SI Methods

Strains, media, and oligonucleotides used in this study. All genetic work was performed in a *MAT***a** *ku80-Δ* NHEJ-deficient background derived from *K. lactis* CBS2359 (1, 2) and in a *MAT***a** *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* background of *S. cerevisiae* (BY4741) (3). Other yeast species used were obtained from the ARS Culture Collection (NRRL) in Peoria, IL, including *K. marxianus* Y-8281, *K. nonfermentans* Y-27343, *Ko. pastoris* Y-1603, *Ko. pseudopastoris* Y-27603, and *Lachancea waltii* Y-8285. *Lachancea thermotolerans* Y-8284 was provided by Gilles Fischer. For growth experiments in *S. cerevisiae* using BY4741-derived strains, a synthetic complete (SC) defined medium lacking uracil was used to maintain the pRS426 vector and its derivatives. The basic growth medium contained 5g/L ammonium sulfate, 1.72g/L yeast nitrogen base, 2g/L synthetic drop-out mix minus uracil, and 20g/L glucose (all reagents from US Biological Life Sciences). The same medium was used for *K. lactis* growth experiments, except the drop-out mix contained uracil. The rich, undefined YPD medium contained 10g/L yeast extract, 20g/L peptone, and 20g/L glucose (all reagents from US Biological Life Sciences). A complete list of strains and oligonucleotides used can be found in Supporting Tables 1 and 2.

***Kluyveromyces lactis* targeted gene replacement.** The *KanMX4* cassette was amplified from the PCR template pUG6 (4) and fused to 250-bp PCR products directly flanking the gene of interest on both sides using splicing-by-overlap extension PCR. Oligonucleotides were purchased from Integrated DNA Technologies and can be found in Table S2. The resulting PCR products were electroporated into *K. lactis* using an Eppendorf Eporator and electroporation cuvettes with a 0.2cm gap width. Preparation of electrocompetent cells was performed as described previously, but without HEPES buffer in the pre-electroporation incubation step (1). Cells were plated directly onto YPD following electroporation and grown overnight at 30°C before replica plating onto YPD plates containing 200 mg/L G418 (US Biological Life Sciences). Complementation of mutants was performed by amplifying the complete intergenic regions surrounding the coding sequences (CDS) of interest, fused to 250-bp PCR products directly flanking the *KlURA3* gene, followed by electroporation, overnight growth at 30ºC and replica plating onto SC plates containing 1 g/L 5'-FOA and 50 mg/mL additional uracil (US Biological Life Sciences). Constitutive gene expression of *PUL1* and *PUL2* was performed by cloning each CDS flanked by the Eg*TEF1* (also known as *MX*) promoter-terminator pair into the multiple cloning site of pIL68, a vector containing a replication sequence functional in *K. lactis* (5), followed by transformation into the appropriate strain and selection on SC plates lacking uracil.

***Saccharomyces cerevisiae* targeted gene replacement.** The *KanMX4* cassette was amplified from the PCR template pUG6 (4) using oligonucleotides containing 40bp of homology immediately upstream and downstream the CDS of the target gene. Cells were transformed using standard lithium acetate/ssDNA chemical transformations (6), and selected on YPD plates containing 200 mg/L G418. Complementation of mutants was performed by amplifying the complete intergenic regions surrounding the CDS of interest using oligonucleotides with 40bp of homology at the 5'-end to the *Sma*I site of pRS426, followed by co-transformation with *Sma*I-digested pRS426 (7) and selection on SC plates lacking uracil.

Pulcherriminic acid extraction. Extraction of pulcherriminic acid was performed similarly to classic protocols (8), with a few modifications. A strain of *Metschnikowia* sp., yHQL305, isolated from leaves from the Standish-Hickey State Recreational Area in California using established protocols (9), was grown to saturation in 50mL minimal medium with 2% (w/v) glucose and 100μM $FeCl_3$. The whole culture was treated with 50% (w/v) KOH to reach a final concentration of 5%(w/v) KOH. The mixture was centrifuged at 1,800x*g* for 10 minutes at 20°C to remove cellular debris and precipitate iron. 45mL of cleared supernatant was transferred to a fresh container, to which 100μL 3.3M $FeCl_3$ was added, followed by 5.5mL of concentrated (36.5-38%) HCl to neutralize the KOH and reduce the pH to approximately 1.

Pulcherrimin typically precipitated at this step, but an overnight incubation at 4°C was sometimes required. The mixture was centrifuged at 1,800x*g* for 10 minutes at 20°C to pellet the pulcherrimin, which was then washed with 1M HCl, dH_2O, and finally 100% ethanol. The pulcherrimin was then allowed to completely dry before weighing. Pulcherriminic acid was re-dissolved to the desired concentration by adding the proper amount of 5%(w/v) KOH, centrifuging the insoluble iron, and transferring the cleared supernatant to a fresh container; aliquots were then stored at -20°C for future experiments.

Growth experiments and PA-treatment. Growth experiments were performed in 96-well plates on a FLUOstar Omega plate reader (BMG Labtech). Experiments using only *S. cerevisiae* and *K. lactis* were performed at 30°C, while experiments using several yeast species were performed at 22°C to permit the growth of species that prefer cooler temperatures. For *K. lactis* experiments, cells were precultured in YPD overnight, OD was normalized across all cultures using fresh YPD, and cells were added to individual wells at a final OD of 0.05. For *S. cerevisiae* experiments, cells were precultured in SC without uracil overnight, OD was normalized across all cultures using fresh SC without uracil, and cells were added to individual wells at a final OD of 0.05. OD_{595} readings were measured over the course of 40-48 hours at 15- or 30-minute intervals and normalized against corresponding uninoculated wells. For PA-treated SC medium, pulcherriminic acid in 5% KOH was added to the medium to a final concentration of 20µM in SC medium (with or without uracil), followed by addition of 0.9 equivalents of 1M HCl. Conditions lacking PA treatment were treated with a blank 5% KOH solution, followed by addition of 0.9 equivalents 1M HCl. Ferric iron supplementations were performed by the addition of $FeCl_3$ (VWR Life Science) to 20µM to demonstrate that PA-treatment was ineffective if excess iron was supplemented (Figure S5C).

Multi-species growth experiments. For comparisons of species that contain or lack *PUL3* and *PUL4*, two species from each of five genera were selected; species containing both genes are listed first, while those lacking *PUL3* or both genes are listed second: *Saccharomyces cerevisiae* and *Saccharomyces mikatae*; *Kluyveromyces marxianus* and *Kluyveromyces nonfermentans*; *Lachancea thermotolerans* and *Lachancea waltii*; *Metschnikowia fructicola* and *Metschnikowia bicuspidata*; *Komagataella pastoris* and *Komagataella pseudopastoris*. For growth assays of multiple species, cells were precultured in YPD for two days at room temperature, individual wells were inoculated at a dilution of 2µl culture per 150µL final volume. OD_{595} readings were measured over the course of 40-48 hours at 15- or 30-minute intervals and normalized against corresponding uninoculated wells.

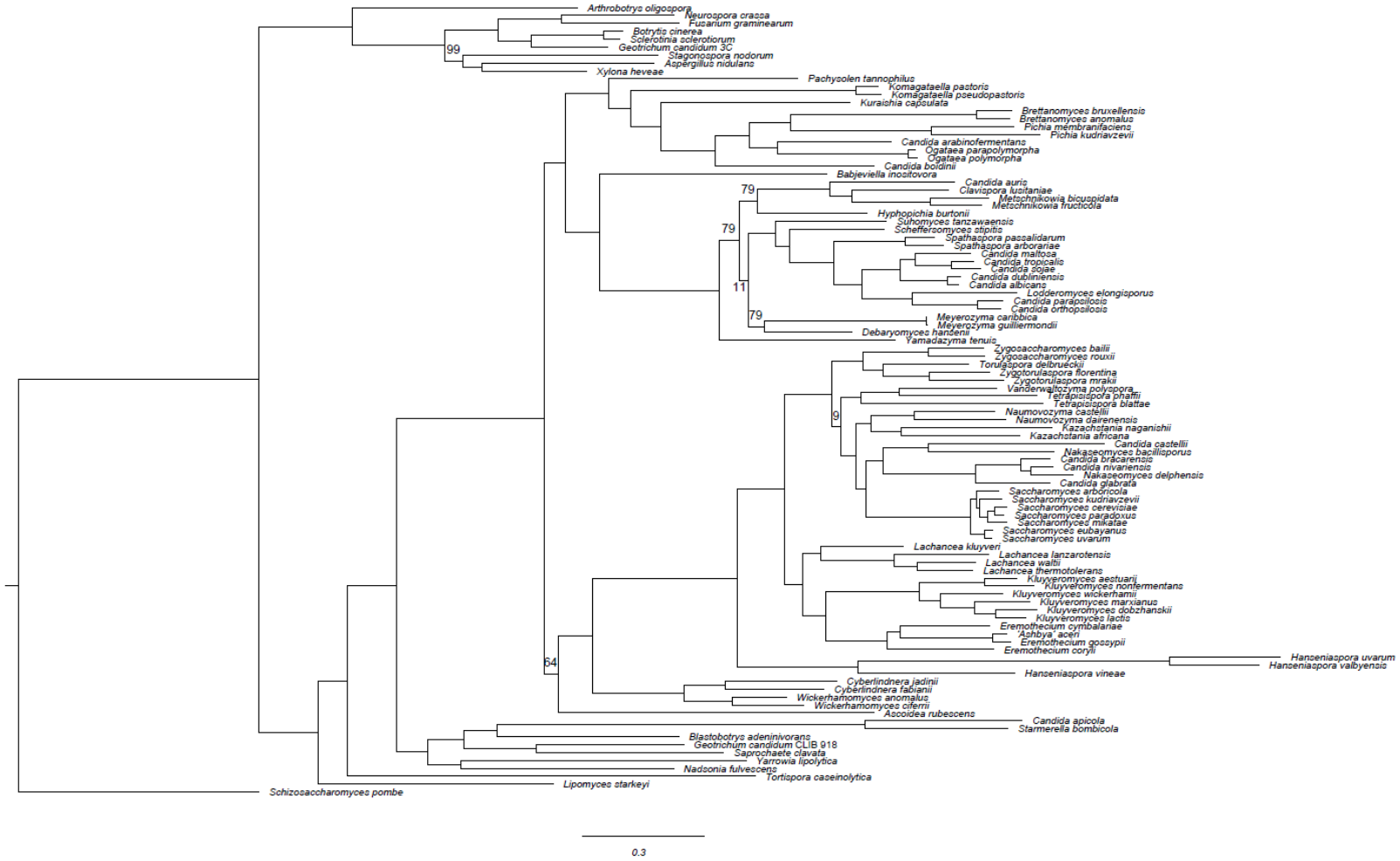
Analysis of growth curve data. Growth data from plate reader experiments was analyzed using the grofit package in R under the 'logistic' model of growth (10). For instances of negligible growth, grofit was unable to calculate parameters for a logistic growth model; therefore, the spline-fitted values were used for these instances. Where technical replicates were performed on a single plate, the median values were used for the biological replicate. Values described in the Results section are mean values of at least three biological replicates. Representative growth curves used in figures were the biological replicates that most closely resembled the mean maximum growth rates and lag times of the three (or more) biological replicates. Growth curve plots were generated using ggplot2 in R (11). Statistical comparisons of growth rates and/or lag phase durations generated in grofit were performed using paired Student's t-tests for a minimum of three biological replicates. All paired comparisons were grown concurrently on the same plate using the same reagents. For the multi-species statistical test, mean growth rate ratios of PA-treated:untreated SC media for three biological replicates of each species were compared between the two groups (containing *PUL3* and *PUL4* vs. lacking *PUL3* or both). A paired Student's t-test was performed, using species paired within each genus.

BLAST search for *PUL* gene homologs outside Saccharomycotina. Amino acid sequences from *K. lactis* (NC_006039.1) for the *PUL1-4* genes were used as query sequences with BLASTp using default parameters against the GenBank non-redundant (nr) protein sequence database. The results of this manuscript represent searches performed on August 16, 2017. Corresponding nucleotide sequences were extracted for each protein entry via the CDS-linked entry.

Analysis of gene neighborhoods. From the list of BLAST hits for *PUL* gene homologs, nucleotide accession numbers were obtained using NCBI's Entrez Programming Utilities. This list of accession numbers was then used to obtain GFF3 files for their corresponding nucleotide sequences. From here, only lines pertaining to coding sequences were extracted, and redundant CDS entries for multi-exon genes were removed. Five CDS preceding and following the BLAST-derived homolog entry were extracted, and protein sequences for these genes were obtained from NCBI's Batch Entrez. To identify *PUL* gene homologs that might have been missed in initial BLAST searches, a local blast database was created using these sequences, and all sequences were searched against this database. These BLAST networks were visualized using cytoscape (12), and networks containing *PUL* gene homologs were extracted for further use. Gene clusters containing homologs of all four *PUL* genes are shown in Figure S10.

Sequence alignment and phylogeny construction The whole-genome phylogeny was constructed from the concatenated alignment of 1,037 BUSCO genes. Bootstrap supports for the phylogeny obtained from the super-alignment of BUSCO genes were calculated with the MPI version of ExaML (13) under the per-site rate category model (PSR) and with the memory saving option enabled. For the individual *PUL* gene phylogenies, nucleotide sequences for each of four genes in the *PUL* cluster were translated, and amino acid multiple sequence alignments were constructed using MAFFT (14), MUSCLE (15), and T-Coffee (16). Resulting alignments were trimmed using trimAl (17) v1.2 with the "-strict" option on, and all other options set to default. Phylogenies were constructed using RAxML (18) v.8.1.20, first identifying the best model using "PROTGAMMAAUTO." The best maximum likelihood tree was obtained from twenty maximum likelihood searches, and 1,000 standard bootstrap replicates were performed. Trees were visualized for figure generation in FigTree (19) v.1.4.3.

Topology testing. For Approximately Unbiased (AU) tests, all reasonable tree topologies were generated using all possible rearrangements of the individual genera represented in the gene alignment. When genera were not monophyletic in the maximum likelihood tree (e.g. *Zygotorulaspora* spp.), they were split up, and when subtrees containing multiple genera had bootstrap support values greater than 70 (e.g. *Candida auris* and *Metschnikowia, Lachancea thermotolerans* and *Kluyveromyces*), these were kept together to keep the numbers of tested trees manageable. Constrained ML trees were constructed in RAxML using the "-g" option to supply a corresponding phylogenetic tree file. Per-site likelihoods were calculated in RAxML, and one-sided Kishino-Hasegawa (KH) tests (20) and AU tests were performed using CONSEL (21) v.1.20. Significance of the resulting *p*-values were determined using a Bonferroni-correction of $p < 0.05$ for 12 alignments ($p < 4.1 \times 10^{-3}$). KH test results, AU test results, and an explanation of these results can be found in Table S3.



Supporting Figure 1 – Complete BUSCO maximum likelihood phylogeny from Figure 1 without collapsed nodes, with branch lengths and bootstrap support values from 100 replicates. The tree was constructed from a concatenated nucleotide alignment of 1,037 BUSCO genes containing 741,755 positions and 100 taxa using RAxML v.8.2.9. Bootstrap supports for the phylogeny obtained from the super-alignment of BUSCO genes were calculated with the MPI version of ExaML (12) under the per-site rate category model (PSR) and with the memory saving option enabled. All nodes have bootstrap support values of 100, except those labeled. Poorly supported nodes do not involve the four newly sequenced species, and alternative topologies are discussed more thoroughly in a recent comprehensive phylogenomic study (21).

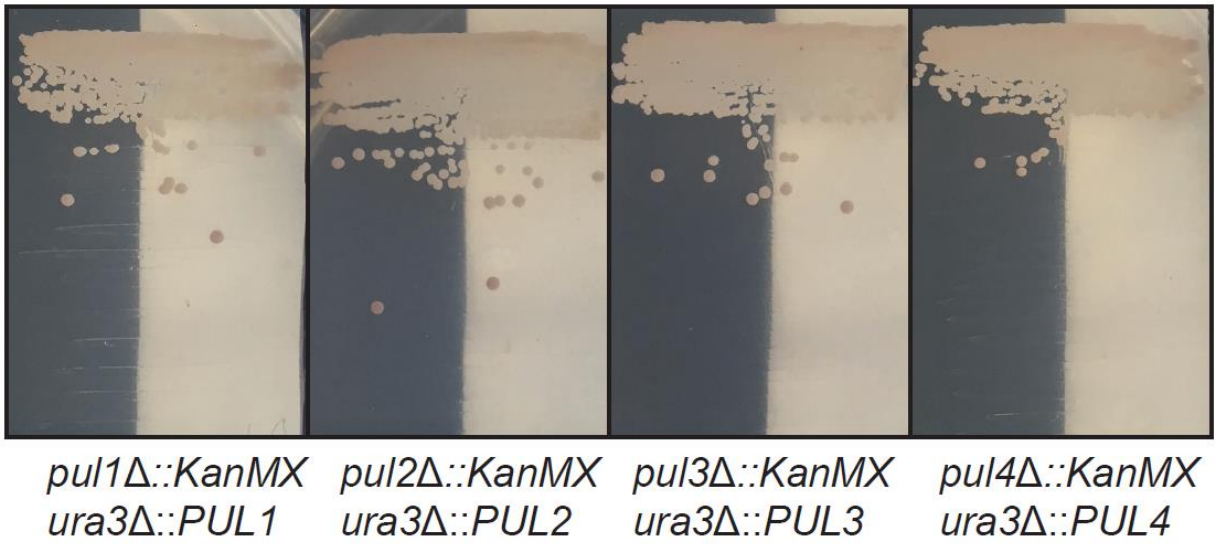


Figure S2 – Complementation of *pul* gene replacement strains were performed by integrating the wild-type gene into the *URA3* locus of *K. lactis*. All complemented strains turn red on synthetic complete medium.

Figure S3 – Cross-feeding experiments were performed by patching a colony of each genotype nearby to one another on SC medium. Strains expressing *PUL1* are on the left and stay white during growth, while strains expressing *PUL2* are on the right and turn pink during growth. Note that, absent this cross-feeding, neither *pul1Δ*, *pul2Δ*, nor *pul4Δ* strains produce pigment (Figure 2A).

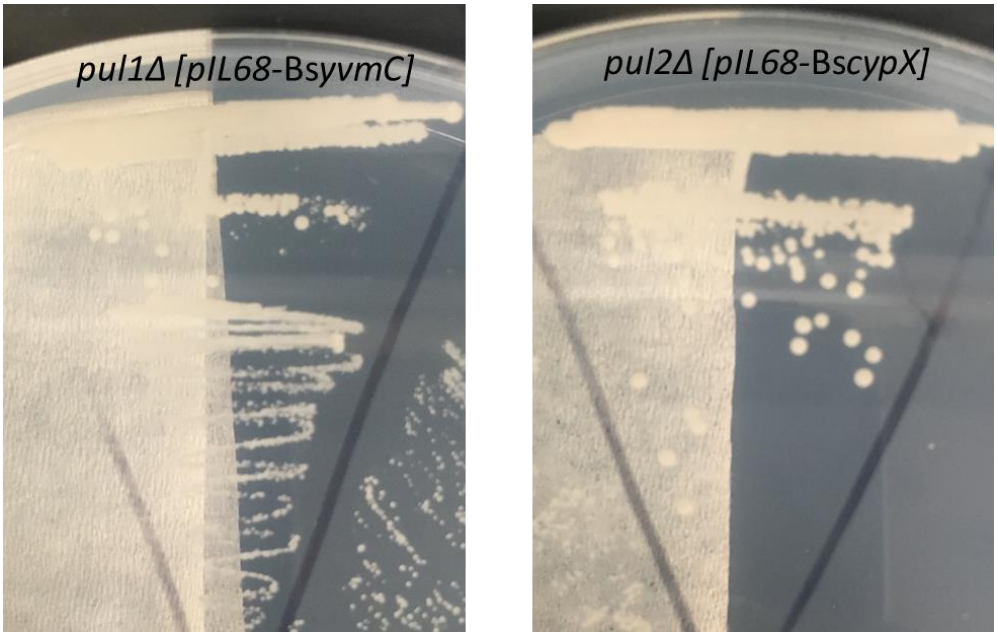


Figure S4 – *K. lactis* pulcherrimin biosynthesis mutants expressing pulcherrimin biosynthesis genes from *Bacillus subtilis*. Left – *pul1Δ* mutant expressing Bs*yvmC*. Right – *pul2Δ* mutant expressing Bs*cypX*. Neither strain shows restoration of pigmentation.

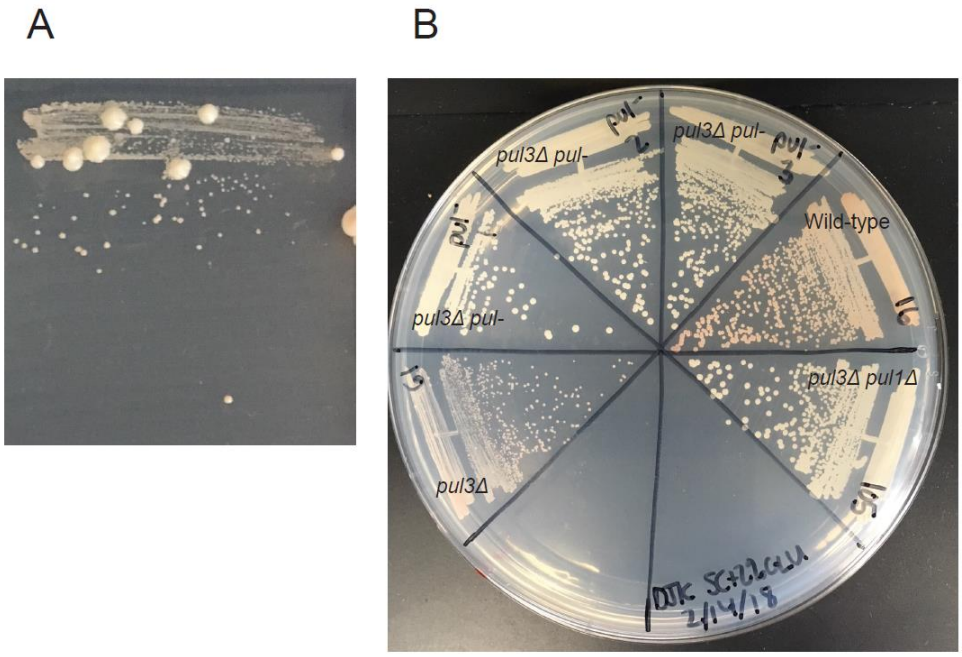


Figure S5 – A) *pul*¯ suppressors forming on SC in the *pul3Δ* mutant. B) The large white Pul¯colonies from (A) were re-streaked onto SC agar plates and grew normally without pulcherrimin production. Counterclockwise from bottom left: *pul3Δ; pul3Δ* suppressor-1; *pul3Δ* suppressor-2; *pul3Δ* suppressor-3; wild-type *K. lactis*; *pul3Δ pul1Δ*.

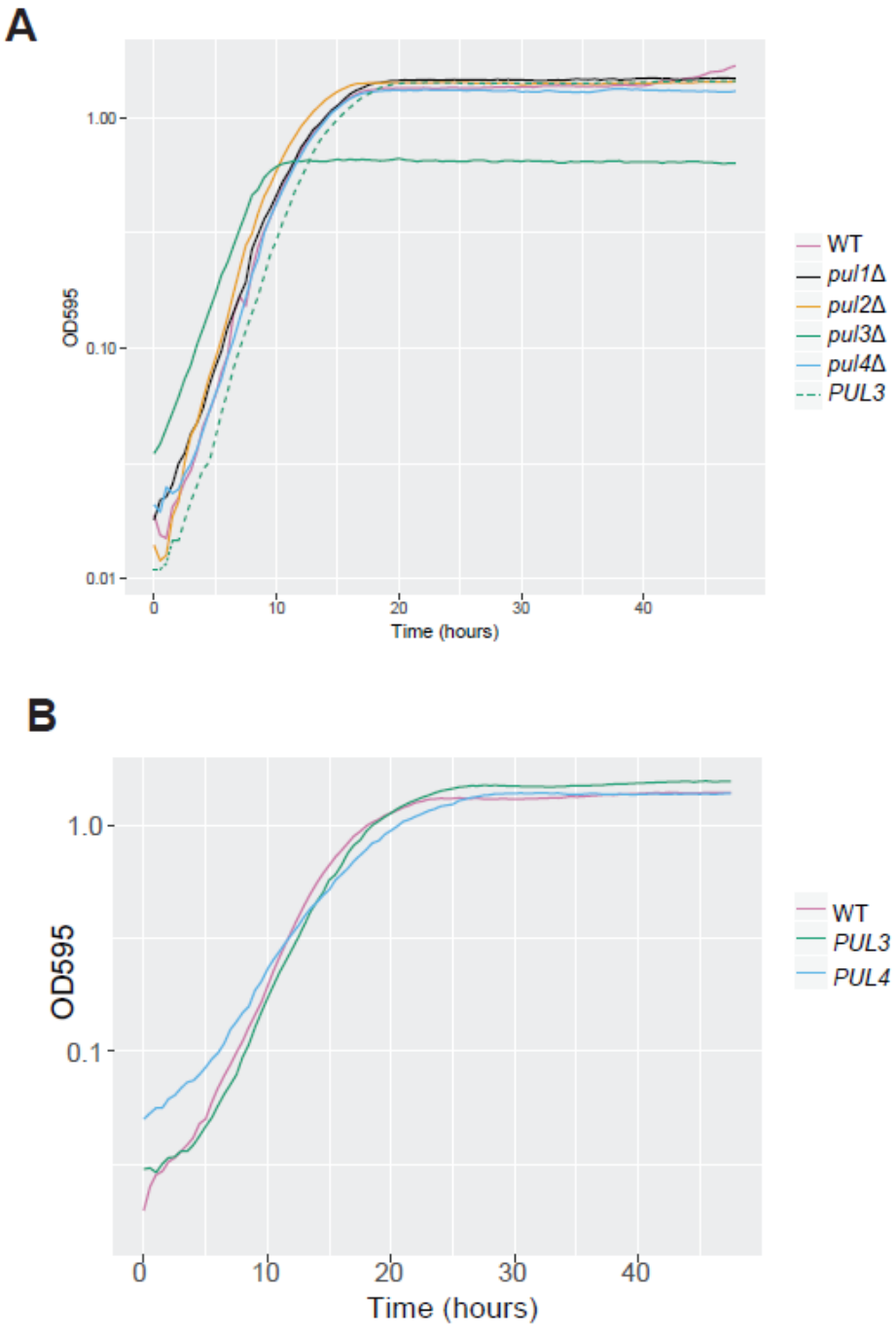


Figure S6 – A) Representative growth curves of *K. lactis pul* mutants in synthetic complete medium without addition of pulcherriminic acid. The *PUL3* strain is a *pul3Δ ura3Δ::PUL3* strain complemented with the wild-type *PUL3* gene. B) Growth of *K. lactis* complemented *pul* mutant strains in PA-treated SC medium.

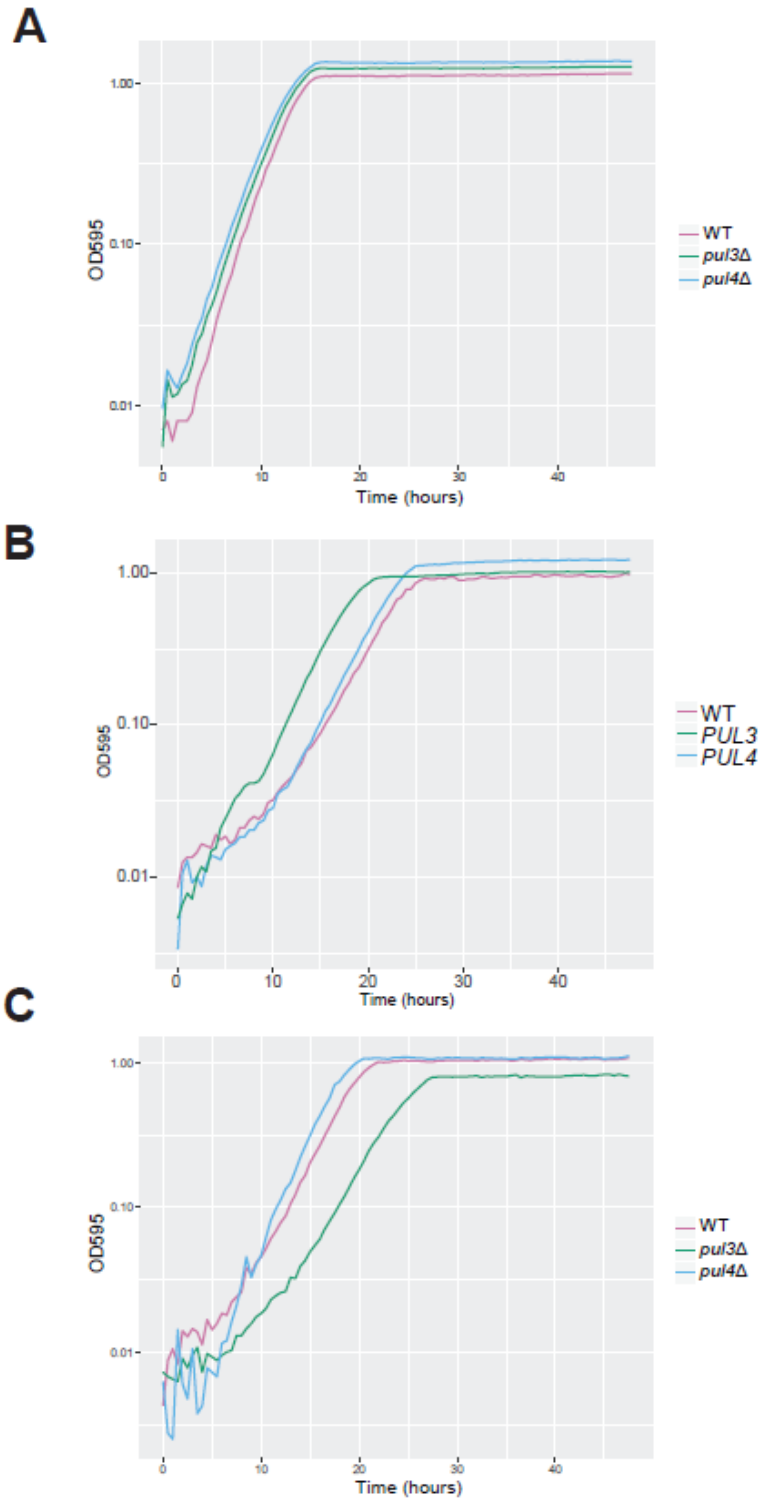


Figure S7 – A) Representative growth curves of *S. cerevisiae pul* mutants in synthetic complete medium without addition of pulcherriminic acid. B) Growth of complemented *S. cerevisiae pul* mutants in synthetic complete medium with addition of pulcherriminic acid. C) Growth of *S. cerevisiae pul* mutants in PA-treated medium, with addition of $FeCl_3$.

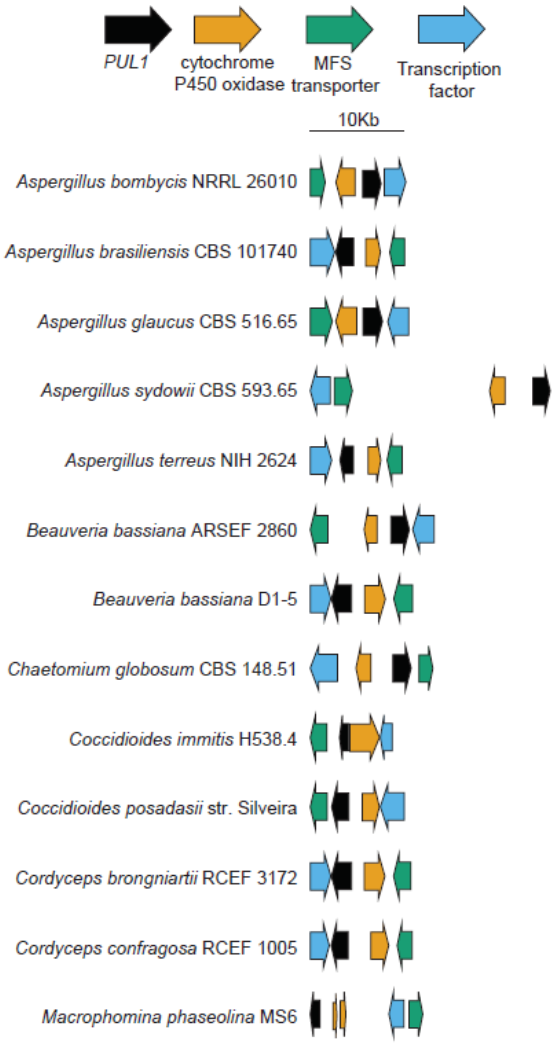
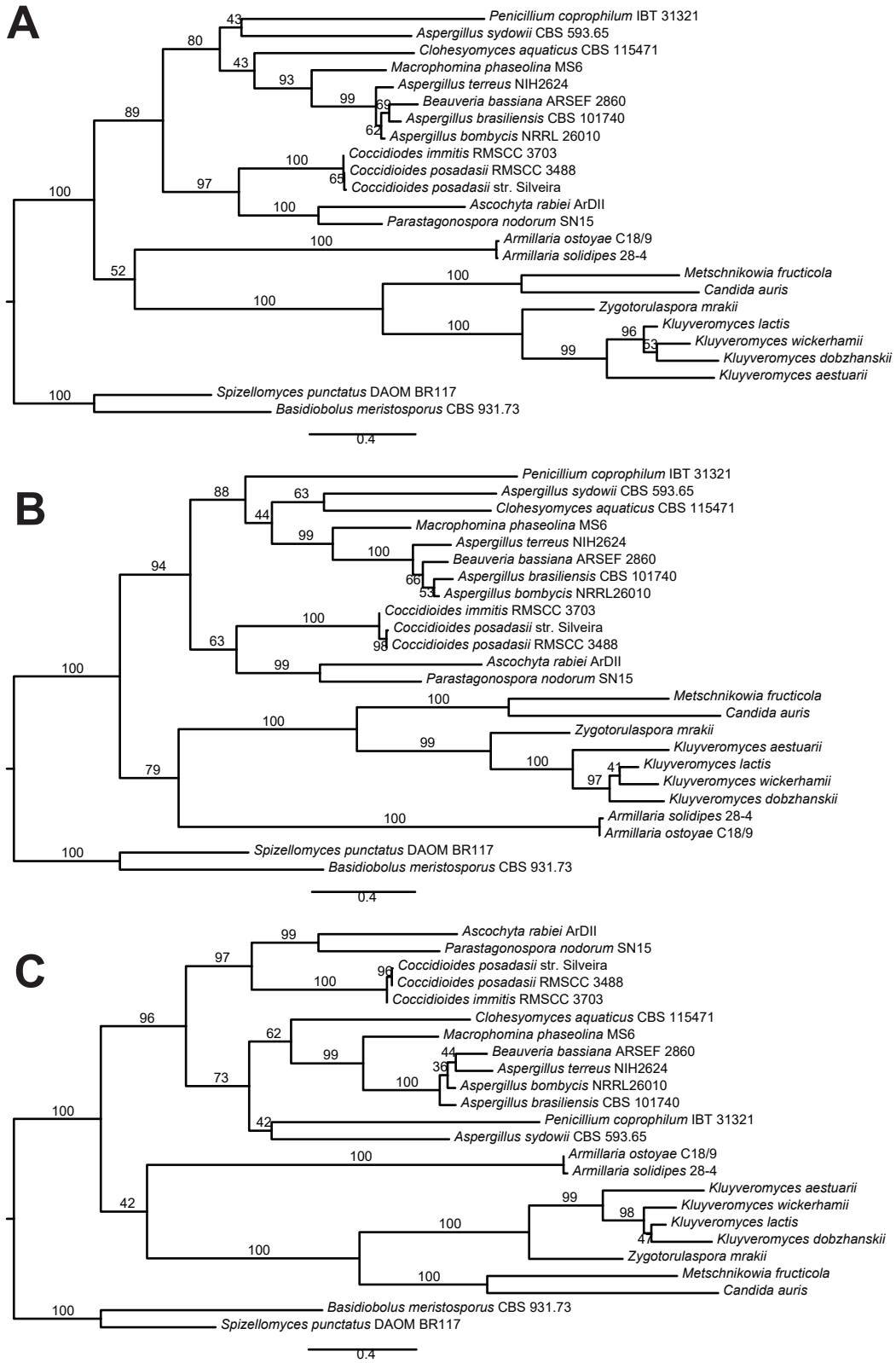


Figure S8 - Gene arrow diagrams of *PUL*cherrimin gene clusters in filamentous fungi and basidiomycetes. Gene neighborhoods of *PUL1* homologs that contained genes with similarity to a cytochrome P450 oxidase, MFS transporter, and transcription factor are shown.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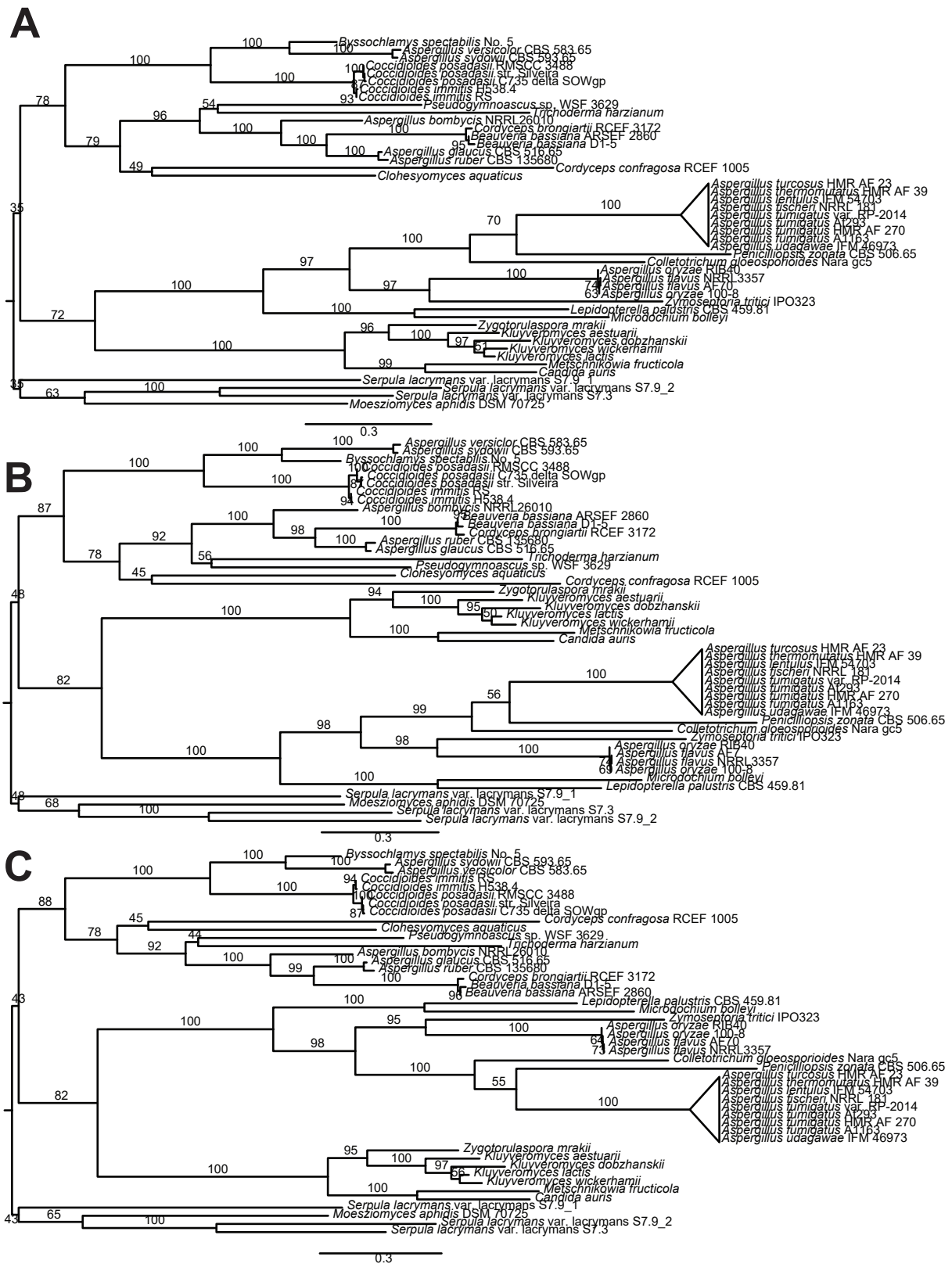
Aspergillus sydowii CBS 593.65
Clohesyomyces aquaticus CBS 115471
Macrophomina phaseolina MS6
Aspergillus terreus NIH2624
Beauveria bassiana ARSEF 2860
Aspergillus brasiliensis CBS 101740
Aspergillus bombycis NRRL 26010
Coccidiodes immitis RMSCC 3703
Coccidioides posadasii RMSCC 3488
Coccidioides posadasii str. Silveira
Ascochyta rabiei ArDII
Parastagonospora nodorum SN15
Armillaria ostoyae C18/9
Armillaria solidipes 28-4
Metschnikowia fructicola
Candida auris
Zygotorulaspora mrakii
Kluyveromyces lactis
Kluyveromyces wickerhamii
Kluyveromyces dobzhanskii
Kluyveromyces aestuarii
Spizellomyces punctatus DAOM BR117
Basidiobolus meristosporus CBS 931.73
0.4
B
Penicillium coprophilum IBT 31321
Aspergillus sydowii CBS 593.65
Clohesyomyces aquaticus CBS 115471
Macrophomina phaseolina MS6
Aspergillus terreus NIH2624
Beauveria bassiana ARSEF 2860
Aspergillus brasiliensis CBS 101740
Aspergillus bombycis NRRL26010
Coccidioides immitis RMSCC 3703
Coccidioides posadasii str. Silveira
Coccidioides posadasii RMSCC 3488
Ascochyta rabiei ArDII
Parastagonospora nodorum SN15
Metschnikowia fructicola
Candida auris
Zygotorulaspora mrakii
Kluyveromyces aestuarii
Kluyveromyces lactis
Kluyveromyces wickerhamii
Kluyveromyces dobzhanskii
Armillaria solidipes 28-4
Armillaria ostoyae C18/9
Spizellomyces punctatus DAOM BR117
Basidiobolus meristosporus CBS 931.73
0.4
C
Ascochyta rabiei ArDII
Parastagonospora nodorum SN15
Coccidioides posadasii str. Silveira
Coccidioides posadasii RMSCC 3488
Coccidioides immitis RMSCC 3703
Clohesyomyces aquaticus CBS 115471
Macrophomina phaseolina MS6
Beauveria bassiana ARSEF 2860
Aspergillus terreus NIH2624
Aspergillus bombycis NRRL26010
Aspergillus brasiliensis CBS 101740
Penicillium coprophilum IBT 31321
Aspergillus sydowii CBS 593.65
Armillaria ostoyae C18/9
Armillaria solidipes 28-4
Kluyveromyces aestuarii
Kluyveromyces wickerhamii
Kluyveromyces lactis
Kluyveromyces dobzhanskii
Zygotorulaspora mrakii
Metschnikowia fructicola
Candida auris
Basidiobolus meristosporus CBS 931.73
Spizellomyces punctatus DAOM BR117
0.4

Figure S9– Gene trees for *PUL1* and homologs. Trees were rooted on branches leading to a basidiomycetous homolog for each gene. Bootstrap values for 1000 replicates shown. A) MAFFT alignment, B) MUSCLE alignment, C) T-Coffee alignment.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Coccidioides posadasii str. Silveira
Coccidioides posadasii C735 delta SOWgp
Coccidioides immitis H538.4
Coccidioides immitis RS
Pseudogymnoascus sp. WSF 3629
Trichoderma harzianum
Aspergillus bombycis NRRL26010
Cordyceps brongiartii RCEF 3172
Beauveria bassiana ARSEF 2860
Beauveria bassiana D1-5
Aspergillus glaucus CBS 516.65
Aspergillus ruber CBS 135680
Cordyceps confragosa RCEF 1005
Clohesyomyces aquaticus
Aspergillus turcosus HMR AF 23
Aspergillus thermomutatus HMR AF 39
Aspergillus lentulus IFM 54703
Aspergillus fischeri NRRL 181
Aspergillus fumigatus var. BP-2014
Aspergillus fumigatus AF293
Aspergillus fumigatus HMR AF 270
Aspergillus fumigatus A1163
Aspergillus udagawae IFM 46973
Penicillopsis zonata CBS 506.65
Colletotrichum gloeosporioides Nara gc5
Aspergillus oryzae RIB40
Aspergillus flavus NRRL3357
Aspergillus flavus AF70
Aspergillus oryzae 100-8
Zymoseptoria tritici IPO323
Lepidopterella palustris CBS 459.81
Microdochium bolleyi
Zygotorulaspora mrakii
Kluyveromyces aestuarii
Kluyveromyces dobzhanskii
Kluyveromyces lactis
Kluyveromyces wickerhamii
Metschnikowia fructicola
Candida auris
Serpula lacrymans var. lacrymans S7.9_1
Serpula lacrymans var. lacrymans S7.9_2
Serpula lacrymans var. lacrymans S7.3
Moesziomyces aphidis DSM 70725
0.3
B
Aspergillus versiclor CBS 583.65
Aspergillus sydowii CBS 593.65
Byssochlamys spectabilis No. 5
Coccidioides posadasii RMSCC 3488
Coccidioides posadasii C735 delta SOWgp
Coccidioides posadasii str. Silveira
Coccidioides immitis RS
Coccidioides immitis H538.4
Aspergillus bombycis NRRL26010
Beauveria bassiana ARSEF 2860
Beauveria bassiana D1-5
Cordyceps brongiartii RCEF 3172
Aspergillus ruber CBS 135680
Aspergillus glaucus CBS 516.65
Trichoderma harzianum
Pseudogymnoascus sp. WSF 3629
Clohesyomyces aquaticus
Cordyceps confragosa RCEF 1005
Zygotorulaspora mrakii
Kluyveromyces aestuarii
Kluyveromyces dobzhanskii
Kluyveromyces lactis
Kluyveromyces wickerhamii
Metschnikowia fructicola
Candida auris
Aspergillus turcosus HMR AF 23
Aspergillus thermomutatus HMR AF 39
Aspergillus lentulus IFM 54703
Aspergillus fischeri NRRL 181
Aspergillus fumigatus var. BP-2014
Aspergillus fumigatus AF293
Aspergillus fumigatus HMR AF 270
Aspergillus fumigatus A1163
Aspergillus udagawae IFM 46973
Penicillopsis zonata CBS 506.65
Colletotrichum gloeosporioides Nara gc5
Zymoseptoria tritici IPO323
Aspergillus oryzae RIB40
Aspergillus flavus AF70
Aspergillus flavus NRRL3357
Aspergillus oryzae 100-8
Microdochium bolleyi
Lepidopterella palustris CBS 459.81
Serpula lacrymans var. lacrymans S7.9_1
Moesziomyces aphidis DSM 70725
Serpula lacrymans var. lacrymans S7.3
Serpula lacrymans var. lacrymans S7.9_2
0.3
C
Byssochlamys spectabilis No. 5
Aspergillus sydowii CBS 593.65
Aspergillus versicolor CBS 583.65
Coccidioides immitis RS
Coccidioides immitis H538.4
Coccidioides posadasii RMSCC 3488
Coccidioides posadasii str. Silveira
Coccidioides posadasii C735 delta SOWgp
Cordyceps confragosa RCEF 1005
Clohesyomyces aquaticus
Pseudogymnoascus sp. WSF 3629
Trichoderma harzianum
Aspergillus bombycis NRRL26010
Aspergillus glaucus CBS 516.65
Aspergillus ruber CBS 135680
Cordyceps brongiartii RCEF 3172
Beauveria bassiana D1-5
Beauveria bassiana ARSEF 2860
Lepidopterella palustris CBS 459.81
Microdochium bolleyi
Zymoseptoria tritici IPO323
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Aspergillus flavus AF70
Aspergillus flavus NRRL3357
Colletotrichum gloeosporioides Nara gc5
Penicillopsis zonata CBS 506.65
Aspergillus turcosus HMR AF 23
Aspergillus thermomutatus HMR AF 39
Aspergillus lentulus IFM 54703
Aspergillus fischeri NRRL 181
Aspergillus fumigatus var. BP-2014
Aspergillus fumigatus AF293
Aspergillus fumigatus HMR AF 270
Aspergillus fumigatus A1163
Aspergillus udagawae IFM 46973
Zygotorulaspora mrakii
Kluyveromyces aestuarii
Kluyveromyces dobzhanskii
Kluyveromyces lactis
Kluyveromyces wickerhamii
Metschnikowia fructicola
Candida auris
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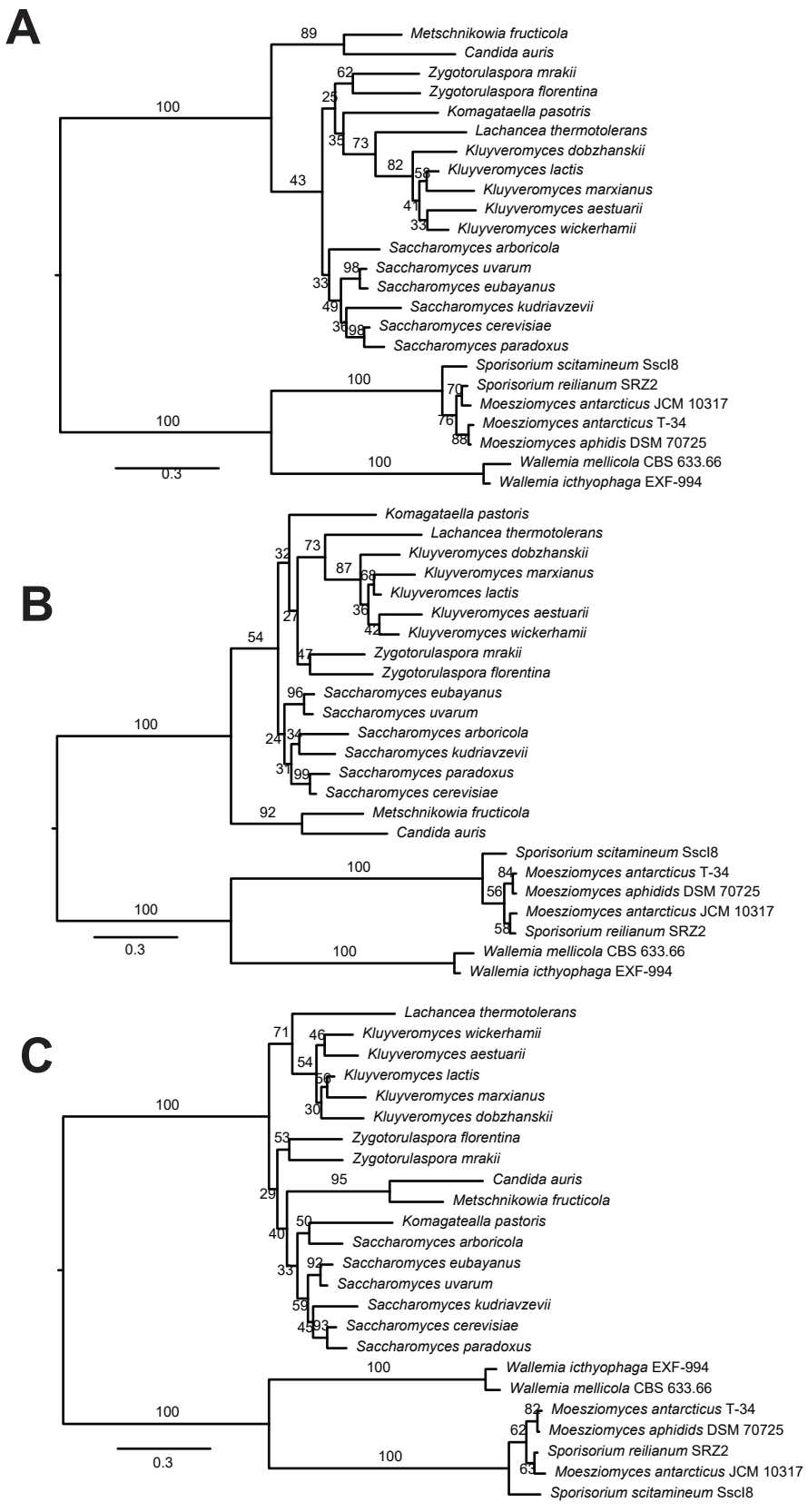
Serpula lacrymans var. lacrymans S7.9_2
Serpula lacrymans var. lacrymans S7.3
0.3

Figure S10 – Gene trees for *PUL2* and homologs. Trees were rooted on branches leading to a basidiomycetous homolog for each gene. Bootstrap values for 1000 replicates shown. A) MAFFT alignment, B) MUSCLE alignment, C) T-Coffee alignment. The *Aspergillus turcosus*/*Aspergillus thermomutatus*/*Aspergillus lentulus*/*Aspergillus fischeri*/*Aspergillus fumigatus*/*Aspergillus udagawae* clade had short branch lengths and poor bootstrap support, so it was collapsed for easier viewing.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Candida auris
Zygotorulaspora mrakii
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Lachancea thermotolerans
Kluyveromyces dobzhanskii
Kluyveromyces lactis
Kluyveromyces marxianus
Kluyveromyces aestuarii
Kluyveromyces wickerhamii
Saccharomyces arboricola
Saccharomyces uvarum
Saccharomyces eubayanus
Saccharomyces kudriavzevii
Saccharomyces cerevisiae
Saccharomyces paradoxus
Sporisorium scitamineum Sscl8
Sporisorium reilianum SRZ2
Moesziomyces antarcticus JCM 10317
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Lachancea thermotolerans
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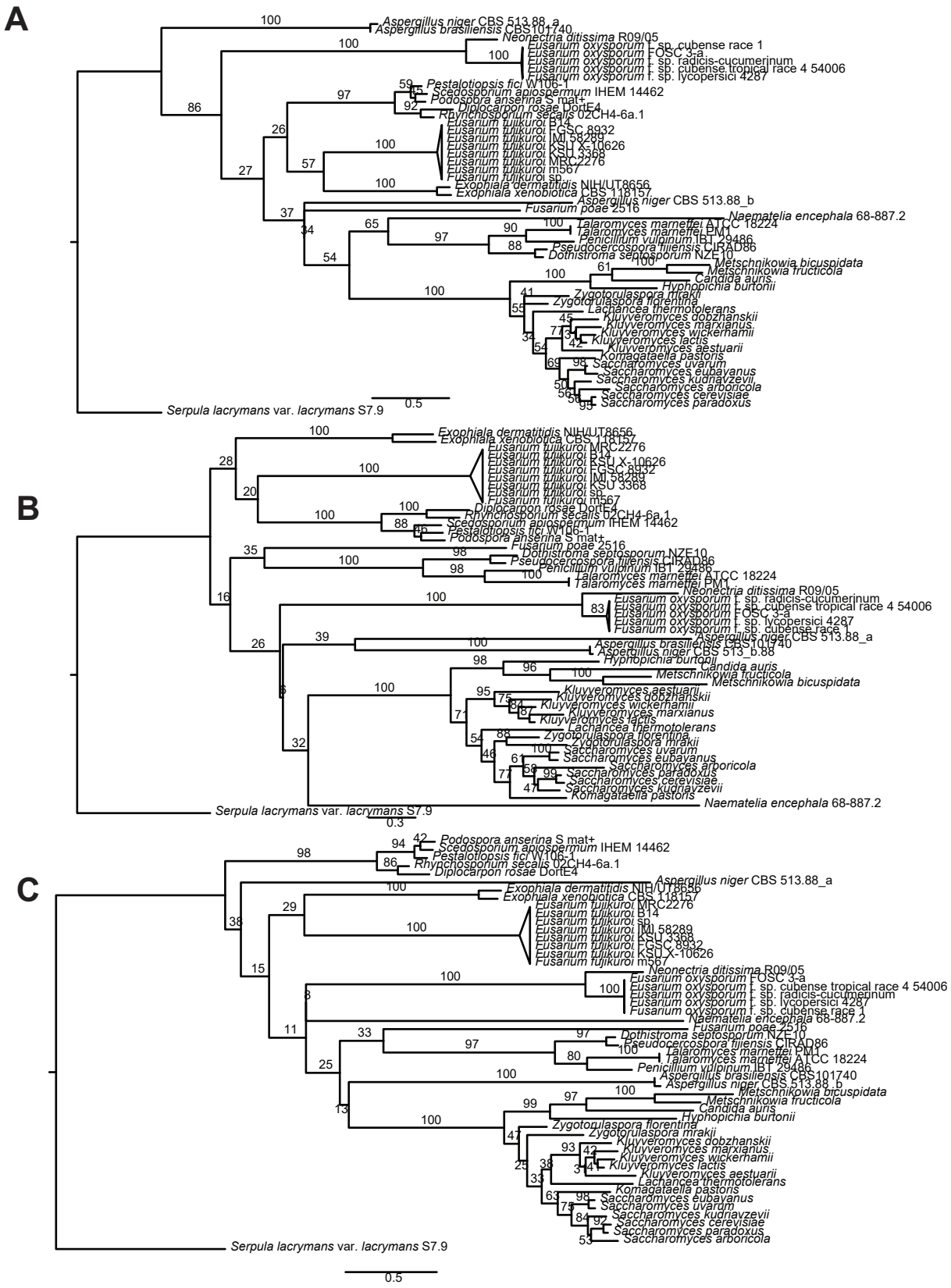
Zygotorulaspora florentina
Saccharomyces eubayanus
Saccharomyces uvarum
Saccharomyces arboricola
Saccharomyces kudriavzevii
Saccharomyces paradoxus
Saccharomyces cerevisiae
Metschnikowia fructicola
Candida auris
Sporisorium scitamineum Sscl8
Moesziomyces antarcticus T-34
Moesziomyces aphidids DSM 70725
Moesziomyces antarcticus JCM 10317
Sporisorium reilianum SRZ2
Wallemia mellicola CBS 633.66
Wallemia icthyophaga EXF-994
0.3
C
Lachancea thermotolerans
Kluyveromyces wickerhamii
Kluyveromyces aestuarii
Kluyveromyces lactis
Kluyveromyces marxianus
Kluyveromyces dobzhanskii
Zygotorulaspora florentina
Zygotorulaspora mrakii
Candida auris
Metschnikowia fructicola
Komagatealla pastoris
Saccharomyces arboricola
Saccharomyces eubayanus
Saccharomyces uvarum
Saccharomyces kudriavzevii
Saccharomyces cerevisiae
Saccharomyces paradoxus
Wallemia icthyophaga EXF-994
Wallemia mellicola CBS 633.66
Moesziomyces antarcticus T-34
Moesziomyces aphidids DSM 70725
Sporisorium reilianum SRZ2
Moesziomyces antarcticus JCM 10317
Sporisorium scitamineum Sscl8
0.3

Figure S11 – Gene trees for *PUL3* and homologs. Trees were rooted on branches leading to a basidiomycetous homolog for each gene. Bootstrap values for 1000 replicates shown. A) MAFFT alignment, B) MUSCLE alignment, C) T-Coffee alignment.


A
Aspergillus niger CBS 513.88_a
Aspergillus brasiliensis CBS101740
Neonectria ditissima R09/05
Fusarium oxysporum f. sp. cubense race 1
Fusarium oxysporum FOSC 3-a
Fusarium oxysporum f. sp. radicis-cucumerinum
Fusarium oxysporum f. sp. cubense tropical race 4 54006
Fusarium oxysporum f. sp. lycopersici 4287
Pestalotiopsis fici W106-1
Scedosporium apiospermum IHEM 14462
Podospora anserina S mat+
Diplocarpon rosae DortE4
Rhynchosporium secalis 02CH4-6a.1
Fusarium fujikuroi B14
Fusarium fujikuroi FGSC 8932
Fusarium fujikuroi IMI 58289
Fusarium fujikuroi KSU X-10626
Fusarium fujikuroi KSU 3368
Fusarium fujikuroi MRC2276
Fusarium fujikuroi m567
Fusarium fujikuroi sp.
Exophiala dermatitidis NIH/UT8656
Exophiala xenobiotica CBS 118157
Aspergillus niger CBS 513.88_b
Fusarium poae 2516
Naematelia encephala 68-887.2
Talaromyces marneffei ATCC 18224
Talaromyces marneffei PM1
Penicillium vulpinum IBT 29486
Pseudocercospora fijiensis CIRAD86
Dothistroma septosporum NZE10
Metschnikowia bicuspidata
Metschnikowia fructicola
Candida auris
Hyphopichia burtonii
Zygotorulaspora mrakii
Zygotorulaspora florentina
Lachancea thermotolerans
Kluyveromyces dobzhanskii
Kluyveromyces marxianus
Kluyveromyces wickerhamii
Kluyveromyces lactis
Kluyveromyces aestuarii
Komagataella pastoris
Saccharomyces uvarum
Saccharomyces eubayanus
Saccharomyces kudriavzevii
Saccharomyces arboricola
Saccharomyces cerevisiae
Saccharomyces paradoxus
Serpula lacrymans var. lacrymans S7.9
0.5
B
Exophiala dermatitidis NIH/UT8656
Exophiala xenobiotica CBS 118157
Fusarium fujikuroi MRC2276
Fusarium fujikuroi B14
Fusarium fujikuroi KSU X-10626
Fusarium fujikuroi FGSC 8932
Fusarium fujikuroi IMI 58289
Fusarium fujikuroi KSU 3368
Fusarium fujikuroi sp.
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Diplocarpon rosae DortE4
Rhynchosporium secalis 02CH4-6a.1
Scedosporium apiospermum IHEM 14462
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Podospora anserina S mat+
Fusarium poae 2516
Dothistroma septosporum NZE10
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Talaromyces marneffei ATCC 18224
Talaromyces marneffei PM1
Neonectria ditissima R09/05
Fusarium oxysporum f. sp. radicis-cucumerinum
Fusarium oxysporum f. sp. cubense tropical race 4 54006
Fusarium oxysporum FOSC 3-a
Fusarium oxysporum f. sp. lycopersici 4287
Fusarium oxysporum f. sp. cubense race 1
Aspergillus niger CBS 513.88_a
Aspergillus brasiliensis CBS101740
Aspergillus niger CBS 513_b.88
Hyphopichia burtonii
Candida auris
Metschnikowia fructicola
Metschnikowia bicuspidata
Kluyveromyces aestuarii
Kluyveromyces dobzhanskii
Kluyveromyces wickerhamii
Kluyveromyces marxianus
Kluyveromyces lactis
Lachancea thermotolerans
Zygotorulaspora florentina
Zygotorulaspora mrakii
Saccharomyces uvarum
Saccharomyces eubayanus
Saccharomyces arboricola
Saccharomyces paradoxus
Saccharomyces cerevisiae
Saccharomyces kudriavzevii
Komagataella pastoris
Naematelia encephala 68-887.2
Serpula lacrymans var. lacrymans S7.9
0.3
C
Podospora anserina S mat+
Scedosporium apiospermum IHEM 14462
Pestalotiopsis fici W106-1
Rhynchosporium secalis 02CH4-6a.1
Diplocarpon rosae DortE4
Aspergillus niger CBS 513.88_a
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Fusarium fujikuroi sp.
Fusarium fujikuroi IMI 58289
Fusarium fujikuroi KSU 3368
Fusarium fujikuroi FGSC 8932
Fusarium fujikuroi KSU X-10626
Fusarium fujikuroi m567
Neonectria ditissima R09/05
Fusarium oxysporum FOSC 3-a
Fusarium oxysporum f. sp. cubense tropical race 4 54006
Fusarium oxysporum f. sp. radicis-cucumerinum
Fusarium oxysporum f. sp. lycopersici 4287
Fusarium oxysporum f. sp. cubense race 1
Naematelia encephala 68-887.2
Fusarium poae 2516
Dothistroma septosporum NZE10
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Talaromyces marneffei ATCC 18224
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Aspergillus niger CBS 513.88_b
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Metschnikowia fructicola
Candida auris
Hyphopichia burtonii
Zygotorulaspora florentina
Zygotorulaspora mrakii
Kluyveromyces dobzhanskii
Kluyveromyces marxianus
Kluyveromyces wickerhamii
Kluyveromyces lactis
Kluyveromyces aestuarii
Lachancea thermotolerans
Komagataella pastoris
Saccharomyces eubayanus
Saccharomyces uvarum
Saccharomyces kudriavzevii
Saccharomyces cerevisiae
Saccharomyces paradoxus
Saccharomyces arboricola
Serpula lacrymans var. lacrymans S7.9
0.5

Figure S12 – Gene trees for *PUL4* and homologs. Trees were rooted on branches leading to a basidiomycetous homolog for each gene. Bootstrap values for 1000 replicates shown. A) MAFFT alignment, B) MUSCLE alignment, C) T-Coffee alignment. The *Fusarium fujikuroi* clade and the *Fusarium oxysporum* clade were collapsed for ease of viewing due to short branch lengths and poor bootstrap support.

Table S1 – Yeast strains and plasmids used in this study

Strain	Genotype, properties	Source or reference
Kluyveromyces lactis		
CBS2359 *ku80*Δ	*MAT***a** *ku80*Δ; NHEJ-deficient strain for efficient gene targeting	Kooistra *et al.* 2004
*pul1*Δ (yHDK46)	*MAT***a** *ku80*-Δ *pul1*Δ::*KanMX4*	This study
*pul2*Δ (yHDK49)	*MAT***a** *ku80*-Δ *pul2*Δ::*KanMX4*	This study
*pul3*Δ (yHDK61)	*MAT***a** *ku80*-Δ *pul3*Δ::*KanMX4*	This study
*pul4*Δ (yHDK58)	*MAT***a** *ku80*-Δ *pul4*Δ::*KanMX4*	This study
*pul1*Δ; *PUL1* (yHDK81)	*MAT***a** *ku80*-Δ *pul1*Δ::*KanMX4* *ura3*Δ::*PUL1*	This study
*pul2*Δ; *PUL2* (yHDK55)	*MAT***a** *ku80*-Δ *pul2*Δ::*KanMX4* *ura3*Δ::*PUL2*	This study
*pul3*Δ; *PUL3* (yHDK82)	*MAT***a** *ku80*-Δ *pul3*Δ::*KanMX4* *ura3*Δ::*PUL3*	This study
*pul4*Δ; *PUL4* (yHDK85)	*MAT***a** *ku80*-Δ *pul4*Δ::*KanMX4* *ura3*Δ::*PUL4*	This study
*pul1*Δ *pul3*Δ (yHDK105)	*MAT***a** *ku80*-Δ *pul1*Δ::*HygMX* *pul3*Δ::*KanMX4*	This study
*pul1*Δ *ura3*-Δ *[pIL68-PUL2]* (yHDK218)	*MAT***a** *ku80*-Δ *ura3*-Δ *pul1*Δ::*HygMX* *[pIL68-URA3-*P_{EgTEF1}*-PUL2-*T_{EgTEF1}*]*	This study
*pul2*Δ *ura3*-Δ *[pIL68-PUL1]* (yHDK221)	*MAT***a** *ku80*-Δ *ura3*-Δ *pul2*Δ::*KanMX4* *[pIL68-URA3-*P_{EgTEF1}*-PUL1-*T_{EgTEF1}*]*	This study
*pul4*Δ *ura3*-Δ *[pIL68-PUL1]* (yHDK224)	*MAT***a** *ku80*-Δ *ura3*-Δ *pul4*Δ::*KanMX4* *[pIL68-URA3-*P_{EgTEF1}*-PUL1-*T_{EgTEF1}*]*	This study
*pul4*Δ *ura3*-Δ *[pIL68-PUL2]* (yHDK227)	*MAT***a** *ku80*-Δ *ura3*-Δ *pul4*Δ::*KanMX4* *[pIL68-URA3-*P_{EgTEF1}*-PUL2-*T_{EgTEF1}*]*	This study
*pul1*Δ *ura3*-Δ *[pIL68-*Bs*yvmC]* (yHDK230)	*MAT***a** *ku80*-Δ *ura3*-Δ *pul1*Δ::*HygMX* *[pIL68-URA3-*P_{EgTEF1}*-*Bs*yvmC-*T_{EgTEF1}*]*	This study
*pul2*Δ *ura3*-Δ *[pIL68-*Bs*cypX]* (yHDK233)	*MAT***a** *ku80*-Δ *ura3*-Δ *pul2*Δ::*KanMX4* *[pIL68-URA3-*P_{EgTEF1}*-*Bs*cypX-*T_{EgTEF1}*]*	This study
Saccharomyces cerevisiae		
BY4741	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	Brachmann *et al.* 1998
*pul3*Δ (yHDK52)	*MAT***a** *his3*-Δ*1 leu2*-Δ*0 met15*-Δ*0 ura3*-Δ*0* *pul3*Δ::*KanMX4*	This study
*pul3*Δ *[pRS426]* (yHDK64)	*MAT***a** *his3*-Δ*1 leu2*-Δ*0 met15*-Δ*0 ura3*-Δ*0* *pul3*Δ::*KanMX4 [pRS426-URA3]*	This study
*pul3*Δ *[pRS426-PUL3]* (yHDK91)	*MAT***a** *his3*-Δ*1 leu2*-Δ*0 met15*-Δ*0 ura3*-Δ*0* *pul3*Δ::*KanMX4 [pRS426-PUL3-URA3]*	This study
*pul4*Δ (yHDK54)	*MAT***a** *his3*-Δ*1 leu2*-Δ*0 met15*-Δ*0 ura3*-Δ*0* *pul4*Δ::*KanMX4*	This study
*pul4*Δ *[pRS426]* (yHDK67)	*MAT***a** *his3*-Δ*1 leu2*-Δ*0 met15*-Δ*0 ura3*-Δ*0* *pul4*Δ::*KanMX4 [pRS426-URA3]*	This study
*pul4*Δ *[pRS426-PUL4]* (yHDK87)	*MAT***a** *his3*-Δ*1 leu2*-Δ*0 met15*-Δ*0 ura3*-Δ*0* *pul4*Δ::*KanMX4 [pRS426-PUL4-URA3]*	This study

Plasmids

pUG6	*KanMX*4, Amp^R	Güldener *et al.* 1996
pRS426	*S. cerevisiae URA3,* Amp^R	Sikorski and Hieter 1989
pIL68	*S. cerevisiae URA3,* Amp^R, *K. lactis* ARS	Liachko and Dunham 2014

Oligo Name	Sequence
Amplification of the *KanMX4* cassette for OEPCR	
oHRW36	5’-CAGCGACATGGAGGCCCAGAATACCC-3’
oHRW37	5’-AGCTCGTTTTCGACACTGGATGGCG-3'
Amplification of flanking sequence to *KlPUL1* for replacement with *KanMX4*	
oHDK56	5’-TGAACACGCTATGCATCAATTTGACAA-3’
oHDK57	5’-GGGTATTCTGGGCCTCCATGTCGCTGTTTTAATTTTCTTGGTGCATGCCATCG-3’
oHDK75	5’-CGCCATCCAGTGTCGAAAACGAGCTTAACTAATATTTTTGCCTAGAATCTATATACCA-3’
oHDK76	5’-GTGCTCTGCTGTGATTTCTTCTCGACT-3’
Amplification of flanking sequence to *KlPUL2* for replacement with *KanMX4*	
oHDK77	5’-CGGTTTGAGGTTATTGTAAAAGACAGG-3’
oHDK78	5’-GGGTATTCTGGGCCTCCATGTCGCTGTTCTTTCCTGTTGCGCTTCAATTGTC-3’
oHDK58	5’-CGCCATCCAGTGTCGAAAACGAGCTGAAATTTTTTTGAAAGTCTGTGTTGAA-3’
oHDK64	5’-ATTGAAGAACGAATGAGCACCTGAAG-3’
Amplification of flanking sequence to *KlPUL3* for replacement with *KanMX4*	
oHDK113	5’-ATAGTTTGCCAACTGCATCGAATG-3’
oHDK114	5’-GGGTATTCTGGGCCTCCATGTCGCTGTGTTAGCCTTTTGTTCTTATCAGGT-3’
oHDK115	5’-CGCCATCCAGTGTCGAAAACGAGCTAGGCAATAAAAGTAGCACCTGATGT-3’

oHDK116	5’-CCGTGACACCAAAGGAATTTTTTCC-3’
Amplification of flanking sequence to *KlPUL4* for replacement with *KanMX4*	
oHDK121	5’-TATATCCAATTCGGTATCTAGGGAGTGA-3’
oHDK122	5’-GGGTATTCTGGGCCTCCATGTCGCTGCAATCAAATGTGCTACACCTAATGTTAGAT-3’
oHDK123	5’-CGCCATCCAGTGTCGAAAACGAGCTTCGGACAGCATAACAATTATCATCGATTTA-3’
oHDK124	5’-CAAATCGCACCCAGAGAGCTGACT-3’
Amplification of the deleted loci for confirmation	
oHDK60	5’-AAAACTCGAGGGTGGAAGTACTTCTAAAAAAACC-3’
oHDK79	5’-AAAAGCGGCCGCTTCTTTCCTGTTGCGCTTCAA-3’
oHDK61	5’-TTTTGCGGCCGCTCAATCAAATGTGCTACACCTAATA-3’
oHDK80	5’-AAAACTCGAGTAACTAATATTTTTGCCTAGAATCTATA-3’
oHDK117	5’-ACAGATCTATATTCAATAAGGTTGCAAAGACT-3’
oHDK118	5’-AAATCTGTTGACTGCGTCAGATTGATTTTAC-3’
oHDK125	5’-GACTGCCTAATCTTTTCTTTTAGTCG-3’
oHDK126	5’-CATTTTCAAGACGCCAATGTTAGTGA-3’
Amplification of loci for integration into *URA3* of *K. lactis*	
oHDK97	5’-TACACACATTACTTGCCTCGA-3’
oHDK98	5’-GTTCTATTAAGTTTCCTGTATAAGGTGCAACTAATTGACGGGA-3’

oHDK99	5’-TCCCGTCAATTAGTTGCACCTTATACAGGAAACTTAATAGAACAAATCAC-3’
oHDK100	5’-AGGAAGTTTGAGAGGGCTTATCG-3’
oHDK101	5’-GGTGCAACTAATTGACGGGAG-3’
oHDK102	5’-TTATACAGGAAACTTAATAGAACAAATCAC-3’
oHDK103	5’-GCGTCAATACACTCCCGTCAATTAGTTGCACCTAACTAATATTTTTGCCTAGAATCTATA-3’
oHDK104	5’-AAATATGTGATTTGTTCTATTAAGTTTCCTGTATAATCAATCAAATGTGCTACACCTAAT-3’
oHDK105	5’-GCGTCAATACACTCCCGTCAATTAGTTGCACCGGTGGAAGTACTTCTAAAAAAACC-3’
oHDK106	5’-AAATATGTGATTTGTTCTATTAAGTTTCCTGTATAATTCTTTCCTGTTGCGCTTCAA-3’
oHDK119	5’-GCGTCAATACACTCCCGTCAATTAGTTGCACCTCGGACAGCATAACAATTATCATC-3’
oHDK120	5’-AAATATGTGATTTGTTCTATTAAGTTTCCTGTATAATATAAAGCAATCCTGGCAATTTAA-3’
oHDK127	5’-GCGTCAATACACTCCCGTCAATTAGTTGCACCGAAATTTTTTTGAAAGTCTGTGTTGA-3’
oHDK128	5’-AAATATGTGATTTGTTCTATTAAGTTTCCTGTATAATTGTTAGCCTTTTGTTCTTATCAG-3’
Amplification of *URA3* locus for confirmation	
oHDK129	5’-TACGGAGACAATCATATGGGAGAAGCAATTGGA-3’
oHDK130	5’-TCTTGTTGTTCCTTACCATTAAGTTGATCCATTG-3’
Amplification of *KanMX4* for replacement of *PUL3* in *S. cerevisiae*	
oHDK81	5’-TGCAGCATCTGCTTCTGGTGCTATAGTGTTCAGTTACATCCAGCTGAAGCTTCGTACGC-3’
oHDK82	5’-ATATATAGCTGGATTTGGACCAGTATATGTCTAAGGAAATGCATAGGCCACTAGTGGATC-3’

Amplification of *KanMX4* for replacement of *PUL4* in *S. cerevisiae*	
oHDK83	5’-AGCTTAATCTAAAACTACAAAAGCGTTCGCAACAAGCAGTCAGCTGAAGCTTCGTACGC-3’
oHDK84	5’-ATTTCTGAATAAAGTGTCAAAAAATCAGCTAGGAAACGGTGCATAGGCCACTAGTGGATC-3’
Confirmation of *PUL3* and *PUL4* loci in *S. cerevisiae*	
oHDK85	5’-GTATTCATGCGGCGGTGCAGTGT-3’
oHDK86	5’-AAAAGAGGCTGACTTTTTGTCGTCCAAG-3’
oHDK87	5’-TGTCATCTAAGATTCAATTAAAGTCGAC-3’
oHDK88	5’-CCTACTACCATGTCATATGGATCT-3’
Amplification of *PUL3* and *PUL4* loci for gap repair into pRS426	
oHDK89	5’-AGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGAACGCTTTTGTAGTTTTAG-3’
oHDK90	5’-CCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTGGTAACAAAATATCAAGCTG-3’
oHDK91	5’-AGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACGATGTAACTGAACACTATAG-3’
oHDK92	5’-CCAAGCGCGCAATTAACCCTCACTAAAGGGAACAAAAGCTAGATACTAAGTTCTTGTGAC-3’
Screening pRS426 and pIL68 for proper insertions	
M13F	5’-CCCAGTCACGACGTTGTAAAACG-3’
M13R	5’-AGCGGATAACAATTTCACACAGG-3’
Cloning *PUL1* and *PUL2* with Eg*TEF1* promoter and terminator into pIL68	
oHDK243	5’- CGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCAGATCTGTTTAGCTTGCCTC-3’

oHDK244	5’- CCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCTCGACACTGGATGGCGGCGT-3’
oHDK167	5’- GGATACAGTTCTCACATCACATCCGAACATAAACAACCATGTACCAACTGCTTTTCCA-3’
oHDK168	5’- AAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGATCAGATTACGAGAGCACCAT-3’
oHDK169	5’- GGATACAGTTCTCACATCACATCCGAACATAAACAACCATGTTAGCTGATATATTAATCC-3’
oHDK170	5’- AAGTTCTTGAAAACAAGAATCTTTTTATTGTCAGTACTGATCACAATGCAGTTAGTTGAA-3’

Cloning Bs*yvmC* and Bs*cypX* with Eg*TEF1* promoter and terminator into pIL68

oHDK226	5’- CAGTTCTCACATCACATCCGAACATAAACAACCATGACCGGAATGGTAACGGAAAGAAGG-3’
oHDK227	5’- TGAAAACAAGAATCTTTTTATTGTCAGTACTGATCATCCTTCAGATGTGATCCGTTTCTC-3’
oHDK259	5’- CACATCACATCCGAACATAAACAACCATGAGCCAATCGATTAAATTGTTTAGTGTGCTTT-3’
oHDK260	5’- AAACAAGAATCTTTTTATTGTCAGTACTGATTATGCCCCGTCAAACGCAACGAG-3’

Table S3 – Topology tests of the *PUL* gene trees.

Gene	Alignment program	Trimmed alignment length	One-sided KH test *p*-value	AU test *p*-value
PUL1	MAFFT	321	0.50	1.0
	MUSCLE	377	0.49	0.99
	T-Coffee	356	0.51	1.0
PUL2	MAFFT	353	0.51	0.93
	MUSCLE	390	0.51	0.92
	T-Coffee	366	0.51	0.92
PUL3	MAFFT	199	0.015	0.083
	MUSCLE	238	0.14	0.06
	T-Coffee	217	0.096	0.084
PUL4	MAFFT	144	0.016	0.076
	MUSCLE	342	0.078	0.22
	T-Coffee	250	0.15	0.25

We tested for HGT within the subphylum by using one-sided Kishino-Hasegawa (KH) tests for significant differences between likelihood values for two trees constructed from the same gene alignment: in our case the likelihoods of the ML gene tree and a tree constrained by the genome-wide species topology (Figure 1). This type of test is generally very liberal: when tests fail to the meet the *p*-value threshold, there is no statistically significant difference between the likelihood values of the two trees, and we conclude the GW tree is as good a representation of the data as the gene tree, but tests exceeding the *p*-value threshold do not necessarily indicate significant differences. We further used the Approximately Unbiased (AU) test to assess the likelihood distribution of a large set of reasonable alternative topologies to the ML tree, including all possible arrangements of the genera analyzed for each gene tree. For the 12 alignments tested, we used a Bonferroni-corrected *p*-value cutoff of 4.1×10^{-3}, and none of the alignments for either test met this threshold, meaning that the genome-wide inferred topology cannot be rejected as a possible representation of the data. We reason that phylogenetic incongruences in the *PUL3* and *PUL4* trees within the Saccharomycotina are more likely to be explained by the result of convergent selective regimes in the conserved, well-aligned regions of the genes.

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