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Supplementary Information for

Evolution of drug resistance in an antifungal-naive chronic *Candida lusitaniae* infection

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Supplemental Materials and Methods:

Strains and culture conditions

Clinical isolates were acquired from sputum and bronchoalveolar lavage fluid samples that were plated on blood agar or CHROMagar Candida media then restreaked on YPD to obtain single isolates which were saved in 25% glycerol. All *C. lusitaniae* strains (Table S2) were streaked onto YPD (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) plates every 8-10 days, from the 25% glycerol stocks stored at -80 ºC, and maintained at room temperature. Overnight cultures were grown in liquid YPD at 30 ºC. Cells were grown with $YNBG_{10}$ or $YNBG_{100}$ (0.67% yeast nitrogen base medium with ammonium sulfate (RPI Corp), either 10 mM or 100 mM dextrose, 1.5% agar as necessary) and RPMI-1640 (Sigma, containing L-glutamine, 165 mM MOPS, 2% glucose) as noted. CHROMagar Candida medium (CHROMagar) was prepared according to manufacturer's instructions. Colony color on CHROMagar Candida medium varied slightly by batch and incubation time, but isolates grouped together by colony color across replicate experiments. All cultures were incubated at 30 ºC unless otherwise noted. *Pseudomonas aeruginosa* strains were streaked onto lysogeny broth (LB) every 8-10 days, from 25% glycerol stocks stored -80 ºC, and maintained at 4 ºC. Cultures were grown overnight in LB at 37 ºC unless otherwise noted.

Species identification

Sequencing of the ITS1 (internal transcribed spacer 1) region amplified from total DNA isolated for a portion of the original right upper lobe sample was previously published (subject 6 in (1)). Additional ITS1 sequencing of individual isolates was performed as follows: DNA was isolated via the MasterPure yeast DNA purification kit

(Epicentre), ITS1 region was amplified from genomic DNA using primers ITS1_F and ITS1_R (2). Amplicon products were concentrated using the DNA Clean and Concentrator Kit (Zymo Research) prior to Sanger cycle sequencing by the Molecular Biology Shared Resource Core at Dartmouth. The resulting sequences were identified using NCBI BLASTn (3) to search for similar sequences among the nucleotide collection database available through NCBI which contains known ITS1 sequences. In addition to *C. lusitaniae*, minor populations of other species were identified but excluded from further analyses, including *Candida parapsilosis* among the Sp1 and LL isolates (11% and 17%, respectively) and *Candida albicans* among the Sp2 isolates (3%).

Whole genome sequencing and variant calling

Genomic DNA was extracted from cultures grown in YPD for ~16 hours using the MasterPure yeast DNA purification kit (Epicentre). To generate the Sp1, UL and LL pools of genomic DNA, 500 ng of DNA from each isolate within the pool was combined, an aliquot of this mixture was used to create the genomic library. Genomic libraries, for single and pooled isolate DNA, were prepared using the KAPA HyperPrep Kit and sequenced using paired-end 150 bp reads on the Illumina NextSeq500 platform, to a depth of 100-150x coverage per sample. Samples sequenced include the twenty clinical isolates identified in Fig. 1C, ATCC 42720 and the Sp1, UL and LL pools.

The pipeline for these analyses is available in a github repository (https://github.com/stajichlab/C_lusitaniae_popseq) and archived under Document Object Identifer DOI: 10.5281/zenodo.1346354. The short read sequences were aligned to the *Candida lusitaniae* ATCC 42720 (4) genome using bwa (0.7.12) (5) and stored as a sorted, aligned read BAM file with Picard (2.14.1, http://broadinstitute.github.io/picard/)

to assign read groups and mark duplicate reads (script 01_bwa.sh). BAM files were processed to realign reads using GATK's RealignerTargetCreator (4.beta.2) and IndelRealigner following best practices of GATK (script 02_realign.sh) (6). Each realigned bam file was processed with GATK's HaplotypeCaller (script 03_GATK_HTC_gatk4.sh). Results were combined using GATK's GenotypeGVCF method to produce a single variant call format (VCF) file of the identified variants (script 04_jointGVCF_call.sh). Low quality SNPs were filtered based on mapping quality (score <40), quality by depth (<2 reads), Strand Odds Ratio (SQR>4.0), Fisher Strand Bias (>200), and Read Position Rank Sum Test (<-20) to produce list of high quality polymorphisms (script 05_filter_vcf.sh). Additional filtering included the removal of fixed SNPs, those that were invariant among the clinical isolates but differed from the reference, the removal of positions that were uncalled by GATK in some isolates, and positions which did not agree between a control set of samples (L17 sequenced in triplicate) (script removedfixed.py). The quality filtered VCF file containing only variants among the clinical isolates was categorized by SnpEff (4.3r) (7) and the ATCC 42720 gene annotation. The reference ATCC 42720 genome was altered to remove mitochondrial fragments inserted into the nuclear assembly and the mitochondrial contig (Supercontig_9) was replaced by a complete mitochondrial genome from strain *C. lusitaniae* CBS 6936 (NC_022161.1). The following regions were masked out due to unusually high coverage and likely mitochondrial origin: (Supercontig_1.2:1869020-1869184,1664421-1664580; Supercontig_1.3:1076192-1076578,1324802-1324956,1353096-1353260; Supercontig_1.6:126390-126604; Supercontig_1.8:29199-29370). Though the analyses described above utilized the published version of the *C.*

lusitaniae ATCC 42720 (4) genome, we resequenced ATCC 42720 in order to identify possible sequencing errors present in the published version. This data was used to correct gene annotations, described below for *CLUG_01938/01939*, and validate that SNPs of interests were correctly identified.

Genome assemblies of the strains was performed with SPAdes (v3.12.0) (8) after trimming and adaptor cleanup of the reads was performed with AdapatorRemoval (v2.0) (9) and quality trimming with sickle (v1.33) (10). *De novo* assemblies were further screened for vector contamination with vecscreen (https://github.com/hyphaltip/autovectorscreen) before submission to Genbank.

Construction of phylogenetic trees

SNPs that varied between clinical isolates were combined into a single multiple alignment (script vcftab_to_fasta.pl) followed by phylogenetic tree inference with IQ-Tree (11) GTR model incorporating ascertainment bias (GTR+ASC) and 100 bootstrap replicates (script make_SNPTree.sh). A phylogeny based on INDELs, created from a VCF file containing INDEL positions notated as matching reference (0) or different than reference (multiple states possible, 1 or 2), was constructed with IQ-TREE (11) using a multistate Morphological model (MORPH) and 100 bootstrap replicates (script make_INDELtree_tableS2.sh). One tree was constructed solely with the lung isolates and using only polymorphic sites segregating in those strains. A second tree was constructed with a dataset that included two outgroup strains ATCC 42720 and CBS 6936 to demonstrate the divergence between the population and other strains.

Copy number variation analysis

CNVs were examined by plotting window-based read coverage of the short-read alignments from each strain. The depth of coverage was constructed with mosdepth (12), manipulated with shell scripts (mosdepth_prep_ggplot.sh) and plotted with R using ggplot2 (13). Heatmap of chromosome 6 coverage was plotted using the heatmap3 (14) package in R (15).

Independent sequencing of *MRR1*

To confirm the presence of SNPs and INDELs in *MRR1*, *MRR1* was amplified from clinical isolate genomic DNA and sequenced. *MRR1* was amplified using primers ED050 and ED051 and sequenced using multiple primers (ED052-ED056) by the Molecular Biology Shared Resource Core at Dartmouth (Sanger cycle sequencing). The resulting sequences were aligned using SnapGene software (GSL Biotech, Chicago, IL) to a manually curated version of *MRR1* which contained mutations identified via whole genome sequencing.

Reannotation of reference genome sequences

For *MRR1*: The protein sequences encoded by the *MRR1* orthologs in *C. albicans* (C3_05920W_A), *Candida dubliniensis* (Cd36_85850*), C. parapsilosis* (Cpar2_807270*), Candida auris* (Qg37_07783*)*, and *C. lusitaniae* (Clug_005*42)* were aligned with PRALINE (16) (Fig. S4A). The first 64 amino acids of *C. lusitaniae* MRR1, based on the annotated start codon in NCBI, shared no homology with Mrr1 from other *Candida* spp. Primers ED068 and ED069, specific to the first 192 nucleotides of *CLUG_00542*, and ED070 and ED071, after nucleotide 193, were used for PCR amplification from genomic DNA and cDNA (created as described in the quantitative RT-PCR section). RNA sequencing (RNA-Seq) read coverage was visualized with CLC

Genomics Workbench 11.0 **(**https://www.qiagenbioinformatics.com/). The reannotation of the *CLUG_00542* start codon changes the gene position on Supercontig 1 to 1,098,284-1,094,487. M65 was used as the start codon when determining the amino acids affected by SNPs and INDELs in *MRR1*.

For *MDR1*: Comparison of the genome sequence surrounding *CLUG_01938* from the clinical isolates and the resequenced ATCC 42720 to the previously published ATCC 42720 (4) genome revealed two sequencing errors (Fig. S8). Manual correction of these SNPs altered the predicted stop codon for *CLUG_01938*, resulting in a new ORF which included both *CLUG_01938* and *CLUG_01939*, referred to as *MDR1*. Clustal Omega (17) was used to align the corrected *C. lusitaniae* Mdr1 sequence with *C. albicans* Mdr1. RNA-Seq read coverage was visualized with CLC Genomics Workbench 11.0 (https://www.qiagenbioinformatics.com/).

RNA sequencing

Overnight cultures were back diluted into $YNBG_{10}$ and grown to exponential (~8 h) twice, in biological duplicate. RNA was harvested from snap-frozen pellets (using liquid nitrogen) using the MasterPure Yeast RNA Purification Kit (Epicentre) and stored at -80 ºC. RNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (NeoPrep) and sequenced using pair-end 75 bp reads on the Illumina NextSeq500 platform. Data analysis pipeline is available in github repository (https://github.com/stajichlab/C_lusitaniae_DHED1_RNAseq/) and archived as DOI: 10.5281/zenodo.1245794. FASTQ files were aligned to the ATCC 42720 (4) genome with the splice-site aware and SNP tolerant short read aligner GSNAP (v 2017-11-15) (18). The alignments were converted to sorted BAM files and read counts computed

with featureCounts (19). Data is archived in Open Science Foundation (https://osf.io/yjwmg/). Genes that had less than one counts per million across all samples (absent genes) were not included for differentially expressed gene analysis. Using the remaining 5,741 genes, we performed differential expression analysis with the EdgeR (20) package in Bioconductor, by fitting a negative binomial linear model in R (15). The resulting P values were corrected for multiple testing with Benjamini-Hochberg to control the false discovery rate.

Quantitative RT-PCR

Overnight cultures were back diluted to an OD_{600} of ~0.1 and grown for 6 hours in $YNBG_{10}$. 7.5 µg RNA (harvested using the MasterPure Yeast RNA Purification Kit (Epicentre)) was DNAse treated with the Turbo DNA-free Kit (Invitrogen). cDNA was synthesized from 500 ng DNAse-treated RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific), following the manufacturer's instructions for random hexamer primer (IDT) and GC rich template. qRT-PCR was performed on a CFX96 Real-Time System (Bio-Rad), using SsoFast Evergreen Supermix (Bio-Rad) with the primers listed in Table S3. Thermocycler conditions were as follows: 95 ºC for 30 s, 40 cycles of 95 ºC for 5 s, 65 ºC for 3 s and 95 ºC for 5 s. Transcripts were normalized to *ACT1* expression.

Drug susceptibility assays

Minimum inhibitory concentration (MIC) was determined using a broth microdilution method as previously described (21) with slight modifications. $2x10^3$ cells were added to a two-fold dilution series of fluconazole prepared in RPMI-1640, starting at an initial concentration of 64 µg/ml, then incubated at 35 ºC for 24-48 hours. The MIC

was defined as the drug concentration that abolished visible growth compared to a drug-free control. No more than a 2-fold difference was observed between MICs recorded at 24 and 48 hours; data from the 24-hour timepoint was reported unless otherwise noted. For comparison to gene expression, FLZ MICs were repeated in $YNBG_{10}$; there was a strong correlation between FLZ MICs in RPMI-1640 and $YNBG_{10}$ (Fig. 6B). 2383 cultures were supplemented with 50 µg/ml uracil and L15 cultures were supplemented with 0.02% casamino acids when grown in $YNBG_{10}$; supplementation did not alter the FLZ MIC of other strains tested.

Growth of *C. lusitaniae* cultures in the presence and absence of 4 µg/ml FLZ were measured on a Synergy Neo microplate reader (Biotek, USA) in a kinetic assay. A starting concentration of ~1 x 10^4 cells in RPMI-1640 (2% dextrose) was incubated at 35 ºC for 18 hours. Following kinetic assay, cultures were plated on YPD +/- 8 µg/ml FLZ to enumerate CFUs. Isolates from the YPD control plates were further patched onto CHROMagar as necessary.

Drug susceptibility on plates was assessed using $YNBG_{100}$ supplemented with different concentrations of antifungals (Fig. S5). Susceptibility was defined as at least a 50% reduction in visible growth after 48h at 30 ºC. Antifungal stocks included: fluconazole (Sigma-Aldrich) at 4 mg/ml in DMSO, caspofungin (Sigma-Aldrich) at 15 mg/ml in dH_2O, Amphotericin B (Sigma-Aldrich) at 2 mg/ml in DMSO and cerulenin (Cayman Chemicals) at 20 mg/ml in DMSO.

Mating and progeny assessment

Cycloheximide resistant (chx^R) derivatives of the clinical isolates were selected for on YPD containing 10 µg/ml cycloheximide (A.G. Scientific, Inc) (22). The 5-FOA

resistant MATa strain 2383 was mated to chx^R derivatives of the MATα clinical isolates on SLAD medium (2% agar, 0.17% YNB without amino acids or ammonium sulfate, 2% glucose, and 50 µM ammonium sulfate) as previously described (22). In brief, following a PBS wash, an OD_{600} of 0.5 of each parental strain was combined and resuspended to a final volume of 1 mL in PBS. 5 µL of each mixture were incubated on SLAD plates at 30°C for 72 hours. Mating spots were scraped up and resuspended in 500 µl H_2O then 50 µl aliquots were plated on double selection medium (1.5% agar, 0.17% YNB without amino acids, 2% glucose, 10 µg/ml cycloheximide, 1 mg/ml 5-FOA, 50 µg/ml uracil) and incubated for 2-4 days at 30°C to select for products of meiosis that contained the resistance markers from both parental strains. Each parental strain alone was used as a control to assess the level of spontaneous resistance due to mutation on the double selection medium. The FLZ MIC and *MRR1* allele was determined for approximately thirty haploid progeny from each cross. *MRR1* allele was determined by amplification of a *MRR1* fragment with SNP specific primers which detected the presence or absence of the SNPs causing the Mrr1 variants H467L (ED062, ED063), L1191H+Q1197* (ED064, ED065) and Y813C (ED066, ED067).

Mutant construction

Mutants were constructed as previously described using an expression free ribonucleoprotein CRISPR-Cas9 method (23). Primers used to create knockout constructs and crRNA are listed in Table S3.

***In vitro* evolution**

Overnight cultures were washed with dH_2O and diluted into fresh $YNBG_{10}$ to an OD_{600} of 0.04. Cultures were grown at 30 ºC on a roller drum for 48 h, before passaging

into fresh $YNBG_{10}$ medium, inoculated to an OD_{600} of 0.04. Each passage yielded approximately 4-5 duplications. CFU were enumerated in triplicate after plating onto YPD agar containing DMSO (control), 4 µg/ml or 16 µg/ml FLZ and incubation at 30 ºC for 36 hours.

Histatin 5 (Hst 5) sensitivity assay

Hst 5 susceptibility was measured as previously described (24) with the following modifications. Briefly, 5 ml of YPD medium was inoculated and grown at room temperature overnight while shaking. Overnight cultures were back diluted into fresh YPD and grown for 4 hours, to reach an OD_{600} of ~1. Cells were washed twice with 10 mM sodium phosphate buffer (NaPB) at pH7.4. Approximately $6x10^3$ cells were incubated in NaPB (control) or NaPB containing 3.75 µM Hst 5 (GenScript) at 30 ºC for 1 hour. CFUs were enumerated after 24-48 hours growth on YPD agar plates. Assays were performed in triplicate for each strain. The percent survival was calculated as [number of colonies from Hst 5-treated cells/number of colonies from control cells] x 100%.

***Pseudomonas aeruginosa* zone of inhibition**

Overnight cultures of *C. lusitaniae* were adjusted to an OD_{600} of 0.1 in dH_2O and spread onto 20 ml YPD agar plates with a sterile swab (approximately $2x10^4$ cells per plate). Cells from overnight cultures of *P. aeruginosa* were resuspended in dH_2O to an OD_{600} of 1. 2 µl of the suspension was applied on top of agar that was inoculated with *C. lusitaniae*. Plates were incubated at 30 ºC for 48 hours before the zone of clearance or inhibition (inhibited *C. lusitaniae* growth) surrounding the *P. aeruginosa* colony was measured, in millimeters. Strains were measured in quintuplicate.

Statistical analyses

Statistical analyses were done using GraphPad Prism 6 (GraphPad Software). Unpaired Student's t-tests (two-tailed) with Welch's correction were used to evaluate the difference in FLZ MIC between mating progeny. One and two-way ANOVA tests were performed across multiple samples with either Tukey's multiple comparison test for unpaired analyses or Sidak's multiple comparison test for paired analyses conducted in a pairwise fashion, for MIC and expression data analysis. Pearson's and Spearman's correlation analyses were performed for comparison of FLZ MIC, *MDR1* expression and zone of inhibition. P values <0.05 were considered as significant for all analyses performed and are indicated with asterisks: *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Data availability

The data supporting the findings in this study are available within the paper and its supplemental information and are also available from the corresponding author upon request. The raw sequence reads from whole genome sequencing and RNA-Seq data have been deposited into NCBI sequence read archive under BioProject PRJNA433226 and SRP133092. Raw and processed RNA-Seq count data are available in Gene Expression Omnibus (GSE111421). Assemblies are deposited and associated with BioProject PRJNA433226 and under accessions QOBD00000000…QOBX00000000.

Code availability

Names of custom codes used for analysis are indicated in where appropriate in above methods. All codes and sequences are available in the indicated github repositories: analysis pipeline and scripts for whole genome sequencing, phylogeny and

CNV analysis are available at https://github.com/stajichlab/C_lusitaniae_popseq and RNA-Seq analysis pipeline and scripts at https://github.com/stajichlab/C_lusitaniae_DHED1_RNAseq. These are archived with Zenodo under DOI: 10.5281/zenodo.1346354 and DOI: 10.5281/zenodo.1245794.

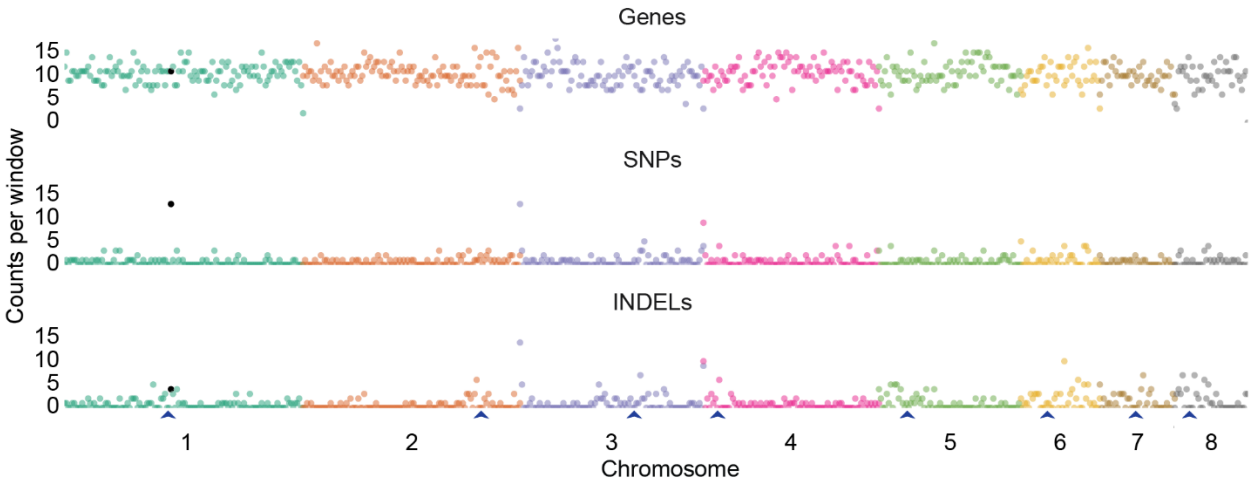


Fig. S1. Distribution of SNPs and INDELs. Number of genes, inter-isolate SNPs and inter-isolate INDELs within non-overlapping 20 kilobase windows for each nuclear chromosome (1-8), highlighted in different colors. Centromeres locations (25) denoted by blue arrowheads above chromosome numbers. The black circle denotes the 20 kilobase window on chromosome 1 that includes *MRR1* (1,094,487...1,098,284). For reference, the chromosome 1 centromere is located at 1,056,291…1,060,764 (25).

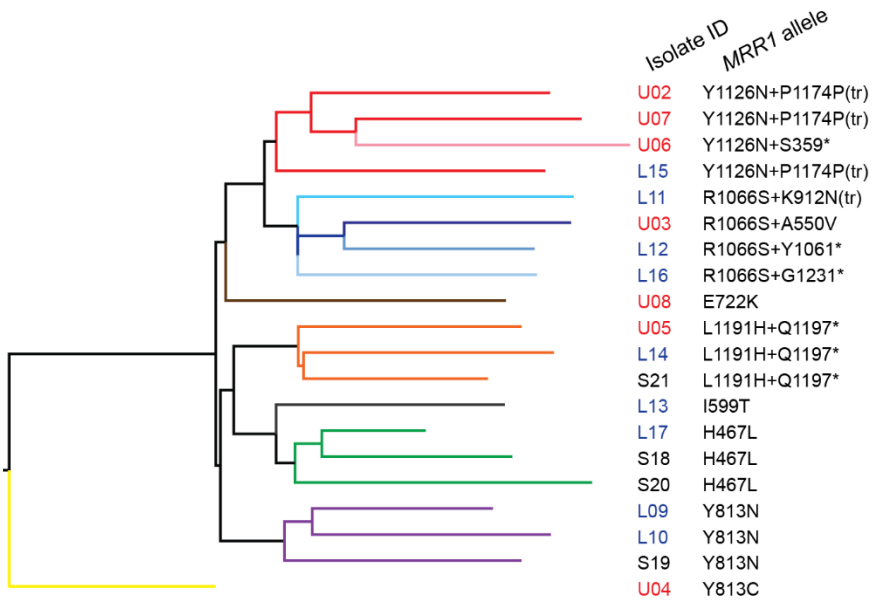
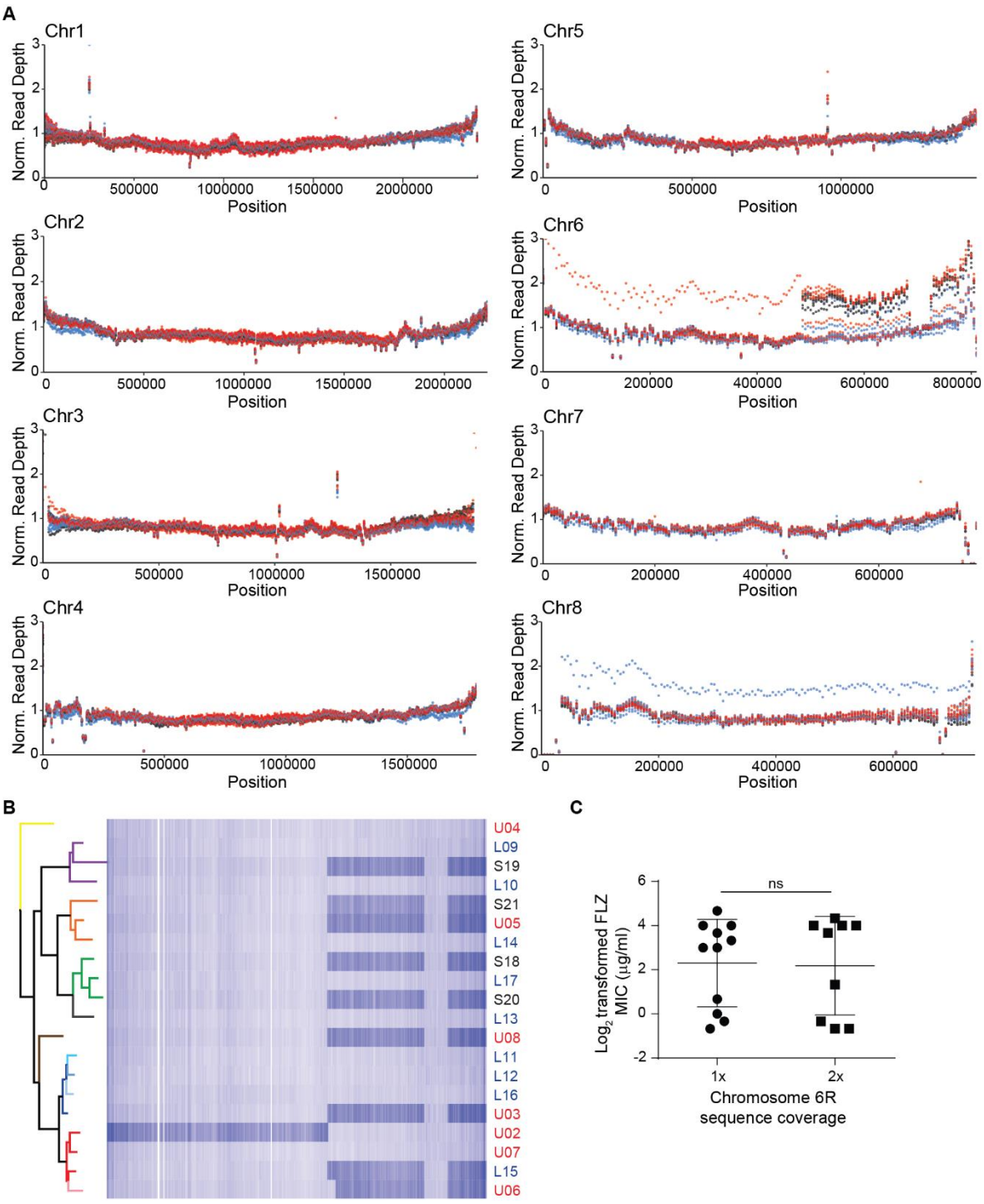


Fig. S2. Phylogenetic tree based on inter-isolate INDELs mirrors genomic relationships seen among SNPs. Maximum-likelihood tree based on the 538 inter-isolate INDELs which vary between the twenty clinical isolates. Branch arms are colored by *MRR1* allele, color scheme matches Fig. 1C. Sample names are color coded by sample of origin: UL BAL (red), LL BAL (blue), Sp1 (black).



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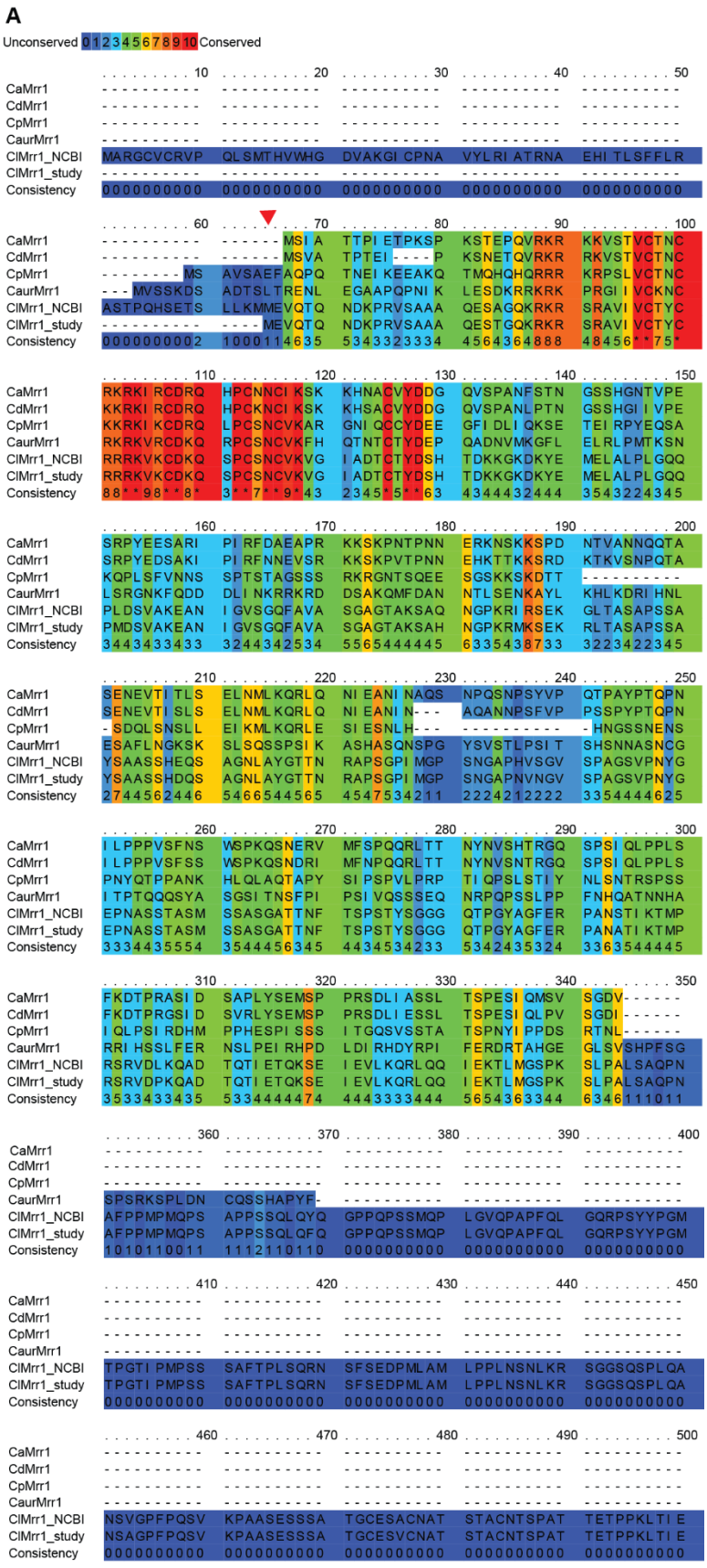
296 **Fig. S3. Copy number variation.** (*A*) Normalized read depth across each nuclear
297 chromosome (Chr 1-8) plotted within non-overlapping 5 kilobase windows. Isolates are
298 colored by sample from which they were isolated: UL (red), LL (blue) and Sp1 (black).
299 (*B*) Heatmap of read depth for chromosome 6 (increased coverage indicated by darker

blue color), clustered by maximum-likelihood tree based on inter-isolate SNPs, left, with branches colored by *MRR1* allele (Fig. 1C). Isolate identifiers, right, are colored by sample of origin, UL (red), LL (blue) and Sp1 (black). (*C*) Log_2 transformed FLZ MICs (μg/ml) of isolates in RPMI at 24 hours grouped by coverage of the right arm of chromosome 6. Mean ± s.d. from three independent replicates shown, ns = not significant, P=0.9018.


A
Unconserved 0 1 2 3 4 5 6 7 8 9 10 Conserved
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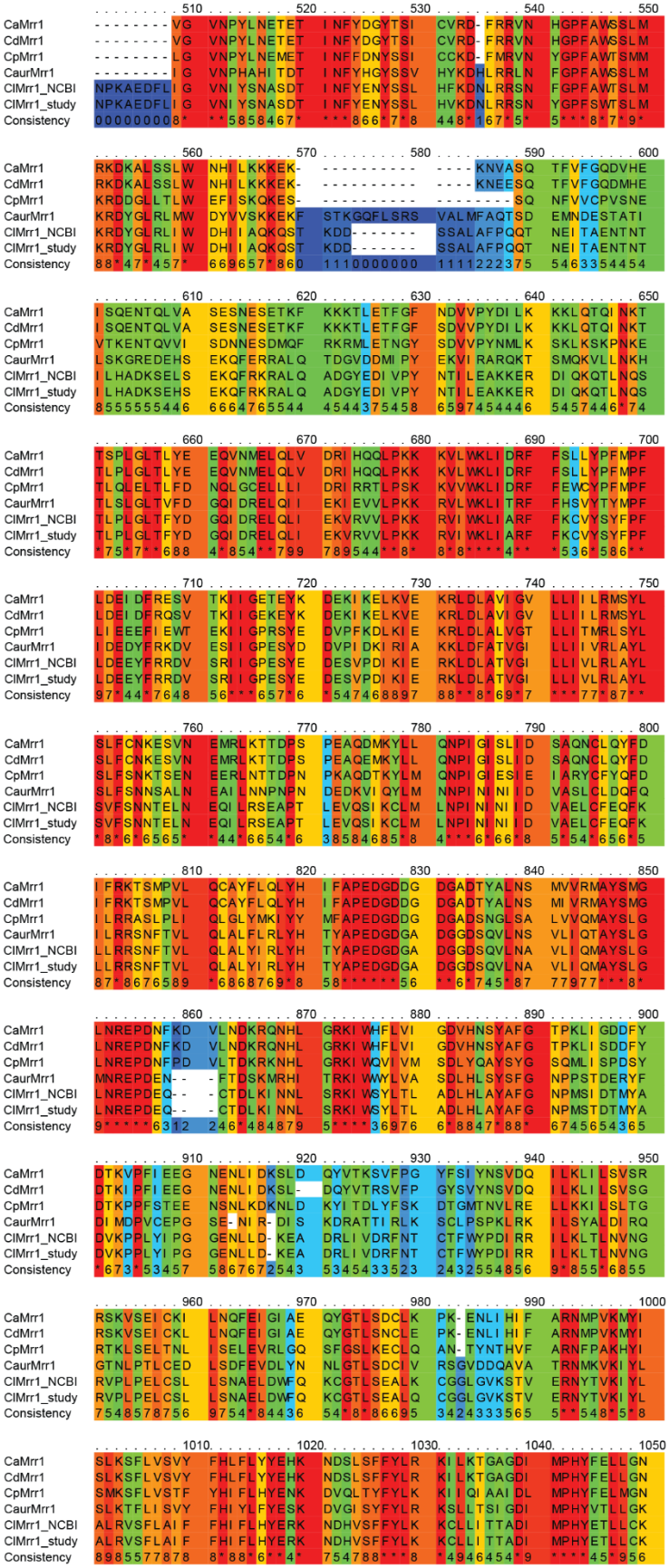
CdMrr1
CpMrr1
CaurMrr1
ClMrr1_NCBI
ClMrr1_study
Consistency


510 520 530 540 550
CaMrr1 --------VG VNPYLNETET INFYDGYTSI CVRD-FRRVN HGPFAWSSLM
CdMrr1 --------VG VNPYLNETET INFYDGYTSI CVRD-FRRVN HGPFAWSSLM
CpMrr1 --------LG VNPYLNEMET INFFDNYSSI CCKD-FMRVN YGPFAWTSMM
CaurMrr1 --------IG VNPHAHITDT INFYHGYSSV HYKDHLRRLN FGPFAWSSLM
ClMrr1_NCBI NPKAEDFLIG VNIYSNASDT INFYENYSSL HFKDNLRRSN FGPFAWTSLM
ClMrr1_study NPKAEDFLIG VNIYSNASDT INFYENYSSL HVKDNLRRSN YGPFSWTSLM
Consistency 000000008* **5858467* ***866*7*8 448*167*5* 5***8*7*9*
560 570 580 590 600
CaMrr1 RKDKALSSLW NHILKKKEK- ---------- ----KNVASQ TFVFGQDVHE
CdMrr1 RKDKALSSLW NHILKKKEK- ---------- ----KNEESQ TFVFGQDMHE
CpMrr1 KRDDGLLTLW EFISKQKES- ---------- --------SQ NFVVCPVSNE
CaurMrr1 KRDYGLRLMW DYVVSKKEKF STKGQQFLSRS VALMFAQTSD EMNDESTATI
ClMrr1_NCBI KRDYGLRLIW DHIIAQKQST KDD------- SSALAFPQQT NEITAENTNT
ClMrr1_study KRDYGLRLIW DHIIAQKQST KDD------- SSALAFPQQT NEITAENTNT
Consistency 88*47*457* 669657*860 1110000000 1111222375 5463354454
610 620 630 640 650
CaMrr1 ISQENTQLVA SESNESETKF KKKTLETFGF NDVVPYDILK KKLQTQINKT
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CpMrr1 VTKENTQVVI SDNNESDMQF RKRMLETNGY SDVVPYNMLK SKLKSKPNKE
CaurMrr1 LSKGREDEHS EKQFERRALQ TDGVDDMIPY EKVIRARQKT SMQKVLLNKH
ClMrr1_NCBI ILHADKSELS EKQFRKRALQ ADGYEDIVPY NTILEAKKER DIQKQTLNQS
ClMrr1_study ILHADKSEHS EKQFRKRALQ ADGYEDIVPY NTILEAKKER DIQKQTLNQS
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660 670 680 690 700
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ClMrr1_NCBI TLPLGLTFYD GQIDRELQLI EKVRVVLPKK RVIWKLIARF FKCVYSYFPF
ClMrr1_study TLPLGLTFYD GQIDRELQLI EKVRVVLPKK RVIWKLIARF FKCVYSYFPF
Consistency *75*7**688 4*854**799 789544**8* 8*8****4** *536*586**
710 720 730 740 750
CaMrr1 LDEIDFRESV TKIIGETEYK DEKIKELKVE KRLDLAVIGV LLIILRMSYL
CdMrr1 LDEIDFRQSV TKIIGEKEYK DEKIKELKVE KRLDLAVIGV LLIILRMSYL
CpMrr1 LIEEEFIEWT EKIIGPRSYE DVPFKDLKIE KRLDLALVGT LLITMRLSYL
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ClMrr1_NCBI LDEEYFRRDV SRIIGPESYE DESVPDIKIE RKLDLATVGI LLIVLRLAYL
ClMrr1_study LDEEYFRRDV SRIIGPESYE DESVPDIKIE RKLDLATVGI LLIVLRLAYL
Consistency 97*44*7648 56***657*6 *54746889 7 88**8*69*7 ***77*87**
760 770 780 790 800
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ClMrr1_NCBI SVFSNNTELN EQILRSEAPT LEVQSIKCLM LNPINININID VAELCFEQFK
ClMrr1_study SVFSNNTELN EQILRSEAPT LEVQSIKCLM LNPINININID VAELCFEQFK
Consistency *8*6*6565* *44*6654*6 38584685*8 4***6*66*8 5*54*656*5
810 820 830 840 850
CaMrr1 IFRKTSMPVL QCAYFLQLYH IFAPEDGDDG DGADTYALNS MVVRMAYSMG
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CpMrr1 ILRRASLPLI QLGLYMKIYY MFAPEDGDDG DGADSNGLSA LVVQMAYSLG
CaurMrr1 ILRRSNFTVL QLALFLRLYH TYAPEDGDGA DGGDSQVLNS VLIQTAYSLG
ClMrr1_NCBI LLRRSNFTVL QLALYIRLYH TYAPEDGDGA DGGDSQVLNA VLIQMAYSLG
ClMrr1_study LLRRSNFTVL QLALYIRLYH TYAPEDGDGA DGGDSQVLNA VLIQMAYSLG
Consistency 87*8676589 *6868769*8 58****** 56 **6*745*87 77977***8*
860 870 880 890 900
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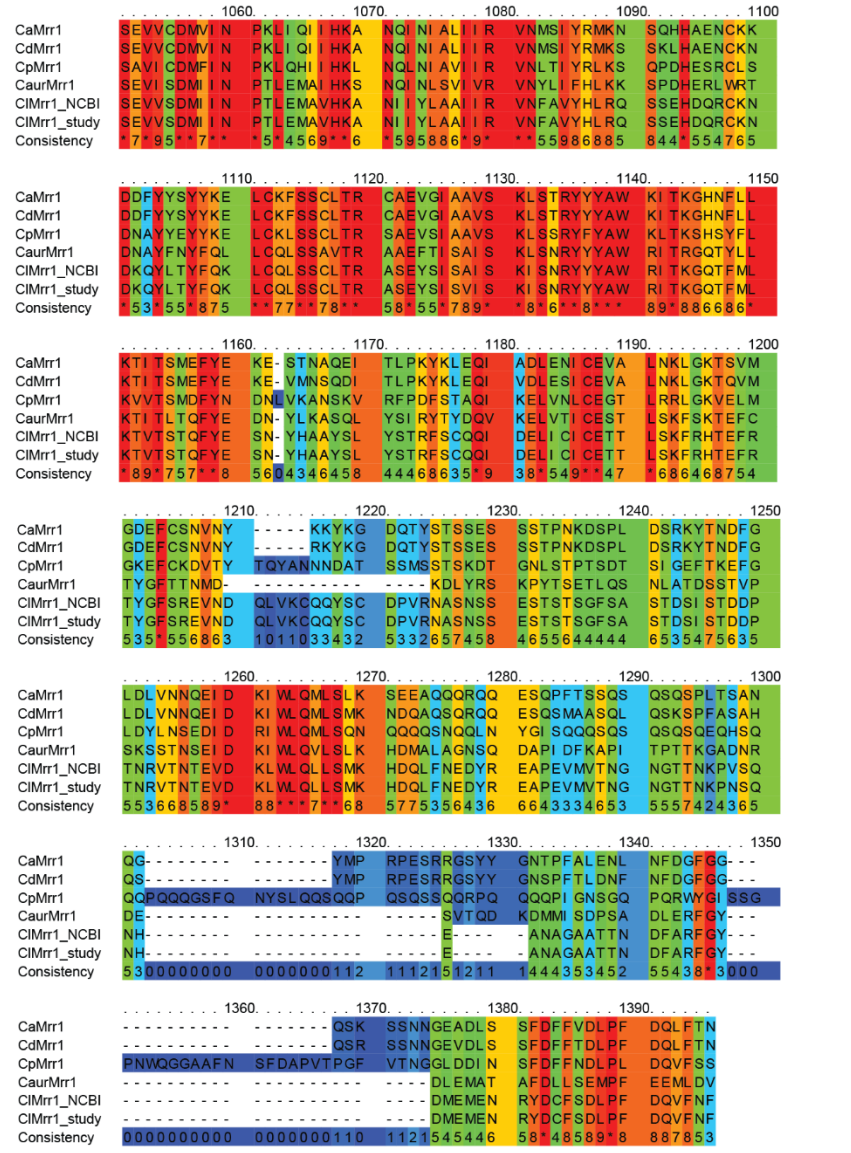
CpMrr1 LNREPDNFPD VLTDKRKNHL GRKIWQVIVM SDLYQAYSYG SQMLISPDSY
CaurMrr1 MNREPDEN-- -FTDSKMRHI TRKIWWYLVA SDLHLSYSFG NPPSTDERYF
ClMrr1_NCBI LNREPDEQ-- -CTDLKINNL SRKIWSYLTL ADLHLAYAFG NPMSIDTMYA
ClMrr1_study LNREPDEQ-- -CTDLKINNL SRKIWSYLTL ADLHLAYAFG NPMSTDTMYA
Consistency 9*****6312 246*484879 5****36976 6*8847*88* 6745654365
910 920 930 940 950
CaMrr1 DTKVPFIEEG NENLIDKSLD QYVTKSVFPG YFSIYNSVDQ ILKLILSVSR
CdMrr1 DTKIPFIEEG NENLIDKSL- DQYVTRSVFP GYSVYNSVDQ ILKLILSVSG
CpMrr1 DTKPPFSTEE NSNLKDKNLD KYITDLYFSK DTGMTNVLRE LLKKILSLTG
CaurMrr1 DIMDPVCEPG SE-NIR-DIS KDRATTIRLK SCLPSPKLRK ILSYALDIRQ
ClMrr1_NCBI DVKPPLYIPG GENLLD-KEA DRLIVDRFNT CTFWYPDIRR ILKLTLNVNG
ClMrr1_study DVKPPLYIPG GENLLD-KEA DRLIVDRFNT CTFWYPDIRR ILKLTLNVNG
Consistency *673*53457 5867672543 5345433523 2432554856 9*855*6855
960 970 980 990 1000
CaMrr1 RSKVSEICKI LNQFEIGIAE QYGTLSDCLK PK-ENLIHIF ARNMPVKMYI
CdMrr1 RSKVSEICKL LNQFEIGIAE QYGTLSNCLE PK-ENLIHIF ARNMPVKMYI
CpMrr1 RTKLSELTNL ISELEVRLGQ SFGSLKECLQ AN-TYNTHVF ARNFPAKHYI
CaurMrr1 GTNLPTLCED LSDFEVDLYN NLGTLSDCIV RSGVDDQAVA TRNMKVKIYL
ClMrr1_NCBI RVPLPELCSL LSNAELDWFQ KCGTLSEALK CGGLGVKSTV ERNYTVKIYL
ClMrr1_study RVPLPELCSL LSNAELDWFQ KCGTLSEALQ CGGLGVKSTV ERNYTVKIYL
Consistency 7548578756 9754*84436 54*8*86695 3424333565 5**548*5*8
1010 1020 1030 1040 1050
CaMrr1 SLKSFLVSVY FHLFLYYEHK NDSLSFFYLR KILKTGAGDI MPHYFELLGN
CdMrr1 SLKSFLVSVY FHLFLYYEHK NDSLSFFYLR KILKTGAGDI MPHYFELLGN
CpMrr1 SMKSFLVSTF YHIFLHYENK DVQLTYFYLK KIIQIAAIDL MPHYFELMGN
CaurMrr1 SLKTFLISVY FHIYLFYESK DVGISYFYLR KSLLTSIGDI MPHYVTLLGK
ClMrr1_NCBI ALRVSFLAIF FHIFLHYERK NDHVSFFYLK KCLLITTADI MPHYETLLCK
ClMrr1_study ALRVSFLAIF FHIFLHYERK NDHVSFFYLK KCLLITTADI MPHYETLLCK
Consistency 8985577878 8*88*6***4* 754788***8 *4946454*9 ****45*956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CdMrr1
CpMrr1
CaurMrr1
ClMrr1_NCBI
ClMrr1_study
Consistency
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1210 1220 1230 1240 1250
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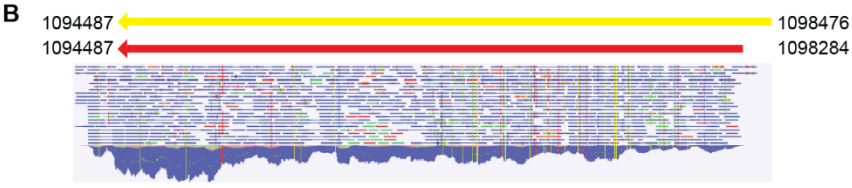
1360 1370 1380 1390


B
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1098476
1094487
1098284

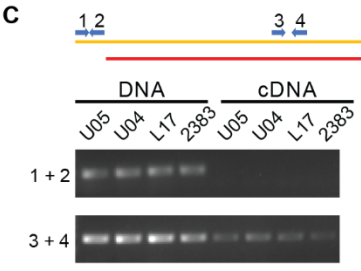
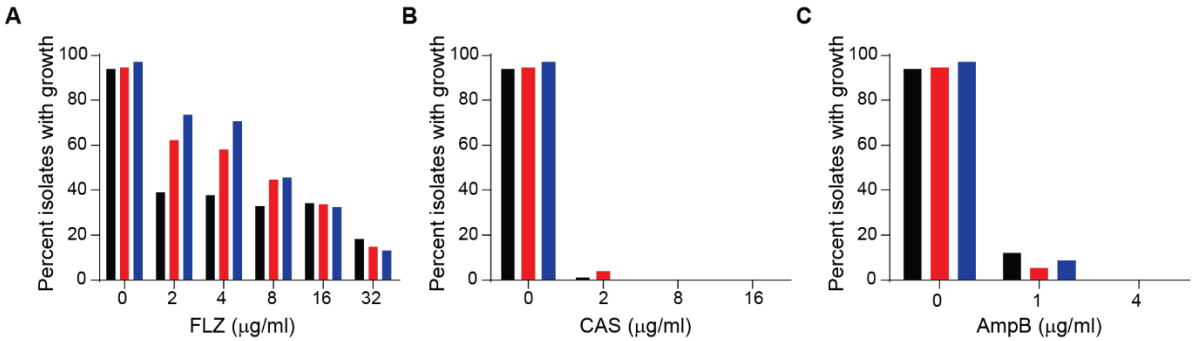

C
1 2
3 4
DNA
cDNA
U05
U04
L17
2383
1 + 2
3 + 4

Fig. S4. Reannotation of *CLUG_00542 as ClMRR1.* We propose that the start codon of *ClMRR1* is currently misannotated in NCBI based on the following evidence and utilized M65 as the start codon (M1) when notating amino acid mutations sites. (*A*) Amino acid sequence alignment of Mrr1 orthologs from *C. albicans* (Ca)*, C. dubliniensis* (Cd)*, C. parapsilosis* (Cp)*, C. auris* (Caur) and *C. lusitaniae* (ClMrr1_NCBI*,* from NCBI database, and ClMrr1_study, annotated with an alternate start codon (M65) and adjusted to contain SNPs shared by all twenty sequenced isolates in this study). Amino acids are colored by sequence conservation between homologs (consistency score), with increased conservation from cool (dark blue) to warm (red) colors, calculated by PRALINE (16). There is no sequence homology between the first 64 amino acids of the ClMrr1_NCBI sequence and that of other *Candida* species. ClMrr1 M65, the corrected start codon, is marked by a red triangle. (*B*) Alignment of RNA-Seq read coverage for *MRR1* from U04 (*MRR1*^{Y813C}). Minimal reads were present to support the current start codon as annotated in NCBI (yellow arrow), whereas read coverage increases around M65 (red arrow), the proposed corrected start codon. (*C*) Schematic of primers to amplify *MRR1*, top, and gel image of PCR products obtained using primer set 1+2 (within disputed 5' region, ED_068 and ED_069) and 3+4 (within gene, ED_070 and ED_071) with DNA or cDNA (representing transcribed mRNA sequence), bottom. No product was obtained from primers 1+2 when using a cDNA template, suggesting this fragment of DNA was not transcribed as part of the *MRR1* mRNA.



329

330 **Fig. S5. Resistance to FLZ, but not other antifungals, was heterogeneous.**

331 Percentage of Sp1 (black, n=82), UL (red, n=74), LL (blue, n=68) isolates capable of

332 growth on increasing concentrations of (*A*) fluconazole (FLZ), (*B*) caspofungin (CAS)

333 and (*C*) amphotericin B (AmpB). Representative data shown, growth on agar plates

334 repeated twice. Presence or absence of growth in FLZ matched what was observed for

335 FLZ MICs.

336

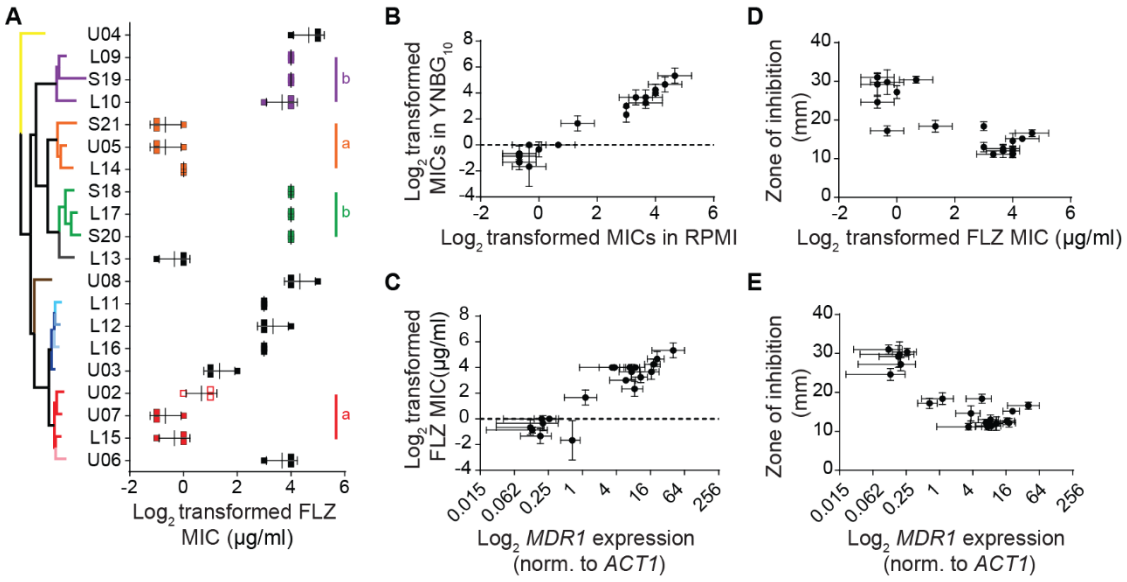


Fig. S6. *MRR1* allele correlates with *MDR1* expression and FLZ and phenazine resistance. Comparisons between the twenty sequenced clinical *C. lusitaniae* isolates. (*A*) Log_2 transformed FLZ MIC (μg/ml) measured in RPMI medium at 24 hours, clustered by maximum-likelihood tree based on inter-isolate SNPs, left, with branches colored by *MRR1* allele (Fig. 1C). Data points for isolates with the same *MRR1* allele are colored the same, a-b $P<0.0001$. Isolate U02, which has a 6L duplication (Fig. S3B), is represented by open symbols. (*B*) Comparison of log_2 transformed FLZ MICs (μg/ml), measured at 24 hours, in RPMI and $YNBG_{10}$ media, Pearson correlation coefficient $r = 0.9677$, $P<0.0001$. (*C*) Comparison of Log_2 transformed FLZ MIC (μg/ml), measured in $YNBG_{10}$ at 24 hours, to *MDR1* expression, normalized to *ACT1* levels, of cells grown in $YNBG_{10}$ for 6 hours. Spearman correlation coefficient $\rho = 0.8414$, $P<0.0001$. (D and E) Comparison of the zone of inhibition around colonies of *P. aeruginosa* strain PA14 on individual *C. lusitaniae* lawns to log_2 transformed FLZ MIC (μg/ml), measured in RPMI medium at 24 hours, Pearson correlation coefficient $r = -0.7563$, $P<0.0001$ (D), and *MDR1* expression, Spearman correlation coefficient $\rho = -0.7868$, $P<0.0001$ (E). Mean ± s.d. for data from three independent replicates of gene expression and FLZ MIC and representative data from five technical replicates for the zone of inhibition (repeated independently three times) is shown for all experiments.

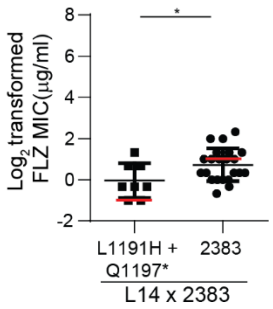


Fig. S7. Mating does not alter FLZ MIC. Log_2 transformed FLZ MICs (µg/ml) for progeny (n=30) obtained by mating the FLZ^S strain 2383 (*MRR1*^{2383}) to the FLZ^S clinical isolates L14 (chx^R, *MRR1*$^{L1191H+Q1197*}$); grouped by *MRR1* allele. Red lines indicate the mean FLZ MIC for the parental strain for each *MRR1* allele. Mean ± s.d. of data from three independent replicates is shown, *P<0.05.

A

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CaMdr1          MHYRFLRDSFVGRVTYHLSKHKYFAHPEEAKDYIVPEKYLADYKPTLADDTSINFEKEEI 60
ClMdr1          MLSKFVRESFFGRLLYHATQHKLFGYAEEKPGYVIPEKYLPGNSVESTDSLD--KLK--- 55
Clug_01938      MLSKFVRESFFGRLLYHATQHKLFGYAEEKPGYVIPEKYLPGNSVESTDSLD--KLK--- 55
Clug_01939      ------------------------------------------------------------ 0

CaMdr1          DNQGEPNSSQSSSSNNTIVDNNNNNDNDVDGDKIVVTWDGDDDPENPQNWPTLQKAFFIF 120
ClMdr1          ---EEPRASLSS--N-----ESSEKSLKKEDDLIIVGWDGEDDPENPYNWPFFYKILFIF 105
Clug_01938      ---EEPRASLSS--N-----ESLEKSLKKEDDLIIVGWDGEDDPENPYNWPFFYKILFIF 105
Clug_01939      ------------------------------------------------------------ 0

CaMdr1          QISFLTTSVYMGSAVYTPGIEELMHDFGIGRVVATLPLTLFVIGYGVGPLVFSPMSENAI 180
ClMdr1          EIGILTAFVYMASAIYTPGVDEIMEKMNINQTLATLPLTMFVFGYGIGPMVFSPMSENAR 165
Clug_01938      EIGILTAFVYMASAIYTPGVDEIMEKMNINQTLATLPLTMFVFGYGIGPMVFSPMSENAR 165
Clug_01939      ------------------------------------------------------------ 0

CaMdr1          FGRTSIYIITLFLFVILQIPTALVNNIAGLCILRFLGGFFASPCLATGGASVADVVKFWN 240
ClMdr1          FGRTSIYIITLFIFFILQIPTALVTDITSLCVLRFIAGFFASPCLATGGASVGDVTAMPY 225
Clug_01938      FGRTSIYIITLFIFFILQIPTALVTDITSLCVLRFIAGFFASPCLATGGASVGDVTAMPY 225
Clug_01939      ------------------------------------------------------------ 0

CaMdr1          LPVGLAAWSLGAVCGPSFGPFFGSILTVKASWRWTFWFMCIISGFSFVMLCFTLPETFGK 300
ClMdr1          IPVSISAWSIAAVCAPSMGPLFGSILVVKGNYHWTFWFVCITSGCAFLVLSWFLPESYGK 285
Clug_01938      IPVSISAWSIAAVCAPSMGPLFGSILVVKGNYHWTFWFVCITSGCAFLVLSWFLPESYRE 285
Clug_01939      ------------------------------------------------------------ 0

CaMdr1          TLLYRKAKRLRAITGNDRITSEGEVENSKMTSHELIIDTLWRPLEITVMEPVVLLINIYI 360
ClMdr1          TILYRKAERLRKLTGNDKITSEGHIENSKMEVHEMAVDILWRPFELIIFEPVVLLINIYI 345
Clug_01938      DHLVQKSREIEKVDWQR---------------------------------------------- 302
Clug_01939      ------------------------------------------------------------ 0

CaMdr1          AMVYSILYLFFEVFPIYFVGVKHFTLVELGTTYMSIVIGIVIAAFIYIPVIRQKFTKPIL 420
ClMdr1          GLVYSIMYTWFEAFPIVFLEIHHFTLIEMGVSYVALMIGIMIGAAFFIPFIYRRFTKKLL 405
Clug_01938      ------------------------------------------------------------ 302
Clug_01939      ----------------------------MGVSYVALMIGIMIGAAFFIPFIYRRFTKKLL  32

CaMdr1          RQEQVFPEVFIPIAIVGGILLTSGLFIFGWSANRTTHWVGPLFGAATTASGAFLIFQTLF 480
ClMdr1          VGEQVQPEVFLPMTILGSILMPIGIFIFGWTSAPDIHWIAPMIGTAVFAAGAFIVFQTLF 465
Clug_01938      ------------------------------------------------------------ 302
Clug_01939      VGEQVQPEVFLPMTILGSILMPIGIFIFGWTSAPDIHWIAPMIGTAVFAAGAFIVFQTLF  92

CaMdr1          NFMGASFKPHYIASVFASNDLFRSVIASVFPLFGAPLFDNLATPEYPVAWGSSVLGFITL 540
ClMdr1          NYLSMSFW-RYLASVFAGNDLFRSIMAGAFPLFGRALFINLKNERFRVGWGSTVLACLCV 524
Clug_01938      ------------------------------------------------------------ 302
Clug_01939      NYLSMSFW-RYLASVFAGNDLFRSIMAGAFPLFGRALFINLKNERFRVGWGSTVLACLCV 151

CaMdr1          VMIAIPVLFYLNGPKLRARSKYAN-  564
ClMdr1          VMVAIPVLFYLNGPKLRARSKYSGF  549
Clug_01938      -------------------------  302
Clug_01939      VMVAIPVLFYLNGPKLRARSKYSGF  176
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B

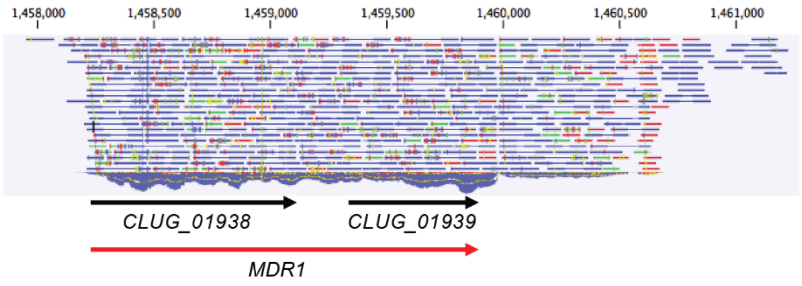

Fig. S8. Reannotation of *CLUG_01938/01939 as ClMDR1.* We propose that *MDR1* is misannotated in NCBI as two genes, *CLUG_01938* and *CLUG_01939*, based on the following evidence. (*A*) Alignment, using Clustal Omega, of amino acid sequences for Mdr1 from *C. albicans* (Ca*,* first) and *C. lusitaniae* (Cl) including our proposed reannotation of ClMdr1 (second) and Clug_01938 (third) and Clug_01939 (fourth) from NCBI. Blue arrows indicate the location of two codons effected by single nucleotide insertions in the ATCC 42720 reference genome available from NCBI which were not present in our resequencing of ATCC 42720 or any of the clinical isolates. Deletion of these nucleotides from the sequence caused a frameshift, elongating the open reading frame of *CLUG_01938* to include *CLUG_01939*. (*B*) Read coverage plot for *MDR1* from RNA-Seq data*,* showing the positions of *CLUG_01938/01939,* black arrows, compared to the proposed corrected *MDR1* sequence, red arrow. Reads are present throughout this entire region, suggesting a continuous transcript rather than two independent transcripts.

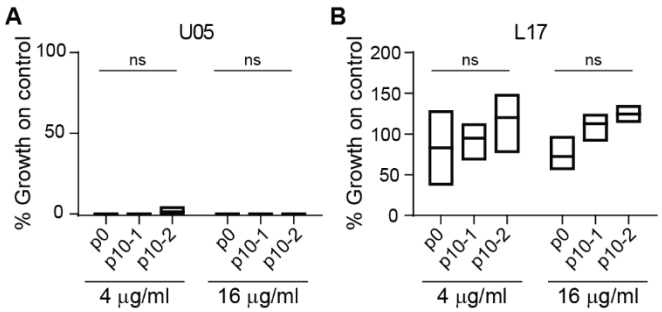

Fig. S9. FLZ resistance profiles are stable following *in vitro* evolution. In duplicate, the (*A*) FLZ^{S} U05 (*MRR1*$^{L1191H+Q1197*}$) and (*B*) FLZ^{R} L17 (*MRR1*^{H467L}) isolates were passaged 10 times in YNBG$_{10}$, accumulating 40+ generations. CFUs for the parental isolate, p0, and evolved isolates, p10, were enumerated (in triplicate) in the presence/absence of either 4 or 16 μg/ml FLZ. Growth on FLZ was normalized to growth on the vehicle only control. ns = not significant.

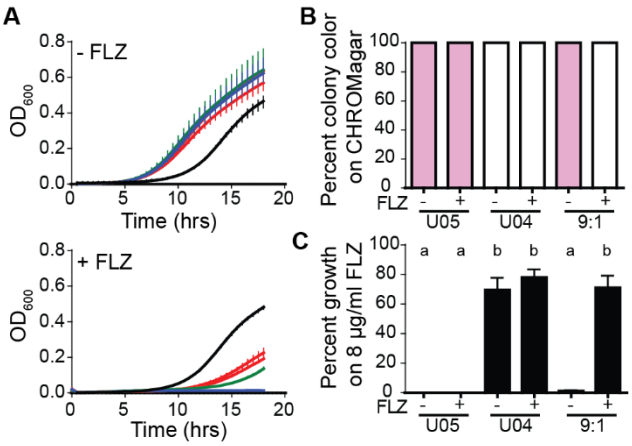

Figure S10. FLZ^{R} subpopulations overtake the population following FLZ exposure. (*A*) Growth in the absence (-) and presence (+) of 4 µg/ml FLZ in RPMI over 18 hours. Mean ± s.d. of three technical replicates is shown for FLZ^{R} U04 (*MRR1*^{Y813C}, black), FLZ^{S} U05 (*MRR1*$^{L1191H+Q1197*}$, blue), a 9:1 mixture of U05:U04 (green) and two replicates of a complex mixture of all UL isolates (n=72, red). Similar results were seen by MIC analysis, see Fig. 4D. (*B*) Comparison of colony color on CHROMagar Candida medium. Following the kinetic assay described in Fig. S10A, cultures were plated on YPD. 10-20 colonies were patched on CHROMagar Candida medium and incubated at 30 ºC for 48 hours. Bar color, pink (U05) and white (U04), represent colony color on CHROMagar Candida medium at 48 hours. (*C*) CFUs were enumerated on YPD +/- 8 µg/ml FLZ plates, after growth as described in Fig. S10A. The CFUs on 8 µg/ml FLZ are plotted as a percent of total CFUs on YPD alone. Mean ± s.d. of three technical replicates is shown, similar results observed on three independent days, a-b, P<0.0001.

Table S1. Differentially expressed genes between FLZ^S and FLZ^R clinical isolates.

Gene	logFC	logCPM	PValue	*C. albicans* ortholog	Annotated features
CLUG_02157	9.1	9.6	3.6E-27		Has domain(s) with predicted zinc ion binding, nucleotide binding, oxidoreductase activity and role in oxidation-reduction process
CLUG_02156	8.6	10.7	2.8E-25		Protein of unknown function
CLUG_02858	6.7	6.7	2.9E-05	*CHA1*	Ortholog(s) have role in filamentous growth
*CLUG_01938**	6.6	8.7	2.1E-22	*MDR1*	Ortholog(s) have fluconazole transporter, drug transmembrane transporter activity, role in fluconazole transport, drug transmembrane transport, drug export, cellular response to drug, pathogenesis, cellular response to oxidative stress
*CLUG_01939**	6.5	8.2	1.4E-20		Protein of unknown function
CLUG_03199	3.9	8.4	5.0E-13		Has domain(s) with predicted nucleotide binding, zinc ion binding, oxidoreductase activity and role in oxidation-reduction process
CLUG_04991	3.9	5.3	1.5E-16		Has domain(s) with predicted catalytic, coenzyme binding, nucleotide binding activity and role in cellular metabolic process
CLUG_03198	3.6	4.2	1.1E-16		Has domain(s) with predicted zinc ion binding, nucleotide binding, oxidoreductase activity and role in oxidation-reduction process
CLUG_01281	3.4	10.7	4.0E-09		Has domain(s) with predicted catalytic, coenzyme binding, nucleotide binding activity and role in cellular metabolic process
CLUG_01282	3.3	8.5	8.6E-12		Has domain(s) with predicted FMN binding, electron carrier, oxidoreductase activity
CLUG_05005	2.6	7.3	4.8E-12		Has domain(s) with predicted hydrolase activity
CLUG_02968	2.5	9.9	1.8E-11	*orf19.7306*	Protein of unknown function
CLUG_00507	2.5	1.4	1.6E-04	*GIT1*	Ortholog(s) have glycerophosphodiester transmembrane transporter activity and role in glycerophosphodiester transport
CLUG_04429	1.7	7.5	1.0E-10		Protein of unknown function
CLUG_05825	1.6	6.7	3.2E-07	*FLU1*	Ortholog(s) have drug transmembrane transporter activity, role in drug transmembrane transport, cellular response to biotic stimulus, peptide transport, spermidine transport, fluconazole transport and plasma membrane localization
CLUG_01393	1.6	5.3	6.5E-07		Has domain(s) with predicted catalytic, coenzyme binding, nucleotide binding activity and role in cellular metabolic process
CLUG_03295	1.3	4.7	6.4E-05	*PTH2*	Protein of unknown function
CLUG_03201	-1.1	5.4	9.1E-05		Protein of unknown function
CLUG_02758	-1.4	4.7	3.3E-05	*OPT2*	Ortholog(s) have oligopeptide transmembrane transporter activity and role in nitrogen utilization, oligopeptide transmembrane transport
CLUG_05131	-1.9	8.2	7.4E-09	*PEX5*	Ortholog(s) have role in fatty acid beta-

	oxidation, protein import into peroxisome matrix

CLUG_01938 and *CLUG_01939* are misannotated and represent one gene, see Fig. S8

Table S2. Strains used in this study.

Strain	Lab #	Relevant genotype	Parental isolate	Source
C. lusitaniae				
U02	DH3097	MATα, *MRR1*$^{Y1126N+P1174P(tr)}$		Dartmouth Hitchcock Medical Center
U03	DH3096	MATα, *MRR1*$^{R1066S+A550V}$		Dartmouth Hitchcock Medical Center
U04 (A04)	DH2949	MATα, *MRR1*^{Y813C}		Dartmouth Hitchcock Medical Center and (23)
U05	DH3087	MATα, *MRR1*$^{L1191H+Q1197*}$		Dartmouth Hitchcock Medical Center
U06	DH3100	MATα, *MRR1*$^{Y1126N+S359*}$		Dartmouth Hitchcock Medical Center
U07	DH3098	MATα, *MRR1*$^{Y1126N+P1174P(tr)}$		Dartmouth Hitchcock Medical Center
U08	DH3105	MATα, *MRR1*^{E722K}		Dartmouth Hitchcock Medical Center
L09	DH3090	MATα, *MRR1*^{Y813N}		Dartmouth Hitchcock Medical Center
L10	DH3091	MATα, *MRR1*^{Y813N}		Dartmouth Hitchcock Medical Center
L11	DH3093	MATα, *MRR1*$^{R1066S+K912N(tr)}$		Dartmouth Hitchcock Medical Center
L12	DH3094	MATα, *MRR1*$^{R1066S+Y1061*}$		Dartmouth Hitchcock Medical Center
L13	DH3104	MATα, *MRR*^{I599T}		Dartmouth Hitchcock Medical Center
L14	DH3088	MATα, *MRR1*$^{L1191H+Q1197*}$		Dartmouth Hitchcock Medical Center
L15	DH3099	MATα, *MRR1*$^{Y1126N+P1174P(tr)}$		Dartmouth Hitchcock Medical Center
L16	DH3095	MATα, *MRR1*$^{R1066S+G1231*}$		Dartmouth Hitchcock Medical Center
L17	DH3101	MATα, *MRR1*^{H467L}		Dartmouth Hitchcock Medical Center
S18	DH3102	MATα, *MRR1*^{H467L}		Dartmouth Hitchcock Medical Center
S19	DH3092	MATα, *MRR1*^{Y813N}		Dartmouth Hitchcock Medical Center
S20	DH3103	MATα, *MRR1*^{H467L}		Dartmouth Hitchcock Medical Center
S21	DH3089	MATα,		Dartmouth Hitchcock

		MRR1$^{L1191H+Q1197*}$		Medical Center
U04 chx^{R}	DH3106	chx^{R}, MATα, *MRR1*^{Y813C}	U04	This study
L17 chx^{R}	DH3107	chx^{R}, MATα, *MRR1*^{H467L}	L17	This study
L14 chx^{R}	DH3108	chx^{R}, MATα, *MRR1*$^{L1191H+Q1197*}$	L14	This study
U04 *mrr1Δ #4*	DH3306	MATα, *mrr1Δ:NAT1*	U04	This study
U04 *mrr1Δ #5*	DH3307	MATα, *mrr1Δ*:NAT1	U04	This study
U04 *mdr1Δ*	DH3112	MATα, *MRR1*^{Y813C}, *mdr1Δ*:NAT1	U04	This study
U05 *mdr1Δ*	DH3114	MATα, *MRR1*$^{L1191H+Q1197*}$, *mdr1Δ:NAT1*	U05	This study
ATCC 42720	DH2387	MATα		(4)
2383	DH2383 (RSY284/CL6936)	MATa, *ura3Δ*		(26)
P. aeruginosa				
PA14	DH122	Wild type		(27)
Δ*phz*	DH933	PA14 with deletion of *phzA1-G1* and *phzA2-G2* operons	PA14	(28)

406
407

408 **Table S3.** Primers used in this study.

Name	Description	Sequence
AB001	Forward to make left flank of knockout construct for *MRR1*	AAG GCG TGT CCT TCA TGT T
AB003	Reverse to make left flank of knockout construct for *MRR1*	AACGTCGTGACTGGGAAAAAT CATTAGCT TCG CTG GAA TTT CTG TTT
AB004	Forward to make right flank of knockout construct for *MRR1*	TAT CCG CTC ACA ATT CCA CTG CTC GGT TCT GGT TCT ATA TG
AB006	Reverse to make right flank of knockout construct for *MRR1*	GAG TAC GTG GAT CTC TAC TTG ATG
AB007	Nested forward to amplify across stitched *MRR1* knockout construct	CTTTGCTTGTTTGGGAAACCTC
AB008	Nested reverse to amplify across stitched *MRR1* knockout construct	TTTCCGGGTTCAATGCCA
AB009	Forward to amplify *NAT1* for *MRR1* knockout construct	AAACAGAAATTCCAGCGAAGCTA ATGATTTTTCCCAGTCACGACGTT
AB010	Reverse to amplify *NAT1* for *MRR1* knockout construct	CATATAGAACCAGAACCGAGCAG TGGAATTGTGAGCGGATA
ED038	Forward to make left flank of knockout construct for *MDR1*	CAGTAGTGTGTTCGTCTCCTTAG
ED039	Reverse to make left flank of knockout construct for *MDR1*	AACGTCGTGACTGGGAAAAATCATTA GCGATTAGGTATTAGATGGATGTTTG
ED042	Nested forward to amplify across stitched *MDR1* knockout construct	CGGCGGAGTTATATCCGTTTC
ED043	Nested reverse to amplify across stitched *MDR1* knockout construct	GGCTTCCGTATTTAAGCTGTACT
ED044	Forward to amplify *NAT1* for *MDR1* knockout construct	CAAACATCCATCTAATACCTAAT CGCTAATGATTTTTCCCAGTCACGACGTT
ED046	Forward to amplify right flank of *MDR1* knockout construct	TAT CCG CTC ACA ATT CCA C GAG TTCACAAGGTAATTGTTCAGG
ED048	Reverse to amplify right flank of *MDR1* knockout construct	CCGACCCTCCCATTCAATC
ED049	Reverse to amplify *NAT1* for *MDR1* knockout construct	CCTGAACAATTACCTTGTGAACT CGTGGAATTGTGAGCGGATA
ED050	Forward upstream of *MRR1* to amplify for sequencing	GTGAACACAATACAATTGAAGAGAAGTC
ED051	Reverse downstream of *MRR1* to amplify for sequencing	GCATCTCCTAATTCGATATTTCATGACT
ED052	Sequencing primer for *MRR1* – region 1	CTGGCTTTGAGCGGCCGGC
ED053	Sequencing primer for *MRR1* – region 2	GGATTTCTTGATTGGCG
ED054	Sequencing primer for *MRR1* – region 3	CAGACGTTGAACCAATC
ED055	Sequencing primer for *MRR1* – region 4	CTTGAAGTGCAGAGTATC
ED056	Sequencing primer for *MRR1* – region 5	GCAATACTTGACATACTTCC
ED057	Sequencing primer for *MRR1* – region 6	GTACTGATTCTATCTCCACC
ED058	Forward for RT-PCR of M*DR1*	TCCATCCATGGGTCCATTATTC
ED059	Reverse for RT-PCR of M*DR1*	CTCAACACAAGGAAAGCACATC
ED060	Forward for RT-PCR of *ACT1*	GGTAGAGACTTGACCGACTACTT
ED061	Reverse for RT-PCR of *ACT1*	CCTTGATGTCACGGACGATTT
ED062	Forward for identification of the SNP	GAAAACTACTCCTCTCTCCT

	causing Mrr1-H467L in progeny	
ED063	Reverse for identification of the SNP causing Mrr1-H467L in progeny	GGAAGTATGTCAAGTATTGC
ED064	Forward for the identification of the SNP causing Mrr1-L1191H+Q1197* in progeny	GGACGAGGAATACTTTAGGC
ED065	Reverse for the identification of the SNP causing Mrr1-L1191H+Q1197* in progeny	GCTAATCATGCTTCATCGAAT
ED066	Forward for the identification of the SNP causing Mrr1-Y813C in progeny	GATCCAAAGCAGGCAGC
ED067	Reverse for the identification of the SNP causing Mrr1-Y813C in progeny	CCAGGCAAGATGAAGATCTG
ED068	Forward for the validation of the annotated 5' region of *MRR1*	CGATTTGCCCAAATGCAGTATATC
ED069	Reverse for the validation of the annotated 5' region of *MRR1*	GGAGTTGAAGCACGGAGAAA
ED070	Forward internal primers for *MRR1*, paired with ED068-69	GACGAGGAATACTTTAGGC
ED071	Reverse internal primers for *MRR1*, paired with ED068-69	GCAAGATTTGCTCGTTTAGC
MDR1 crRNA	crRNA for *MDR1*	AGTCCTTGCTTGGCCACAGG
MRR1 crRNA	crRNA for *MRR1*	TTCATCACTAAAGATGATGG

Dataset S1. Positions and predicted effects of biallelic SNPs.

Dataset S2. Positions and predicted effects of INDELs.

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