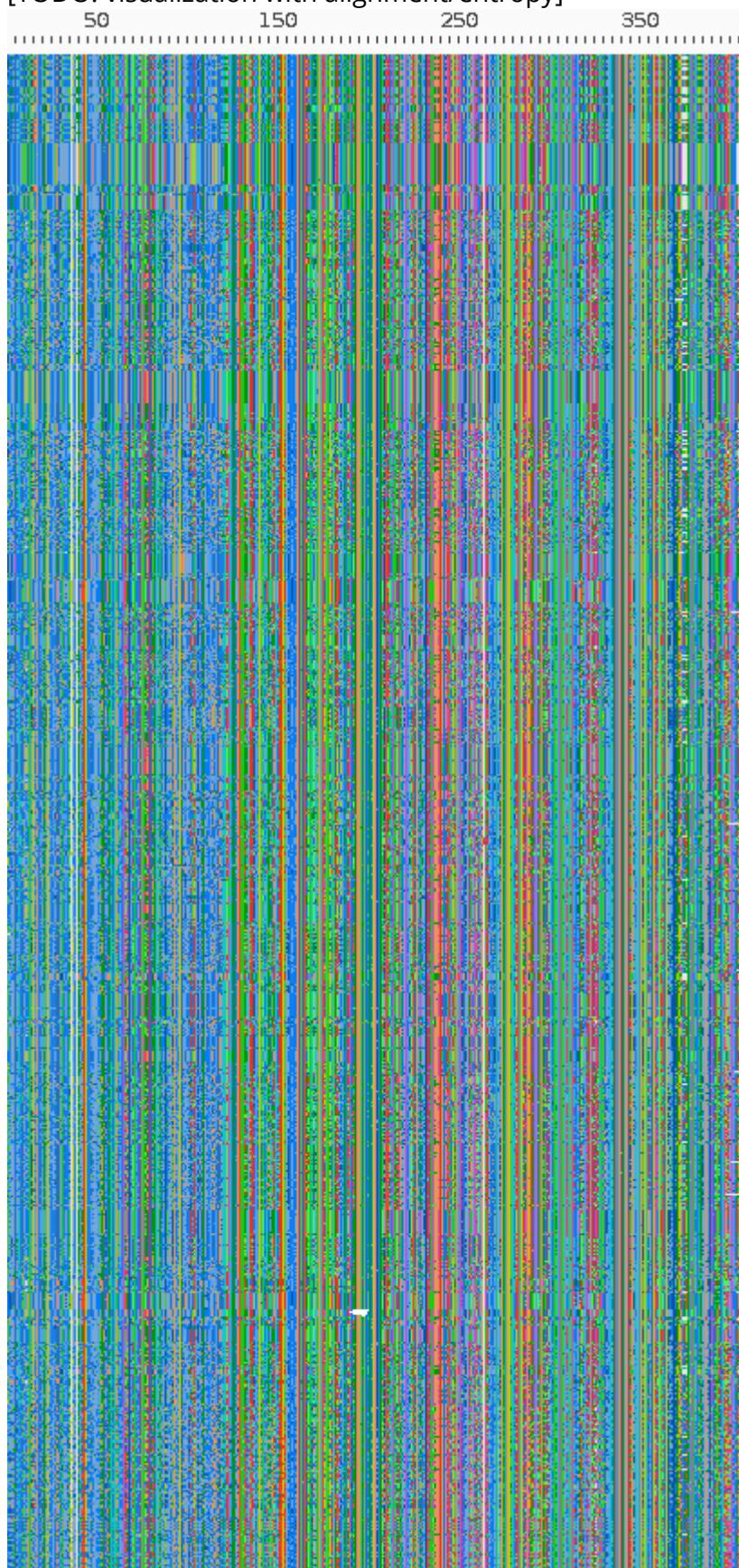


Figure 4: Clade containing putative MCR 5 and ICR-Mc clades pruned from Phylogenetic relationship of 32 canonical (labels prefixed with lcl_canon_in yellow), and 54 prevalence (labels prefixed with lcl_prev_in tan) MCR family sequences, 595 NCBI non-redundant sequences (labels prefixed with lcl_prev_in blue), and 91 UBA sourced sequences (labels prefixed with lcl_prev_in green). Each MCR variant is coloured based on its primary numerical value. If the sequence is not reported to be MCR family it is coloured in grey.

[TODO: visualization with plasmid distribution]

MCR Alignment

[TODO: visualization with alignment/entropy]



Phylogenetic analysis of KPC

18 canonical sequences, and the 8 prevalence sequences (clustered according to Table 1), non-redundant sequences, and 7 UBA sequences were combined in the phylogeny in Figure 2. In investigating the phylogenetic relationship of KPC also resulted in UBA sequences which were too distant from the canonical sequences to make a conjecture regarding their relationship to KPC family. In Figure 5 the 7 resulting UBA sequences branch with class A beta-lactamases as reported by the non-redundant BLAST.

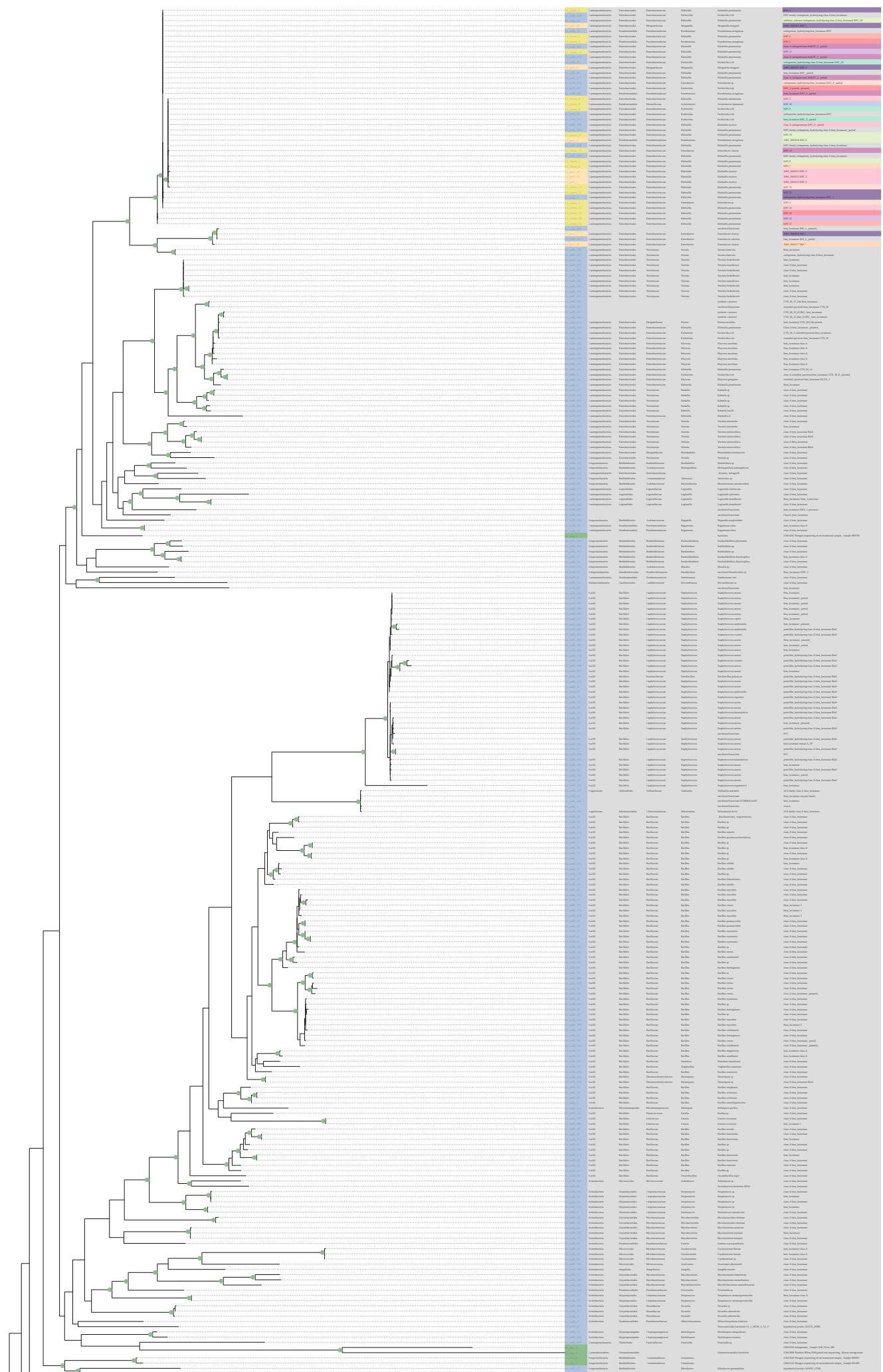




Figure 5: Phylogenetic relationship (lcl_canon) KPC family sequences along with 14 prevalence (lcl_prev) sequences.

Phylogenetic analysis of NDM

The phylogenetic relationships of the NDM family were investigated. The 14 canonical sequences were used to query the same databases as in the phylogenetic analyses of MCR.

The genes retained for the phylogeny were the 14 canonical sequences, 3 prevalence sequences ((clustered as per table 1)), 7 non-redundant sequences, 1 UBA sequence, and 1 out-group.

This total of 27 resulted in the phylogeny in Figure 6.

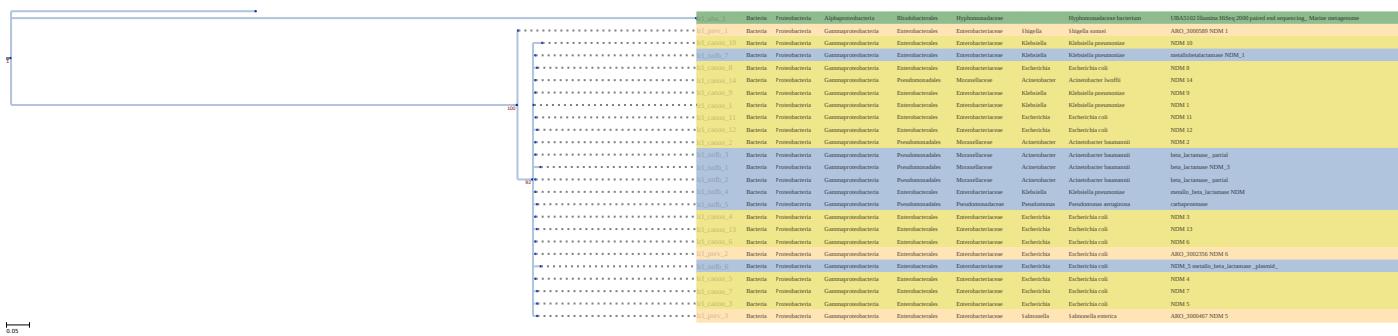


Figure 6: Phylogenetic relationship (lcl_canon) NDM family sequences along with 14 prevalence (lcl_prev) sequences.

The 7 sequences resulting from the NCBI data included 3 genes reported to be NDM-1, NDM-3, and NDM-5. The other 4 genes were reported to be general beta lactamase/carbapenemase hits. These sequences form a closely related multifurcation (due to poor support values) with the prevalence and canonical data. The only phylogenetically resolved gene in this clade was a prevalence hit for NDM-1 found in *Shigella sonnei* representing only itself in the cd-hit cluster. Interestingly other representatives from other purported NDM families are more closely related to each other, including the canonical NDM-1 sequence from *Klebsiella pneumoniae*. The one UBA BLAST result branches far from the clade containing the canonical indicating that under the coverage queried, there are no reasonably detectable NDM homologues in the UBA data. The alignment of this UBA under this relaxed query coverage of 60% is already pushing the limits of a “good alignment”, and reducing this further would produce meaningless results.

Phylogenetic analysis of OXA-48

In investigating the phylogenetic relationship of OXA-48, the result was similar to that of NDM. There were multiple BLAST results for UBA sequences, but the hits were too phylogenetically dissimilar to draw a conjecture about their relationship to the OXA family. 6 canonical sequences, and the 5 prevalence sequences (clustered according to Table 1), 11 non-redundant sequences, and 112 UBA sequences were combined in the phylogeny in Figure 7.

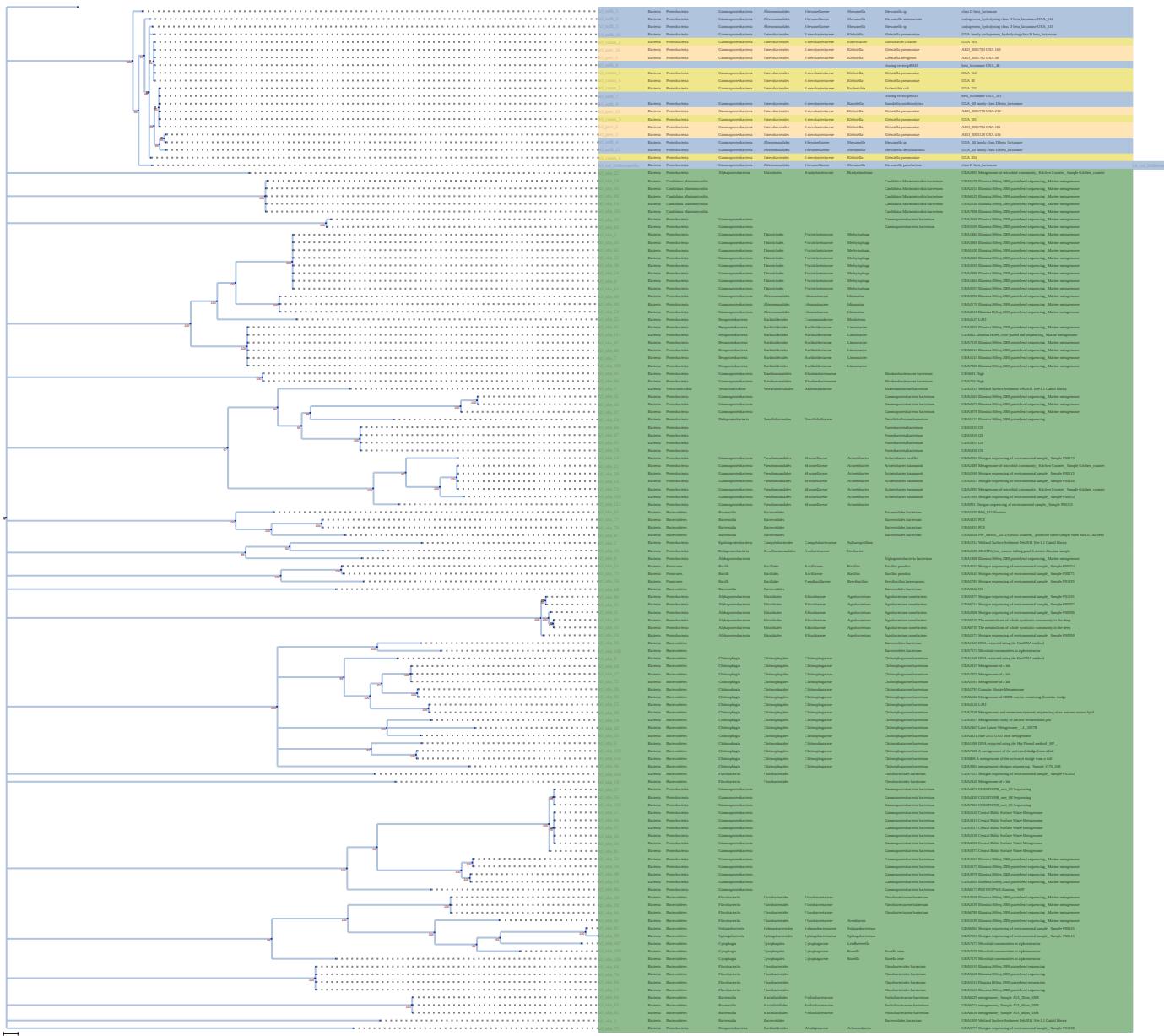
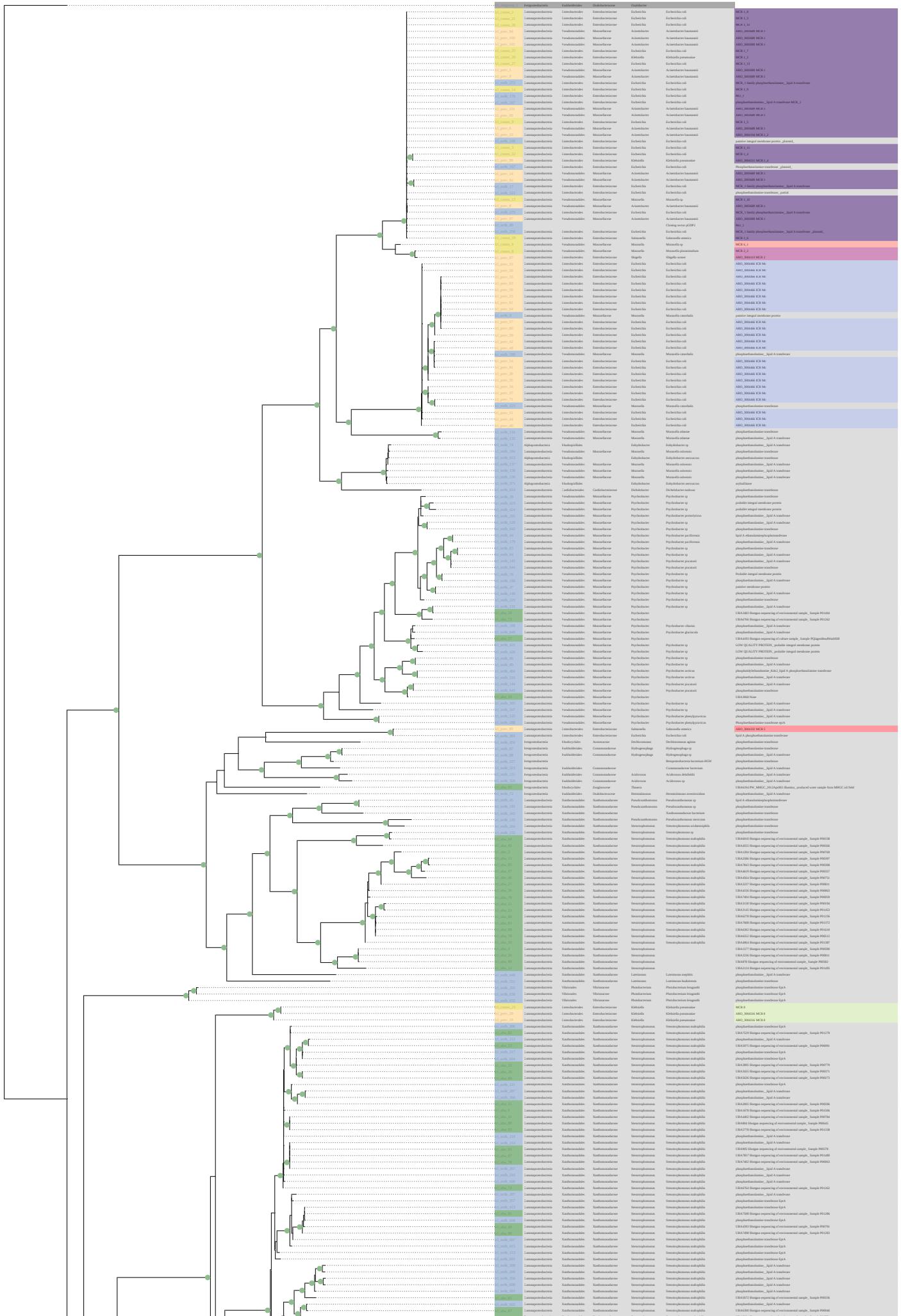
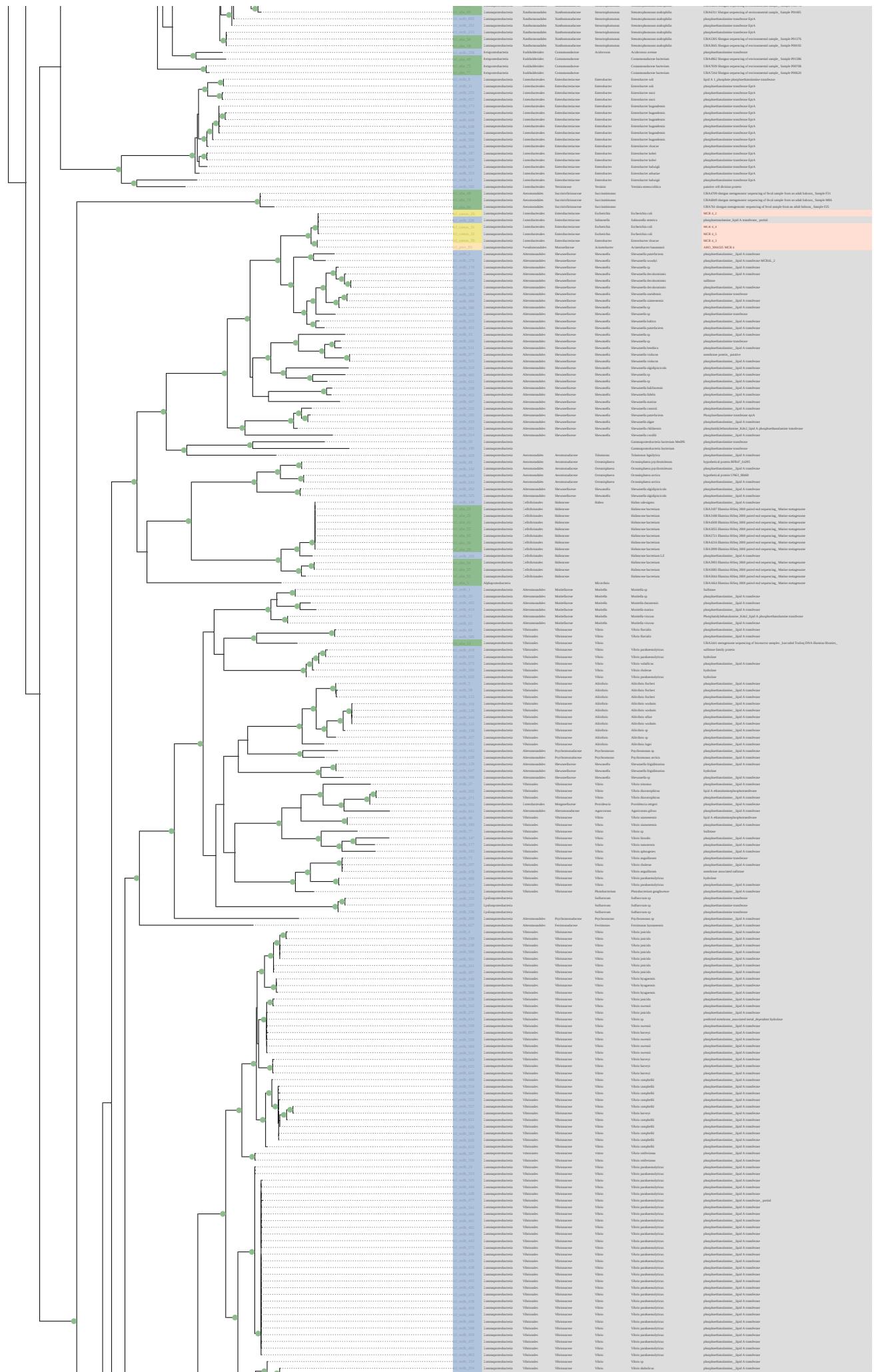


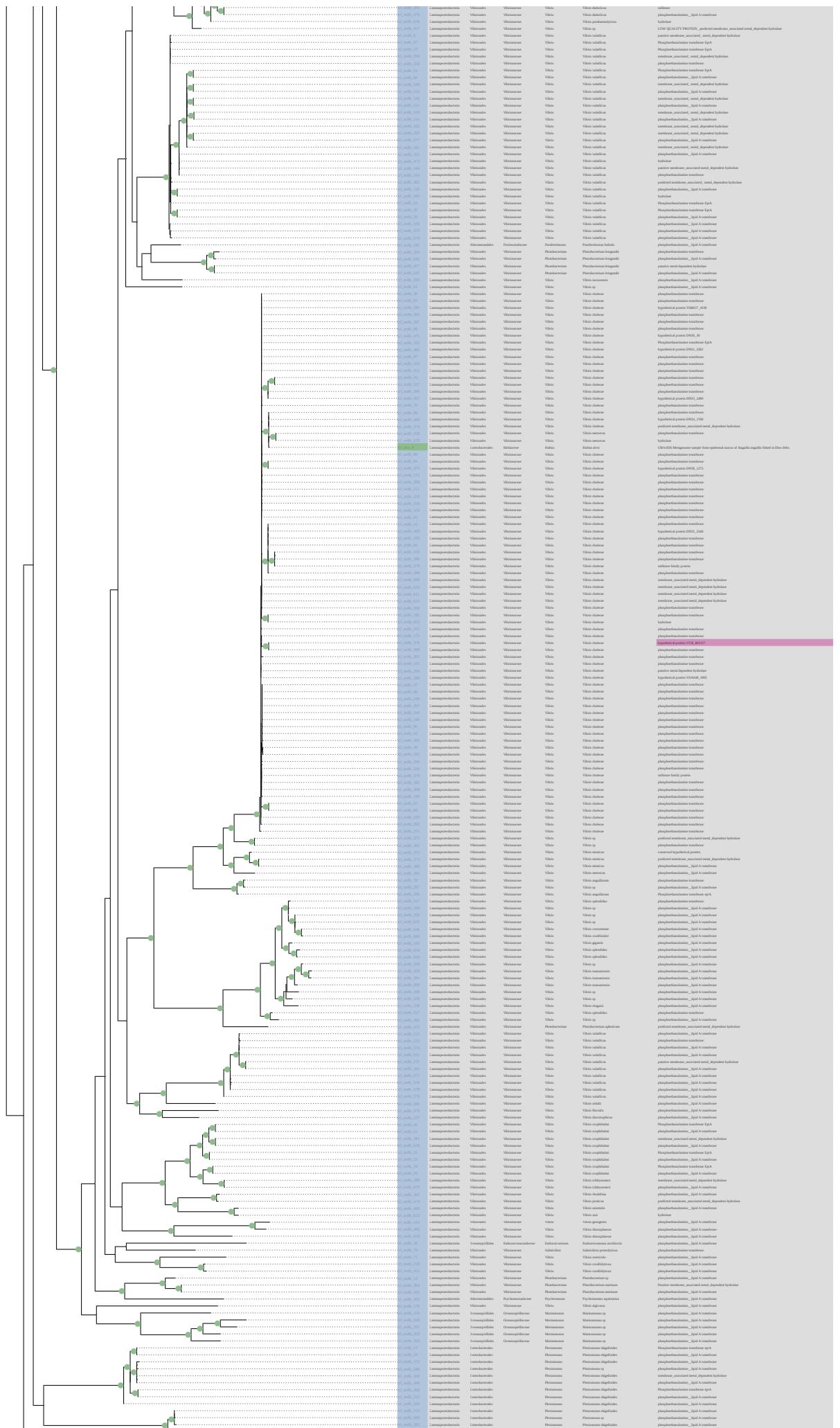
Figure 7: Phylogenetic relationship (lcl_canon) OXA-48 family sequences along with 14 prevalence (lcl_prev) sequences.

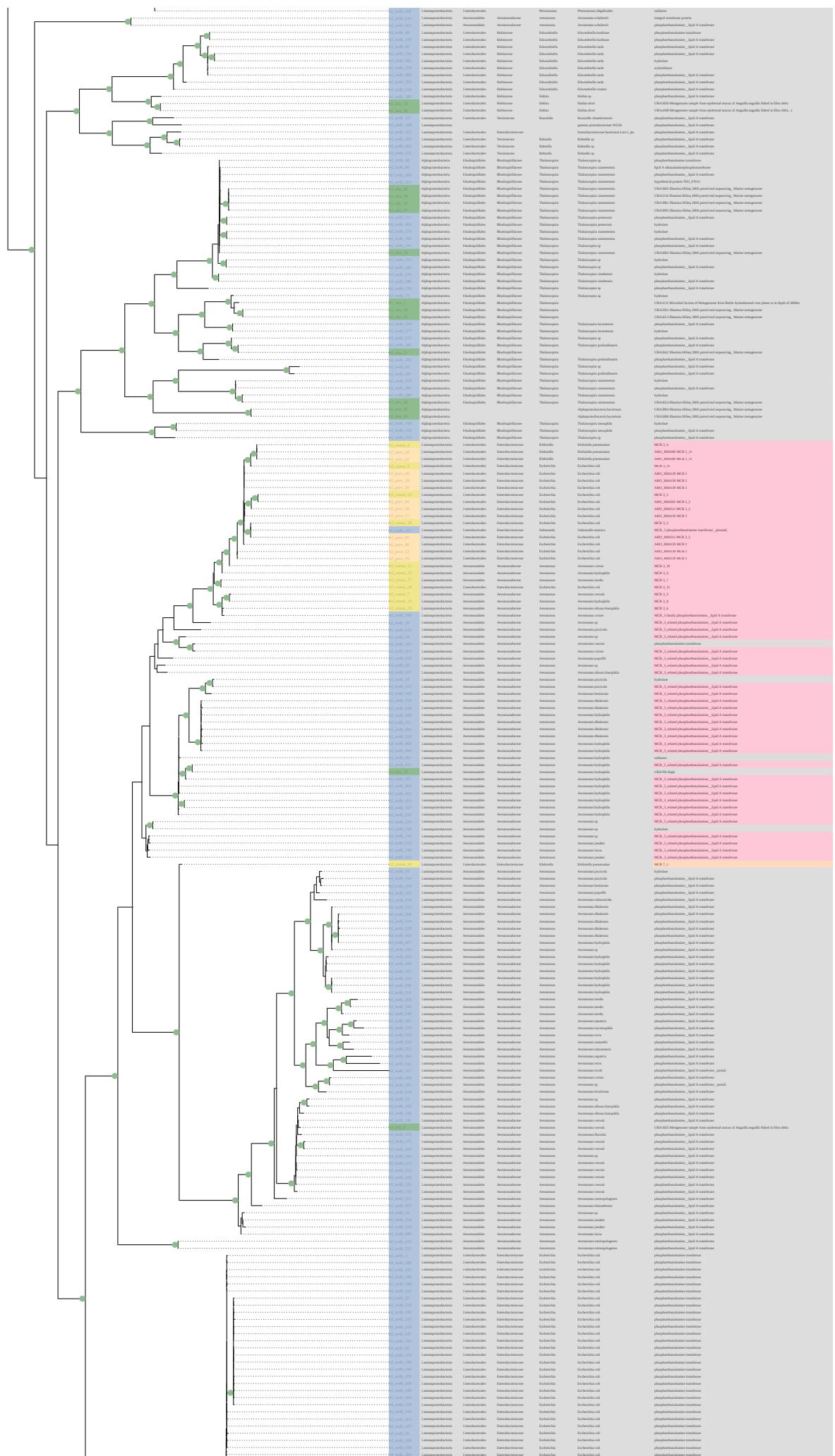
The OXA-48 prevalence hits added further diversity to the reference OXA-48-like sequences. OXA-436 [25](#) was found, clustered with no other gene, in the prevalence data, and OXA-514 and OXA-515 were found in the non-redundant data.

Supplemental









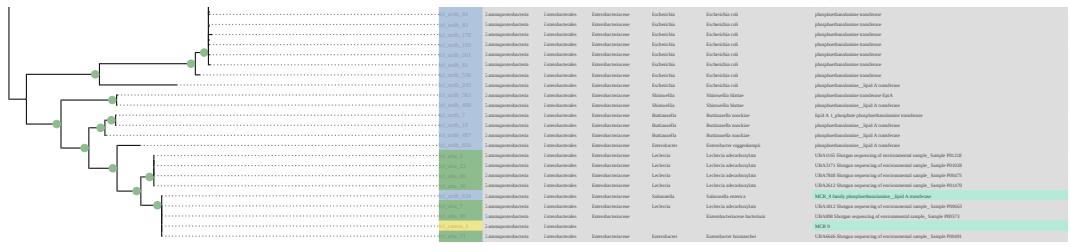


Figure 8: Phylogenetic relationship of 32 canonical (labels prefixed with `Icl_canon_` in yellow), and 54 prevalence (labels prefixed with `Icl_prev_` in tan) MCR family sequences, 595 NCBI non-redundant sequences (labels prefixed with `Icl_prev_` in blue), and 91 UBA sourced sequences (labels prefixed with `Icl_prev_` in green). Each MCR variant is coloured based on its primary numerical value. If the sequence is not reported to be MCR family it is coloured in grey.

Discussion

- TODO: Discuss the following:
 - We found a lot of intermingled MCRs, not so much with the others (plasmids etc.)
 - Why were some UBAs not covered? (plasmids)
 - What should we do with naming, especially as new variants are discovered and fill in the tree?
 - Taxonomic anomalies and how they may be explained
 - There were many clinical samples represented that *did* yeild MCRs, but the recovery could be bad

Conclusion

S1: MCR 3.11, 6.1, 2.2, 3.6, 3.10, 3.5, 1.10, 1.9, 3.9, 3.8, 3.7, 3.2, 3.1, 1.6, 1, 2, 3, 1.2, 1.3, 1.4, 8, 4, 5, 7.1

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