High sorbic acid resistance of *Penicillium roqueforti* is mediated by the SORBUS gene cluster

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Abstract

Penicillium roqueforti is a major food-spoilage fungus known for its high resistance to the food preservative sorbic acid. Here, we demonstrate that the minimum inhibitory concentration of undissociated sorbic acid (MIC_u) ranges between 4.2 and 21.2 mM when 34 *P. roqueforti* strains were grown on malt extract broth. A genome-wide association study revealed that the six most resistant strains contained the 180 kbp gene cluster SORBUS, which was absent in the other 28 strains. In addition, a SNP analysis revealed five genes outside the SORBUS cluster that may be linked to sorbic acid resistance. A partial SORBUS knock-out (>100 of 180 kbp) in a resistant strain reduced sorbic acid resistance to similar levels as observed in the sensitive strains. Whole genome transcriptome analysis revealed a small set of genes present in both resistant and sensitive *P. roqueforti* strains that were differentially expressed in the presence of the weak acid. These genes could explain why *P. roqueforti* is more resistant to sorbic acid when compared to other fungi, even in the absence of the SORBUS cluster. Together, the MIC_u of 21.2 mM makes *P. roqueforti* among the most sorbic acid-resistant fungi, if not the most resistant fungus, which is mediated by the SORBUS gene cluster.

Author summary

Chemical preservatives, such as sorbic acid, are often used in food to prevent spoilage by fungi, yet some fungi are particularly well-suited to deal with these preservatives. First, we investigated the resistance of 34 *Penicillium roqueforti* strains to various food preservatives. This revealed that some strains were highly resistant to sorbic acid, while others are more sensitive. Next, we used DNA sequencing to compare the genetic variation between these strains and discovered a specific genetic region (SORBUS) that is unique to the resistant strains. Through comparative analysis with other fungal species the SORBUS region was studied in more detail and with the use of genetic engineering tools we removed this unique region. Finally, the mutant lacking the SORBUS region was confirmed to have lost its sorbic acid resistance. This finding is of particular interest as it suggests that only some, not all, *P. roqueforti* strains are potent spoilers and that specific genetic markers could help in the identification of resistant strains.

Introduction

Fungi are responsible for 5–10 % of all food spoilage [1] resulting in the production of off-flavors, discoloration and acidification [1,2]. Some species also produce mycotoxins, such as PR toxin and roquefortine C in the case of *Penicillium roqueforti* [3]. Toxins partially contribute to the high incidence of food-borne diseases affecting up to 30 % of the people in industrialized countries every year [4].

Food spoilage by filamentous fungi is believed to be mainly caused by spores that are spread through the air, water or other vectors like insects [5]. Preservation techniques such as pasteurization, fermentation, cooling or the addition of preservatives are used to reduce spoilage [6]. Some of the most applied preservatives are weak organic acids such as benzoic, propionic and sorbic acid. *Paecilomyces variotii, Penicillium paneum, Penicillium carneum* and *P. roqueforti* are among the few filamentous fungi capable of spoiling products containing weak acids, and are therefore called preservative-resistant moulds [7]. Weak-acid preservatives inhibit microbial growth, but their mode-of-action is not completely understood. According to the classical 'weak-acid preservative theory' the antimicrobial activity of weak acids is derived from their undissociated form that can pass the plasma membrane. These weak acids dissociate in the cytosol due to its neutral pH, and inhibit growth through acidification of the cytoplasm [8]. The inhibitory activity of sorbic acid at pH 6.5 and the correlation of sorbic acid resistance with ethanol tolerance in *Saccharomyces cerevisiae* suggest that this weak acid can also act as a membrane-active compound [8].

In *A. niger*, sorbic acid resistance is mediated by the phenylacrylic acid decarboxylase gene *padA*, and the putative 4-hydroxybenzoate decarboxylase gene, known as the cinnamic acid decarboxylase (*cdcA*) gene [9,10]. These genes encode proteins that catalyze the conversion of sorbic acid into 1,3-pentadiene. Genes *padA* and *cdcA* are regulated by the sorbic acid decarboxylase regulator (SdrA), which is a Zn2Cys6-finger transcription factor [10]. These three genes are present on the same genetic locus in *A. niger* with *sdrA* being flanked by *cdcA* and *padA.* Orthologs of *cdcA* and *padA* are also clustered in *S. cerevisiae*, but this is not the case for the *sdrA* homologue [10,11]. Inactivation of *cdcA, padA* or *sdrA* results in reduced growth of *A. niger* on sorbic acid and cinnamic acid [9]. The fact that growth is not abolished and the fact that *padA* transcript levels are less affected than that of *cdcA* on sorbic acid upon deletion of *sdrA* suggests that an additional regulator is involved [9]. Indeed, the

weak-acid regulator WarA is also involved in sorbic acid resistance as well as other weak acids such as propionic and benzoic acid [12].

Weak-acid resistance in fungi is subject to intra- and inter-strain heterogeneity. Intra-strain heterogeneity has been observed in *Zygosaccharomyces bailii* with the existence of a small sub-population of cells that are more resistant (MIC = 7.6 mM) to sorbic acid than the sensitive population (MIC = 3 mM) [13], while inter-strain resistance has been observed in *P. roqueforti* with the existence of sorbic acid-resistant and sorbic acid-sensitive strains [14]. Genome analysis of *P. roqueforti* strains have yielded clues about adaptive divergence in this species. Genomic islands with high identity are present in distant *Penicillium* species, while they are absent in closely related species, supporting the hypothesis of recent horizontal gene transfer events [15,16]. For instance, the presence of two large genomic regions, *Wallaby* and *CheesyTer* correlates with faster growth and functions relevant in a cheese matrix, respectively [16,17]. Noteworthy, the *CheesyTer* and *Wallaby* regions are only present in cheese isolates other than Roquefort, indicating that the Roquefort isolate population is potentially more closely related to the 'ancestral' *P. roqueforti* populations [18].

In this study, sorbic acid resistance of 34 *P. roqueforti* strains was assessed. A genome-wide association analysis revealed the presence of the 180 kbp gene cluster SORBUS in the six most resistant strains. A partial SORBUS knockout in such a strain showed a reduced sorbic acid resistance similar to that of the other 28 sensitive *P. roqueforti* strains.

Results

Weak-acid sensitivity screening

Weak-acid sensitivity of 34 *P. roqueforti* wild-type strains was assessed on MEA plates supplemented with 5 mM propionic, sorbic or benzoic acid, which corresponds to 4.42, 4.25 and 3.07 mM undissociated acid, respectively. Three strains had been isolated from blue-veined cheeses such as Roquefort and the other 31 strains had been isolated from non-cheese environments (mostly related to spoiled food) (Table S1). The colony surface area was determined after five days of growth (Fig 1). The inhibitory effect of propionic acid at the tested concentration was limited for most strains, since 26 out of 34 strains grew to > 80 % of the colony surface area reached under control conditions. Strains DTO012A1 and DTO012A8 even showed an increased colony size (up to 120 %) when compared to the control. The inhibitory effects of sorbic and benzoic acid were more pronounced. MEA supplemented with

potassium sorbate reduced colony area for all 34 *P. roqueforti* strains. The surface area under sorbic acid stress ranged from 0 to 80 % of the surface area reached under control conditions. Strains DTO006G1, DTO006G7, DTO013E5 and DTO013F2 showed the highest sorbic acid resistance, followed by DTO046C5. Benzoic acid was the most inhibitory compound, resulting in a maximum colony surface area between 0 and 20 % of the control. The most benzoic acid-resistant strains were DTO013F2 and DTO013E5. As these strains were also among the most sorbic acid-resistant strains, similar resistance mechanisms may be involved to cope with benzoic and sorbic acid stress.

Sorbic acid resistance was further analyzed by determining the MIC_u values of sorbic acid for the 34 *P. roqueforti* strains. The four strains with the highest sorbic acid resistance on MEA (Fig 1), were also among the strains (DTO006G1, DTO006G7, DTO012A1, DTO012A8, DTO013E5 and DTO013F2) that showed the highest MIC_u (Fig 2). DTO013E5 and DTO013F2 were the most resistant even showing growth at the highest tested undissociated sorbate concentration of 21.2 mM, indicating a MIC_u > 21.2 mM. The other strains showed a distinctly lower MIC_u, ranging between 4.2 mM and 9.95 mM. Only strain DTO012A9 showed an intermediate resistance to sorbic acid with an average MIC_u of 13.72 mM undissociated sorbic acid.

Genome statistics and phylogeny

The genomes of the 34 *P. roqueforti* strains were sequenced. Scaffold count varied between 45 and 1358, assembly length between 26.53 and 31.74 Mb and GC content between 46.85 and 48.44 % (Table 1). The number of predicted genes varied between 9633 and 10644, the number of genes with PFAM domains between 73.11 and 75.38 %, and the number of secondary metabolism gene clusters between 32 and 36. All strains had a BUSCO completeness of >99 %, indicating high quality assembly and gene predictions, except for DTO012A8 with a completeness of 94.83 %. This and the high scaffold count of the DTO012A8 assembly (1358 scaffolds) indicates that its genome assembly is not complete. The strain was kept in the downstream analysis as most other metrics did not differ much compared to the other strains (Table 1). Figure 3 presents a phylogenetic tree of the 34 *P. roqueforti* strains based on 6923 single-copy orthologous genes. Three of the six sorbic acid-resistant strains (DTO012A2, DTO013F2, DTO013E5 and DTO012A8) are similar with DTO012A8 being closely

138 related to those strains. The two other resistant strains are also similar and more distantly
139 related.
140
141 *Sorbic acid resistance correlates with a genomic cluster containing genes regulating sorbic acid*
142 *decarboxylation*
143 The 34 *P. roqueforti* strains were divided into two groups based on their sorbic acid resistance,
144 a group of six resistant (R-type) strains ('a', Fig 2) and a group of 28 sensitive (S-type) strains
145 ('b-d', Fig 2). Whole genome comparison methods are often based on differences in variants
146 (SNPs), however these methods do no reveal larger missing regions or genes between strains.
147 Hence, a GWAS method was developed to compare whole-genome assemblies based on the
148 MUMmer software. With this method 57 genes unique for the R-type group were identified,
149 of which 51 were present on scaffold 43 of DTO006G7 (Table 2). In addition to the 51 unique
150 genes in this scaffold, it contains 19 genes which are also found completely or in part in some
151 of the S-type strains. The genomic alignment shows the genes on scaffold 43, which is present
152 in the R-type strains (Fig 4). The first 80 kbp of scaffold 43 (protein IDs g12000-g12029) mainly
153 contains hypothetical proteins without predicted function, while the remaining region
154 between 80-180 kbp (g12030 – g12069) contains multiple regions homologous to genes
155 previously reported as related to weak-acid resistance in *A. niger*. Predicted genes orthologous
156 to *padA*, *cdcA* and *sdrA* of *A. niger* were found alongside each other (g12064-g12066) with
157 respective identities (based on BLAST) of 87 %, 83 % and 53 %. Additional orthologs of *cdcA*,
158 named *cdcB* (g12056) and *cdcC* (g12040) were identified on the same gene cluster as well,
159 with identities of 72 % and 71%, respectively, when compared to *cdcA* of *A. niger*. Another
160 locus (g2591) outside of this cluster also contains a protein homologous to *cdcA* (82 % identity)
161 that is also present in the S-type strains. Similarly, two *padA* orthologs with high BLAST
162 similarities to *padA* of *A. niger* (63 % and 58 %) were identified on the R-type specific cluster
163 and named *padB* (g12032) and *padC* (g12057). In contrast, no homologs of *A. niger sdrA* and
164 *padA* were found outside of the cluster, while 1 and 3 homologs were present inside cluster,
165 respectively. In addition, a transcription factor (g2820 in DTO006G7) orthologous to *warA* was
166 identified outside of the cluster in the genomes of all strains. These results indicate that the
167 R-type strains contain a gene cluster similar to the sorbic acid resistance gene cluster described
168 in *A. niger*, but considerably expanded [9,10]. For further reference, we name this cluster (i.e.,
169 scaffold 43 of strain DTO006G7) SORBUS after the tree *Sorbus aucuparia* as sorbic acid has

been first isolated from its berries by August Hoffman [19,20]. While SORBUS as a whole is only present in the R-type strains (DTO006G1, DTO006G7, DTO012A2, DTO012A8, DTO013F2, DTO013E5), some S-type strains share up to 5 kbp parts of the sequence, especially in the first 80 kbp of the cluster (Fig 4). It should be noted that the R-type strains were all isolated from non-cheese environments. Based on alignments of the sequencing reads to DTO006G7 we confirmed that out of 35 previously sequenced *P. roqueforti* strains [18], none of the 17 cheese strains contained the SORBUS cluster, while two out of the 18 non-cheese strains contained the SORBUS cluster (Table S1).

PLINK [21] was used to identify which SNPs correlate to sorbic acid resistance. This method allowed for the quantitative use of $\log_{10}(MIC_u)$ values as input for analysis, as opposed to the approach described above. The SNPs are visualized in a Manhattan plot (Fig 5). SNPs located on genes with a $-\log_{10}(P) > 5$ and either a high or moderate impact (SNPeff) were selected (Table 3). This resulted in 338 SNPs in 41 genes. Out of these SNPs, 29 had a 'high' impact according to SNPeff and were located in 17 genes. Only six out of these 17 genes with high impact variants (g7017, g8100, g8106, g9942, g9943, g9976) were not located in the SORBUS cluster, the other 11 genes were either among the non-unique genes present on SORBUS, or genes of which less than 90 % of the sequence was found in S-type strains. With a BLAST analysis (protein-protein) and PFAM annotations the functions of the predicted proteins were assessed. This revealed that gene g8100 contains an ankyrin-repeat containing domain and gene g9943 is homologous to a zinc finger C3H1-type domain-containing protein, the putative function of the other three genes could not be assessed (hypothetical proteins). In all cases, the five genes showed high homology (> 99 %) to other *Penicillium* species. The PLINK analysis also revealed SNPs in two genes encoding proteins with a putative transmembrane transporter (g216) or cation transporter (g296) function.

To investigate the evolutionary origin of this cluster, presence of five PFAM domains (from g12060, g12061 and g12063-g12065) that are present on the SORBUS cluster was analysed in 32 Aspergilli and Penicillia as well as the 34 *P. roqueforti* strains (Table 4). These PFAM domains encode a putative GPR1/FUN34/yaaH family (g12060), a flavin reductase like domain (g12061), 3, 4-dihydroxy-2-butanone 4-phosphate synthase (g12063), a flavoprotein (g12064, *padA*) and a UbiD domain (g12065, *cdcA*). These domains are selected as they are clustered and because of their predicted role. The first domain has been associated with acetic acid sensitivity in *S. cerevisiae* [22], while the latter four domains are part of the gene cluster

described in *A. niger* [9]. Genes containing these domains were aligned using MAFFT and alignments were used to construct a phylogenetic tree per domain. Based on these phylogenetic trees the relatedness of the five genes of the SORBUS cluster was assessed. This revealed that the SORBUS genes g12061, g12064 and g12065 cluster more closely with several *Aspergillus* species (Table 4), whereas the PFAM domains from g12060 and g12063 did not cluster with any of the species included. In addition, the PFAMs present in the core genome (present both in S- and R-type strains) aligned more closely to *P. digitatum* or *P. oxalicum*. Furthermore, two genes with homology to transposase-like proteins (g12052 and g12055) and several reverse-transcriptase domains were identified in the SORBUS cluster.

Genome-wide expression profiles of a sorbic acid-sensitive and sorbic acid-resistant strain

A genome-wide expression profile was performed on a sorbic acid-sensitive strain (DTO377G3) and a sorbic acid-resistant strain (DTO006G7) grown on MEB in the presence or absence of 3 mM sorbic acid. The sequence reads were aligned to the assemblies of these two *P. roqueforti* strains. Gene expression values were calculated and differentially expressed genes were identified (Table S4). The expression profiles of the biological replicates were similar, demonstrated by their clustering in the PCA plot (Fig 6). Combined, PC1 and PC2 explain 90 % of the variation observed. The samples treated with sorbic acid separate from the control samples on Y-axis while the differences between the strains are separated by on the X-axis.

Genes that are either up- or down-regulated in both the R-type and S-type strain when exposed to sorbic acid might be involved in a general response to sorbic acid stress in *P. roqueforti*. Venn diagrams were constructed revealing that 33 genes were significantly up-regulated in both the S-type and R-type strain (Fig 7A). An enrichment analysis revealed that the functional annotation terms ‘secretion signal’ and ‘small secreted protein’ are over-represented in these genes. Among the 21 shared down-regulated genes (Fig 7B) the NmrA-like family and NAD(P)H-binding domains were over-represented. Table S5 lists all genes present in both shared pools. The expression of the SORBUS genes (g12000-g12069) was analysed. This revealed that nine out of the 70 genes were significantly differentially expressed (Table 2), including two out of the three genes homologous to *cdcA* that were lower expressed ($\log_2$FC = -1.5) in the presence of sorbic acid. On the SORBUS cluster g12061 (with a flavin-reductase like domain) was the highest expressed gene, reaching 2000 FPKM in the control condition.

Partial knock-out confirms role of SORBUS in sorbic acid resistance

Strains *P. roqueforti* DTO013F2Δ*kusA* SC1 and SC2 were obtained lacking part of the SORBUS cluster. Nanopore sequencing was used to investigate how much of the SORBUS cluster was removed in these two transformants (Fig 8). This revealed that the deletions were larger than the 93 kbp region. A 105 and a 131 kbp part of the cluster was removed in DTO013F2 Δ*kusA* SC1 and SC2, respectively. Detailed analysis of the nanopore reads revealed that the smaller fragments within the SORBUS cluster present in SC1 and SC2 strains consisted of highly dissimilar sequences, indicating these are parts of repetitive DNA (Fig 8). Both SC strains had a reduced resistance to sorbic acid when compared to DTO013F2 and DTO013F2 Δ*kusA* with a MIC_u similar to the S-type strain DTO377G3 (Fig 9). This shows that part of the SORBUS cluster is involved in sorbic acid stress mitigation.

Discussion

P. roqueforti is often encountered as a spoilage organism of food and feed. This can partly be attributed to its ability to grow at refrigeration temperatures [23], low O_2 levels [24] and/or its resistance to preservatives, such as sorbic acid [25]. The inhibitory effect of propionic, benzoic and sorbic acid on *P. roqueforti* growth was assessed and benzoic acid was found to have the strongest inhibitory effect on 34 *P. roqueforti* strains. Previous studies report that *P. roqueforti* is resistant to benzoic acid. Growth was observed up to levels of 3000 ppm sodium benzoate [26,27], while we observed growth at 610 ppm (5 mM). Propionic acid hardly affected the growth of *P. roqueforti*, which is not surprising since it has already been reported that this fungus germinates on potato dextrose agar containing 0.5 M propionic acid (pH 5.6) with an estimated MIC of 0.79 M [23]. The sorbic acid resistance of *P. roqueforti* has also been assessed previously [14,25–30], reporting MIC values ranging from 0 – 40 mM sorbic acid (i.e. a MIC_u = 0 – 20 mM). This is similar to the range (MIC_u = 4.2 – 21.3 mM) found in this study. In fact, a resistant and sensitive group (R- and S-types) consisting of six and 28 strains, respectively, were found in this study. S-type and R-type strains showed a resistance up to 11.9 mM and 21.2 mM undissociated sorbic acid, respectively.

The R-type but not the S-type strains were found to contain a gene cluster (SORBUS) containing 70 genes, of which 51 genes are unique for the R-type strains. Even though the R-type strain DTO006G7 was used with the least fragmented Illumina assembly, the limits of the

SORBUS cluster could not be established based on these results alone. The Oxford Nanopore of the DTO013F2 Δ*kusA* assembly revealed that SORBUS is part of a 2.3 Mb contig. Genes homologous to sorbic acid degradation-associated genes in *A. niger* (*sdrA*, *cdcA*, *padA*, *warA*) were identified in the *P. roqueforti* genome [9,12]. A total of 1, 4, 3 and 1 orthologs were found, respectively, and only *warA* and one *cdcA* ortholog were not located on the SORBUS cluster. Two genes with putative transmembrane transport (g216) or cation transporter (g296) function were identified in the PLINK analysis. The encoded proteins might also be involved in sorbic acid stress mediation alongside the SORBUS cluster, because in addition to decarboxylation, sorbic acid stress could be mediated by an efflux pump or through removal of protons from the plasma membrane by H^+-ATPase [12,31].

As mentioned, only six out of the 34 strains assessed in this study were found to contain the SORBUS cluster. This might be explained by the ecology of *P. roqueforti. P. roqueforti* is found in forest soil and wood, but is also associated with lactic acid bacteria (e.g. in silage). Frisvad & Samson [32] already speculated that these micro-organisms may have very well co-evolved, because all the metabolics produced by lactic acid bacteria (e.g. lactic and acetic acid, CO_2) are tolerated by *P. roqueforti* [33]. The elevated levels of weak acids might act as selection pressure to maintain SORBUS in *P. roqueforti* strains which grow in this niche environment. In contrast, this selection pressure is not present in cheese which might explain that none of *P. roqueforti* strains in the 'cheese' population contain the SORBUS cluster [18]. It should be noted that the S-type sequence fragments that align with SORBUS mostly consists of proteins annotated as transposase-like proteins or reverse transcriptase, which might explain why these fragments are found in the S-type strains and it suggests that SORBUS has been obtained from a different species by horizontal gene transfer. This is supported by the phylogenetic analysis on PFAM domains of five SORBUS genes, as the results show that three out of the five SORBUS specific genes are more closely aligned to *Aspergillus* species than *Penicillium* species. The gene cluster SORBUS was also present in two of 35 previously sequenced *P. roqueforti* strains [18] and their sorbic acid resistance could be determined to validate the role of SORBUS in sorbic acid resistance.

Transcriptome analysis of the R-type *P. roqueforti* strain DTO006G7 revealed that two of the three *cdcA* paralogs (*cdcA* and *cdcC*) are significantly down-regulated during growth in the presence of sorbic acid. This is in contrast with the results previously described [9], where the authors found a > 500-fold change for *cdcA* when *A. niger* was cultivated on sorbic acid.

298 This difference might be caused by the difference in medium type, because that study [9] used
299 sorbic acid as the sole carbon source, whereas in our experiments sorbic acid was used as a
300 stressor in a nutrient-rich medium. This indicates that the sorbic acid content in the medium
301 does not increase gene expression of the loci leading to increased resistance, suggesting that
302 either these genes are constitutively expressed or expression is induced based on a different
303 compound present in MEB.

304 Two partial SORBUS knockout strains in the DTO013F2 Δ*kusA* R-type strain showed
305 reduced sorbic acid resistance to a level similar to that of the S-type strains. In contrast to the
306 DTO013F2 Δ*kusA* strain, the SORBUS knockout strains were not repaired through homology
307 directed repair using the donor DNA nor non-homologous end-joining. This might be due to
308 the size of the fragment. Possibly, an alternative DNA repair mechanism such as
309 microhomology-mediated end joining as described in *A. fumigatus* is employed in the
310 DTO013F2 Δ*kusA* strain [34].

311 Despite the absence of a full SORBUS cluster in the S-type strains and the deletion strain, their
312 MIC is still relatively high when compared to other fungal species such as *A. niger* (as shown
313 in Fig 10) or *A. fumigatus* [12]. This suggests that along with the genes on the SORBUS cluster,
314 other proteins are involved in sorbic acid stress mitigation. Our transcriptomics analysis
315 revealed 21 down-regulated and 33 up-regulated genes that were similarly expressed both in
316 a S-type and a R-type strain when exposed to sorbic acid. One of these up-regulated genes is
317 the cation transporter (g1689, Table S4) which could have H^+ ATPase activity in the plasma
318 membrane to counteract the acidification caused by the undissociated sorbic acid in the
319 cytosol [31].

320 In conclusion, the results presented in this study demonstrate that weak-acid
321 resistance varies between *P. roqueforti* strains and the SORBUS cluster contributes to a high
322 sorbic acid resistance. Yet, even in the absence of this cluster the resistance is still relatively
323 high, implying that other mechanisms are also involved in resistance to this weak acid.

324

Methods

Strain and cultivation conditions

All fungal strains (Table S1) were provided by the Westerdijk Fungal Biodiversity Institute. Conidia were harvested with a cotton swab after seven days of growth at 25 °C on malt extract agar (MEA, Oxoid, Hampshire, UK) and suspended in 10 mL ice-cold ACES buffer (10 mM N(2-acetamido)-2-aminoethanesulfonic acid, 0.02 % Tween 80, pH 6.8). The conidia suspension was passed through a syringe containing sterilized glass wool and washed twice with ACES buffer after centrifugation at 4 °C for 5 min at 2,500 *g*. The spore suspension was set to $2 \cdot 10^8$ spores mL^{-1} using a Bürker-Türk haemocytometer (VWR, Amsterdam, The Netherlands) and kept on ice until further use.

Weak acid growth assay

Conidial suspension (5 µL) was inoculated in the centre of MEA plates containing 5 mM potassium sorbate, benzoic acid, or sodium propionate (all from Sigma). Medium was set at pH 4.0 using HCl, which corresponds to undissociated concentrations of 4.26, 3.07 and 4.42 mM of these acids, respectively. The absence of preservative was used as a control. Cultures were photographed after 5 days and colony surface area was measured using a manual threshold in ImageJ.

Sorbic acid resistance

Conidial suspensions of the *P. roqueforti* strains were diluted to 10^7 spores mL^{-1} and mixed in a 1:99 ratio with MEB pH 4.0 with and without 25 mM potassium sorbate. 300 µL of the resulting mixture was added in a well of a 96 wells plate (Greiner Bio-One, Cellstar 650180, www.gbo.com). Serial dilutions were made by mixing 225 µL MEB with potassium sorbate and 75 µL MEB without potassium sorbate, resulting in wells with potassium sorbate concentrations of 25, 18.75, 14.06, 10.55, 7.91, 5.93, 4.45 and 0 mM. This corresponded to undissociated sorbic acid concentrations of 21.22, 15.92, 11.94, 8.95, 6.72, 5.04, 3.78 and 0 mM, respectively. The undissociated sorbic acid concentrations were determined using the Henderson-Hasselbach equation.

The 96-wells plates were sealed with parafilm and incubated for 28 days at 25 °C using biologically independent replicates. After 28 days, growth was assessed and the undissociated minimal inhibitory concentration (MIC_u) was determined for each strain. The MIC_u was defined

as the lowest undissociated concentration in which no hyphal growth was observed. An one-way ANOVA followed by a Tukey's HSD test was used to test for significant differences in MIC_u ($P < 0.05$).

DNA extraction, genome sequencing, assembly and annotation

DNA extraction was performed as described [35] and Illumina NextSeq500 2x150 bp paired-end technology was used for sequencing (Utrecht Sequencing Facility, useq.nl). The reads were trimmed on both ends when quality was lower than 15 using bbduk from the BBMap tool suite (BBmap version 37.88; https://sourceforge.net/projects/bbmap/). The trimmed reads were assembled with SPAdes v3.11.1 applying kmer lengths of 21, 33, 55, 77, 99 and 127 and the –careful setting was used to reduce the number of indels and mismatches [36]. Genes were predicted with Augustus version 3.0.3 [37] using the parameter set that was previously generated for *P. roqueforti* [35]. Functional annotation of the predicted genes was performed as described [38]. Repetitive sequences in the assembly were masked using RepeatMaker [39], RepBase library [40] and RepeatScout [41]. The Short Read Archive (SRA) numbers of the datasets in this study are listed in Table S1 under accession numbers. The genomes, gene predictions and functional annotations can be accessed interactively at https://fungalgenomics.science.uu.nl. [Available upon publication].

Genomic phylogeny and analysis

Single-copy orthologous groups were identified and aligned using OrthoFinder v2.5.2 [42]. A maximum likelihood (ML) inference was performed using RAxML [43]☐ under the PROTGAMMAAUTO model. The number of bootstraps used was 200 (Average WRF = 0.43 %) and *Penicillium rubens* Wisconsin 54-1255 [44] was used to root the tree. The phylogenetic tree was visualized using iTOL v5 [45].

Genome-wide association study

A genome-wide association study (GWAS) was performed based on the sorbic acid resistance screening and the whole-genome sequences. First, *P. roqueforti* strains were grouped into a resistant (R-type) or sensitive (S-type) group. DTO006G7 was selected from the R-group as reference for the analysis, because the assembly of this strain was the least fragmented in this group. Next, the program nucmer from the MUMmer suite (http://mummer.sourceforge.net/,

version 4.0) was used to perform whole-genome alignment. Each genome was aligned to the reference and regions and genes unique for the R-type isolates were identified with the BEDtools package. The genome alignment was visualized with pyGenomeTracks [46]. A gene was considered absent when 90 % or more of its sequence was not found in the genome of a strain. The best practices recommended by GATK (Genome Analysis Toolkit) were used to obtain single nucleotide polymorphisms (SNPs) for each strain. In short, the sequence reads were aligned to the reference genome (DTO006G7) using Bowtie2 (version 2.2.9) and PCR duplicates were removed with Picard tools (MarkDuplicates; version 2.9.2). For variant calling, the HaplotypeCaller (GATK, version 3.7) was used with the following parameters: -stand_call_conf 30, -ploidy 1 and -ERC. The single-sample variant files (GVCFs) were joined into a GenomicsDB before joint genotyping. The variants were annotated using SNPeff (version 4.3) based on their predicted biological effect, such as the introduction of an early stop-codon or a synonymous annotation. The number of SNPs and their putative impact ('low', 'moderate' or 'high', as defined by SNPeff) was listed for each gene per genome. The SNPs were then correlated to sorbic acid resistance using PLINK v1.9 [21] with parameters '–maf 0.5 –allow-extra-chr'. The resulting association files were analysed and visualized using R.

RNA extraction and sequencing

A genome-wide transcriptome analysis was performed on the sorbic acid sensitive *P. roqueforti* strain DTO377G3 and the sorbic acid-resistant *P. roqueforti* strain DTO006G7. Erlenmeyer flasks containing 50 mL MEB (pH 4.0) were inoculated with 100 µl ACES containing 10^7 conidia and incubated for 48 h at 25 °C and 200 rpm. Mycelium was harvested using a sterilized miracloth filter and equally divided in Erlenmeyer flasks with 50 mL MEB (pH 4.0) or 50 mL MEB containing 3 mM potassium sorbate (pH 4.0). Growth was continued for another four hours, after which the mycelium was harvested using a sterilized Miracloth filter and frozen in liquid nitrogen. Total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen) and purified by on-column DNase digestion according to the manufacturer's protocol. RNA was sequenced with Illumina NextSeq2000 2x50 bp paired-end technology (Utrecht Sequencing Facility; useq.nl). The transfer experiment and subsequent RNA-sequencing was performed in biological triplicates. The transcript lengths, counts per gene and read mapping were determined using Salmon v1.5.2 with --validateMappings [47]. The transcript abundance of reads was quantified using custom constructed indices for DTO006G7 and DTO377G3.

DESeq2 [48] was used for pairwise comparisons of the samples and the identification of differentially expressed genes. Genes with low read counts (<10) were excluded from the analysis and a gene was considered differentially expressed when the adjusted p-value was < 0.05. In addition, genes were considered up- or down-regulated when they had a $\log_2$ fold change of > 2 or < -2, respectively. A Fisher Exact test as implemented in PyRanges [49] was employed to identify over- and under-representation of functional annotation terms in sets of genes. To correct for multiple testing the False Discovery Rate method was used, with a P-value < 0.05 as cut off.

The sequence reads are available in the Short Read Archive under BioProject PRJNA796729 [available upon publication].

Plasmid construction and generation knockout strain

A *kusA* deficient DTO013F2 strain was constructed using the protocol and plasmid pPT22.4 as described [50]. Deletion was confirmed through diagnostic PCR and nanopore sequencing (Fig S1). CRISPR/Cas9 technology was employed to remove a 93 kbp region (from 84 to 177 kbp) of the SORBUS cluster in DTO013F2 Δ*kusA* . Two single-guide RNAs (sgRNAs) were designed to perform a simultaneous double restriction in the 93 kbp SORBUS region. Transformation procedures were performed as described, with some modifications [50]. In short, plasmid pFC332 [51] was used as a vector to express the sgRNA, *cas9* and a hygromycin selection marker (for primer sequences used in this study see Table S2). The 5' and 3' flanking regions of sgRNA were amplified using plasmids pTLL108.1 and pTLL109.2 as template [52]. The amplified products were fused and introduced into pFC332 using Gibson assembly (NEBuilder HiFi DNA Assembly Master Mix, New England Biolabs, MA, USA). The vectors containing the sgRNA were then transformed into competent *Escherichia coli* TOP10 cells for multiplication overnight. Plasmids were recovered using Quick Plasmid Miniprep Kit (ThermoFisher, Waltham, MA, USA) and digested using SacII to verify the presence of the sgRNA. In addition, the correct integration of the sgRNA was confirmed with sequencing. To construct donor DNA, two 1 kbp homologous regions located at scaffold 43 at nucleotide position 83573 to 84629 and 177760 to 178854 were amplified and fused using a unique 23 nucleotide sequence GGAGTGGTACCAATATAAGCCGG with a PAM site for further genetic engineering.

Transformation was performed as described with adjustments [53]. In short, *P. roqueforti* conidia were incubated 48 h at 25 °C in 100 mL potato dextrose broth at 200 rpm.

453 The mycelium was washed in SMC and incubated for 4 h at 37 °C in lysing enzymes from
454 *Trichoderma harzianum* (Sigma) dissolved in SMC. Protoplasts were resuspended in 1 mL STC
455 and kept on ice after centrifuging for 5 min at 3000 g. To 100 µL of this suspension, 2 µg donor
456 DNA, 2 µg of each pFC332 vector containing sgRNA (pTF and pSdrA) and 1.025 mL of freshly
457 made PEG solution was added (see Table S3 for the vectors used in this study). After 5 min, 2
458 mL STC was added and the protoplasts were mixed with 20 mL liquid MMS containing 0.3 %
459 agar and 200 µg hygromycin mL^{-1} (InvivoGen, San Diego, CA, USA). The mixture was poured
460 on MMS containing 0.6 % agar and 200 µg hygromycin mL^{-1}. Transformants were grown for 7-
461 14 days at 25 °C and then single streaked on MM containing 100 µg mL^{-1} hygromycin until
462 sporulating colonies appeared. Next, the plasmid was removed by a single streak on MM
463 plates without antibiotic. Finally, transformants were single streaked on MM, MM containing
464 100 µg hygromycin mL^{-1} and MEA plates to confirm that transformants lost the plasmids.
465 To verify the transformants, genomic DNA from DTO013F2 Δ*kusA* and two DTO013F2
466 Δ*kusA* Δ*SORBUS* strains was sequenced with Oxford Nanopore MinION technology (FLO-
467 MIN106) at the Utrecht Sequencing Facility (useq.nl). The reads were assembled using Canu
468 v2.2 using the option for raw nanopore data and guided by a genome size of 28 Mbp [54]. In
469 addition, the nanopore reads were aligned to the DTO006G7 assembly using Minimap2 [55].
470 Only reads aligning once and that had a mapping quality > 60 were selected using samtools.
471 The sequencing data generated with the MinION technology is deposited in the SRA archive
472 under the following accession numbers: SRR17178875, SRR17178876 and SRR17178877.
473
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478 184.034.019).
479

Figure captions & Tables

Fig 1. Weak-acid screening shows variabe resistances to multiple acids.

Average colony size (cm^2) of 34 *P. roqueforti* strains after five days of growth on MEA (pH 4.0) (grey) or MEA (pH 4.0) supplemented with 5 mM propionic acid (orange), sorbic acid (blue) or benzoic acid (green). Strains DTO006G1, DTO006G7, DTO013E5 and DTO013F2 are relatively resistant to sorbic acid. Error bars indicate standard deviation of biologically independent replicates.

Fig 2. Sorbic acid resistance screening reveals 6 resistant *P. roqueforti* strains.

Average undissociated MIC_u values of sorbic acid of 34 *P. roqueforti* strains (mM ± standard deviation). Each bar graph represents biological triplicates. Error bars indicate standard deviation and letters indicate significant difference in MIC_u ($p < 0.05$).

Fig 3. Phylogeny of the *P. roqueforti* strains.

Phylogenetic tree of the 34 *P. roqueforti* strains used in this study. Sorbic acid resistance (MIC_u) is indicated in blue-yellow shading. The tree is based on 6923 single-copy orthologous genes and was constructed using RAxML. *P. rubens* [44] was used as outgroup. Bootstrap values <100 are indicated. Strains containing the SORBUS cluster are highlighted in blue.

Fig 4. Genome comparison reveals unique gene cluster in R-type strains.

Genomic alignment of 33 *P. roqueforti* strains to the SORBUS cluster (scaffold 43 of DTO006G7). Predicted genes are indicated with arrows, repetitive DNA is indicated in red, the sequence read coverage is indicated in the green tracks, and blue bars indicate (partial) overlap with DTO006G7. The complete SORBUS cluster is only present in the R-type strains (DTO006G1, DTO006G7, DTO012A2, DTO012A8, DTO013F2 and DTO013E5).

Fig 5. Manhattan plot shows SNPs associated with sorbic acid resistance.

Scaffolds are listed on the x-axis, while the y-axis display the significance of the association ($-\log_{10}$(p-value)). Yellow, orange and red dots indicate 'low', 'moderate' or 'high' impact SNPs as determined by SNPeff, respectively. The GeneIDs associated with the SNPs with a $-\log_{10}$(p-value) > 7.5 are indicated. The SORBUS cluster is located between the dashed lines.

Fig 6. Principle component analysis demonstrates clustering of sorbic acid-exposed strains. Each dot represents a biological replicate. PC1 and PC2 together describe 90% of the variation. The sample grown on sorbic acid separate on the Y-axis and the two strains are separated on the X-axis.

Fig 7. Venn diagram of differentially expressed genes in the presence of sorbic acid. Up- (A) or down-regulated (B) in R-type DTO006G7 and S-type DTO377G3 when exposed to sorbic acid compared to the control in the absence of this weak acid. Genes were considered up- or down-regulated when the $\log_2$FC > 2, p-value < 0.05 and FPKM >10.

Fig 8. Schematic overview of SORBUS sequences of the knockouts strains.
Strains DTO006G7, DTO013F2 and knock-out strains DTO013F2 Δ*kusA,* SC1 and SC2 are depicted. Predicted genes are indicated with arrows, repetitive DNA is indicated in red, the sequence read coverage is indicated in the green tracks, and blue bars indicate (partial) overlap with DTO006G7. The orange bar indicates the targeted knock-out region (target), flanked by the 5' and 3' ends (in blue). Dotted lines and scissors indicate the loci targeted by the sgRNAs.

Fig 9. Sorbic acid resistance of five *P. roqueforti* strains and *A. niger* N402.
The average MIC_u (mM ± standard deviation) is given for the fungal strains. Each bar graph represents average value of biological independent triplicates. Error bars indicate standard deviation and letters indicate significant difference in MIC_u ($p < 0.05$).

Table 1. Genome assembly and annotation statistics. The number of scaffolds, assembly length, GC content and genes of the 34 sequenced *P. roqueforti* strains are listed. In addition, the number of genes with a PFAM domain, the number of secondary metabolism gene clusters and the BUSCO completeness are listed. The type column indicates if the strain is sorbic acid-resistant (R) or sorbic acid-sensitive (S).

	Assembly			Annotation			Annotation quality	Type
Strain	Scaffolds	Total assembly length (Mbp)	Assembly GC content (%)	Genes	Genes with PFAM (total, %)	Secondary metabolism gene cluster	BUSCO2 completeness (%)	R/S
DTO002I6	134	29.48	47.75	10135	7539 (74.39%)	33	99.66%	S
DTO003C3	353	30.28	47.82	10362	7612 (73.46%)	36	99.66%	S
DTO003H1	382	30.29	47.82	10349	7607 (73.5%)	36	99.66%	S
DTO006G1	109	27.97	48.18	9975	7468 (74.87%)	33	99.66%	R
DTO006G7	100	27.97	48.18	9982	7471 (74.84%)	33	99.66%	R
DTO012A1	158	29.37	48.11	10280	7557 (73.51%)	33	100%	R
DTO012A2	73	27.07	48.26	9786	7362 (75.23%)	33	99.66%	S
DTO012A6	93	27.29	48.32	9817	7376 (75.13%)	33	99.66%	S
DTO012A7	62	27.33	48.07	9748	7335 (75.25%)	34	99.66%	S
DTO012A8	1358	29.6	47.88	10253	7483 (72.98%)	32	94.83%	R
DTO012A9	581	29.84	47.89	10341	7613 (73.62%)	34	99.31%	S
DTO013E5	165	29.27	48.11	10252	7537 (73.52%)	33	100%	R
DTO013F2	657	31.74	47.49	10644	7739 (72.71%)	33	100%	R
DTO013F5	304	29.56	48.04	10313	7591 (73.61%)	35	99.66%	S
DTO027I6	177	29.3	48.12	10325	7560 (73.22%)	32	99.66%	S
DTO032C6	141	28.45	48.13	10008	7441 (74.35%)	33	100%	S
DTO039G3	45	27.22	48.14	9764	7352 (75.3%)	32	99.66%	S
DTO046C5	143	28.8	48.15	10098	7488 (74.15%)	33	99.66%	S
DTO070G2	60	26.75	48.39	9724	7324 (75.32%)	33	100%	S
DTO081F9	47	27.24	48.13	9760	7349 (75.3%)	32	99.66%	S
DTO101D6	141	28.96	48.07	10109	7497 (74.16%)	33	99.31%	S
DTO102I9	186	28.53	47.92	9978	7439 (74.55%)	32	99.66%	S
DTO126G2	174	28.06	48.02	9939	7431 (74.77%)	33	99.31%	S

DTO127F7	59	26.54	48.43	9639	7315 (75.89%)	35	99.66%	S
DTO127F9	54	26.53	48.44	9633	7311 (75.9%)	36	99.66%	S
DTO130C1	537	30.48	47.79	10375	7612 (73.37%)	36	99.66%	S
DTO163C3	239	30	48.16	10485	7653 (72.99%)	33	99.66%	S
DTO163F5	284	30.9	46.85	10010	7441 (74.34%)	33	100%	S
DTO163G4	104	28.45	48.12	10006	7441 (74.37%)	33	100%	S
DTO265D5	66	26.76	48.44	9751	7350 (75.38%)	33	100%	S
DTO369A1	156	28.29	47.97	9945	7437 (74.78%)	32	99.66%	S
DTO375B1	62	27.2	48.2	9804	7378 (75.25%)	33	99.31%	S
DTO377G2	101	28.07	48.14	9962	7444 (74.72%)	33	99.31%	S
DTO377G3	84	26.97	48.24	9762	7335 (75.14%)	33	99.66%	S

Average	217.32	28.55	48.05	10038.65
Min	45	26.53	46.85	9633
Max	1358	31.74	48.44	10644

540

Table 2. Genes (DTO006G7) located on the SORBUS cluster. Fold change (log_2FC) of the sorbic acid samples compared to the control is given. Numbers in bold are significantly differently expressed (adjusted p-value < 0.05) and the mean expression (FPKM) of three biological replicates is given per condition (control and sorbic acid). Rows highlighted in grey indicate genes that are not unique for the R-type strains.

GeneId	Name	Functional annotation (PFAM or description)	Expression (FPKM)		log_2FC	p-value (Adj.)
			Control	Sorbic acid		
12000		FAM167	4	8	0.69	0.66
12001		hypothetical protein	16	86	**2.18**	**0.00**
12002		NEMP	2	2	-0.27	0.89
12003		BTB/POZ domain	16	23	0.48	0.35
12004		hypothetical protein	59	40	-0.56	0.16
12005		hypothetical protein	0	0	1.05	0.59
12006		Reverse transcriptase	0	0	-	-
12007		DUF3723	0	0	-	-
12008		hypothetical protein	0	0	-	-
12009		Hly-III related protein	0	0	-	-
12010		Cyclin like F-box	1	1	0.72	0.32
12011		Cyclin like F-box	17	24	0.47	0.25
12012		Reverse transcriptase	3	7	**1.02**	**0.03**
12013		Endonuclease/Exonuclease/phosphatase family	1	4	**1.40**	**0.00**
12014		Probable transposable element	1	3	0.83	0.51
12015		Aldo/keto reductase family	463	365	-0.34	0.36
12016		Reverse transcriptase	26	20	-0.35	0.57
12017		Telomere-associated recq-like helicase	13	15	0.23	0.57
12018		Cyclin like F-box	29	25	-0.23	0.41
12019		hypothetical protein	0	0	-1.04	0.83
12020		hypothetical protein	1	1	0.22	0.96
12021		Centrosomin N-terminal motif 1	0	0	2.51	0.24
12022		hypothetical protein	3	7	1.02	0.09
12023		Cyctochrome C mitochondrial import factor	1	0	-0.84	0.73

12024		hypothetical protein	8	10	0.21	0.86
12025		hypothetical protein	5	6	0.25	0.60
12026		Winged helix-turn helix	0	0	-	-
12027		hypothetical protein	15	10	-0.57	0.23
12028		Pronucleotidyl transferase	0	0	-0.14	0.95
12029		Pronucleotidyl transferase	1	0	-0.83	0.64
12030		hypothetical protein	11	15	0.42	0.58
12031		hypothetical protein	0	0	-	-
12032	*padB*	Flavoprotein	450	288	-0.62	0.15
12033		PHF5-like protein	3	3	0.11	0.91
12034		Pyridoxamine 5'-phosphate oxidase	370	287	-0.36	0.48
12035		Mitochondrial carrier protein	11	12	0.06	0.96
12036		Mitochondrial carrier protein	12	17	0.42	0.65
12037		GTP cyclohydrolase II	10	2	**-1.86**	**0.01**
12038		Potassium channel tetramerisation domain	26	30	0.18	0.82
12039		hypothetical protein	17	10	-0.58	0.58
12040	*cdcC*	UbiD	134	65	**-1.02**	**0.01**
12041		Pyridoxamine 5'-phosphate oxidase	2	1	-0.54	0.90
12042		hypothetical protein	9	11	0.27	0.76
12043		GTP cyclohydrolase II	5	1	**-1.63**	**0.03**
12044		GPR1/FUN34/yaaH family	47	9	**-2.05**	**0.00**
12045		Mitochondrial carrier protein	1	1	-0.61	0.73
12046		hypothetical protein	12	12	-0.03	0.97
12047		Helix-turn-helix domain/endonuclease	8	8	0.03	0.95
12048		Flavonol reductase/cinnamoyl-CoA reductase	53	56	0.07	0.93
12049		Flavonol reductase/cinnamoyl-CoA reductase	9	8	-0.26	0.63
12050		Tannase	427	352	-0.27	0.55
12051		hypothetical protein	0	1	1.76	
12052		Transposase-like protein	6	7	0.13	0.84
12053		Reverse transcriptase	0	0	0.31	0.89
12054		Zinc finger transcription factor	26	13	-0.98	0.07
12055		Transposase-like protein	0	0	1.64	
12056	*cdcB*	UbiD	211	142	-0.51	0.65

12057	*padC*	Flavoprotein	78	75	-0.07	0.90
12058		Pyridoxamine 5'-phosphate oxidase like	348	504	0.50	0.28
12059		GTP cyclohydrolase II	25	24	-0.04	0.96
12060		GPR1/FUN34/yaaH family	3	3	-0.39	0.67
12061		Flavin reductase like domain	2028	1532	-0.40	0.42
12062		hypothetical protein	272	295	0.08	0.86
12063		3, 4-dihydroxy-2-butanone 4-phosphate synthase	65	34	-0.85	0.25
12064	*padA*	Flavoprotein	92	45	-0.97	0.08
12065	*cdcA*	UbiD	65	22	**-1.53**	**0.00**
12066	*sdrA*	Hypothetical transcription factor	2	1	-0.76	0.71
12067		hypothetical protein	0	0		
12068		NACHT domain	4	8	0.85	0.09
12069		Histone H3	3	6	**0.95**	**0.00**

546

547 **Table 3.** Genes (DTO006G7) containing SNPs associated with sorbic acid resistance. Only SNPs
548 with a $-\log_{10}$(p-value) > 5 and a moderate or high impact as determined by SNPeff are listed.
549 Grey shading indicates overlap with sequence repeats.

	SNP impact			
GeneID	**Moderate**	**High**	**Effect**	**PFAM annotation**
g103	1	0		hypothetical protein
g216	1	0		ABC transporter transmembrane region
g235	1	0		STAG domain
g296	5	0		Cation transporter/ATPase, N-terminus
g312	1	0		Probable molybdopterin binding domain
g313	1	0		DDHD domain
g314	1	0		hypothetical protein
g315	1	0		FAD binding domain
g7015	1	1	Stop lost & splice region variant	hypothetical protein
g8100	0	1	Stop gained	Ankyrin repeats (3 copies)
g8101	3	0		DDE superfamily endonuclease
g8104	7	0		hypothetical protein
g8105	10	0		hypothetical protein
g8106	3	1	Stop gained	hypothetical protein
g8107	26	0		Ankyrin repeats (3 copies)
g12002	2	0		NEMP
g12005	9	1	Frameshift variant	hypothetical protein
g12006	42	2	Frameshift variant & Stop gained	Reverse transcriptase
g12007	8	0		Protein of unknown function (DUF3723)
g12013	48	3	Frameshift variants	Endonuclease/Exonuclease/phosphatase family
g12014	8	1	Stop lost & splice region variant	Probable transposable element
g12016	2	0		Reverse transcriptase
g12019	11	0		hypothetical protein
g12025	9	1	Stop gained	hypothetical protein
g12028	18	1	Stop lost & splice region variant	Pronucleotidyl transferase
g12029	26	6	Stop gained, Stop lost & splice variant	Pronucleotidyl transferase
g12030	16	2	Frameshift variant & Stop lost	hypothetical protein

g12046	1	0		hypothetical protein
g12052	4	1	Stop gained	Transposase-like protein
g12053	1	0		Reverse transcriptase
g12054	4	0		hypothetical protein
g12055	6	2	Frameshift variant & Stop lost	Transposase-like protein
g12069	7	1	Frameshift variant	Histone H3
g9749	7	0		hypothetical protein
g9942	1	2	Stop gained	hypothetical protein
g9943	2	2	Stop gained	AAA domain
g9944	8	0		hypothetical protein
g9945	4	0		ATPase family associated with various cellular activities (AAA)
g9961	1	0		hypothetical protein
g9966	2	0		Endonuclease-reverse transcriptase
g9976	0	1	Start lost	hypothetical protein
TOTAL	309	29		

Table 4. Number of genes containing PFAM domains corresponding to g12060, g12061 and g12063-g12065 (PF01184, PF01613, PF00926, PF02441, PF01977) based on phylogenetic trees constructed with their respective PFAM domains. Top six strains are R-type *P. roqueforti* strains containing the SORBUS cluster. C (CORE) indicates if the domains aligned closely to the PFAMs not unique for the SORBUS cluster or did not align to *P. roqueforti*, S (SORBUS) indicates the number of PFAM domains which aligned closely to PFAMs originated from SORBUS.

	g12060 PF01184		**g12061 PF01613**		**g12063 PF00926**		**g12064 PF02441 (*padA*)**		**g12065 PF01977 (*cdcA*)**	
Strain	**C**	**S**	**C**	**S**	**C**	**S**	**C**	**S**	**C**	**S**
DTO013F2	7	1	4	1	1	1	5	3	2	3
DTO013E5	7	1	3	1	1	1	5	3	2	3
DTO012A8	7	1	3	1	1	1	5	3	2	3
DTO012A1	7	1	3	1	1	1	5	3	2	3
DTO006G7	7	1	4	1	1	1	5	3	2	3
DTO006G1	7	1	4	1	1	1	5	3	2	3
DTO377G3	7	0	4	0	1	0	5	0	2	0
DTO377G2	7	0	4	0	1	0	5	0	2	0
DTO375B1	7	0	3	0	1	0	5	0	2	0
DTO369A1	7	0	4	0	1	0	5	0	2	0
DTO265D5	7	0	3	0	1	0	5	0	2	0
DTO163G4	7	0	3	0	1	0	5	0	2	0
DTO163F5	7	0	3	0	1	0	5	0	2	0
DTO163C3	7	0	4	0	1	0	5	0	2	0
DTO130C1	7	0	3	0	1	0	5	0	2	0
DTO127F9	7	0	3	0	1	0	5	0	2	0
DTO127F7	7	0	3	0	1	0	5	0	2	0
DTO126G2	7	0	4	0	1	0	5	0	2	0
DTO102I9	7	0	4	0	1	0	5	0	2	0
DTO101D6	7	0	3	0	1	0	5	0	2	0
DTO081F9	7	0	4	0	1	0	5	0	2	0
DTO070G2	7	0	3	0	1	0	5	0	2	0
DTO046C5	7	0	3	0	1	0	5	0	2	0
DTO039G3	7	0	4	0	1	0	5	0	2	0
DTO032C6	7	0	3	0	1	0	5	0	2	0
DTO027I6	7	0	4	0	1	0	5	0	2	0
DTO013F5	7	0	4	0	1	0	5	0	2	0

DTO012A9	7	0	3	0	1	0	5	0	2	0
DTO012A7	7	0	3	0	1	0	5	0	2	0
DTO012A6	7	0	3	0	1	0	5	0	2	0
DTO012A2	7	0	3	0	1	0	5	0	2	0
DTO003H1	7	0	3	0	1	0	5	0	2	0
DTO003C3	7	0	3	0	1	0	5	0	2	0
DTO002I6	7	0	3	0	1	0	5	0	2	0
Penicillium roqueforti FM164	8	0	3	0	1	0	5	0	2	0
Penicillium oxalicum	4	0	2	0	1	0	2	0	0	0
Penicillium digitatum	1	0	2	0	1	0	4	0	1	0
Paecilomyces variotii	8	0	6	0	1	0	4	1	2	0
Paecilomyces variotii DTO217A2	7	0	7	0	1	0	5	1	3	0
Paecilomyces niveus	9	0	8	0	1	0	5	1	2	0
Aspergillus wentii	4	0	5	0	1	0	4	1	2	0
Aspergillus violaceofuscus	4	0	5	1	1	0	4	0	2	0
Aspergillus vadensis	3	0	5	0	1	0	4	1	2	1
Aspergillus uvarum	4	0	5	1	1	0	3	0	2	0
Aspergillus tubingensis	3	0	6	0	1	0	4	0	2	1
Aspergillus sclerotiicarbonarius	3	0	5	1	1	0	4	1	2	0
Aspergillus sclerotioniger	3	0	4	1	1	0	3	0	1	0
Aspergillus piperis	3	0	5	0	1	0	4	1	2	0
Aspergillus aureofulgens	3	0	5	0	1	0	4	1	2	0
Aspergillus niger N402	3	0	7	0	1	0	5	1	3	0
Aspergillus niger ATCC 1015	3	0	7	0	1	0	5	1	3	0
Aspergillus neoniger	4	0	5	0	1	0	4	1	2	1
Aspergillus niger (lacticoffeatus)	3	0	5	0	1	0	4	1	2	0
Aspergillus japonicus	4	0	5	1	1	0	4	0	3	0
Aspergillus indologenus	4	0	5	1	1	0	4	0	2	0
Aspergillus ibericus	3	0	5	1	1	0	4	1	1	0
Aspergillus heteromorphus	3	0	5	0	1	0	3	1	2	0
Aspergillus fumigatus	3	0	3	0	1	0	2	0	0	0
Aspergillus flavus	5	0	4	0	1	0	4	0	2	0
Aspergillus fijiensis	4	0	5	1	1	0	4	1	4	0
Aspergillus eucalypticola	3	0	5	0	1	0	4	1	2	1
Aspergillus ellipticus	3	0	5	0	1	0	6	2	7	0
Aspergillus costaricaensis	4	0	5	0	1	0	4	0	2	1
Aspergillus brunneovolaceus	4	0	6	1	1	0	4	1	4	0
Aspergillus aculeatinus	4	0	5	1	1	0	4	0	3	0

Arthroderma benhamiae	2	0	1	0	0	1	2	0	0	0

557

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699

Control
Propionic acid
Sorbic acid
Benzoic acid
Area (cm^2)
15
10
5
0
DTO002I6
DTO003C3
DTO003H1
DTO006G1
DTO006G7
DTO012A1
DTO012A2
DTO012A6
DTO012A7
DTO012A8
DTO012A9
DTO013E5
DTO013F2
DTO013F5
DTO027I6
DTO032C6
DTO039G3
DTO046C5
DTO070G2
DTO081F9
DTO101D6
DTO102I9
DTO126G2
DTO127F7
DTO127F9
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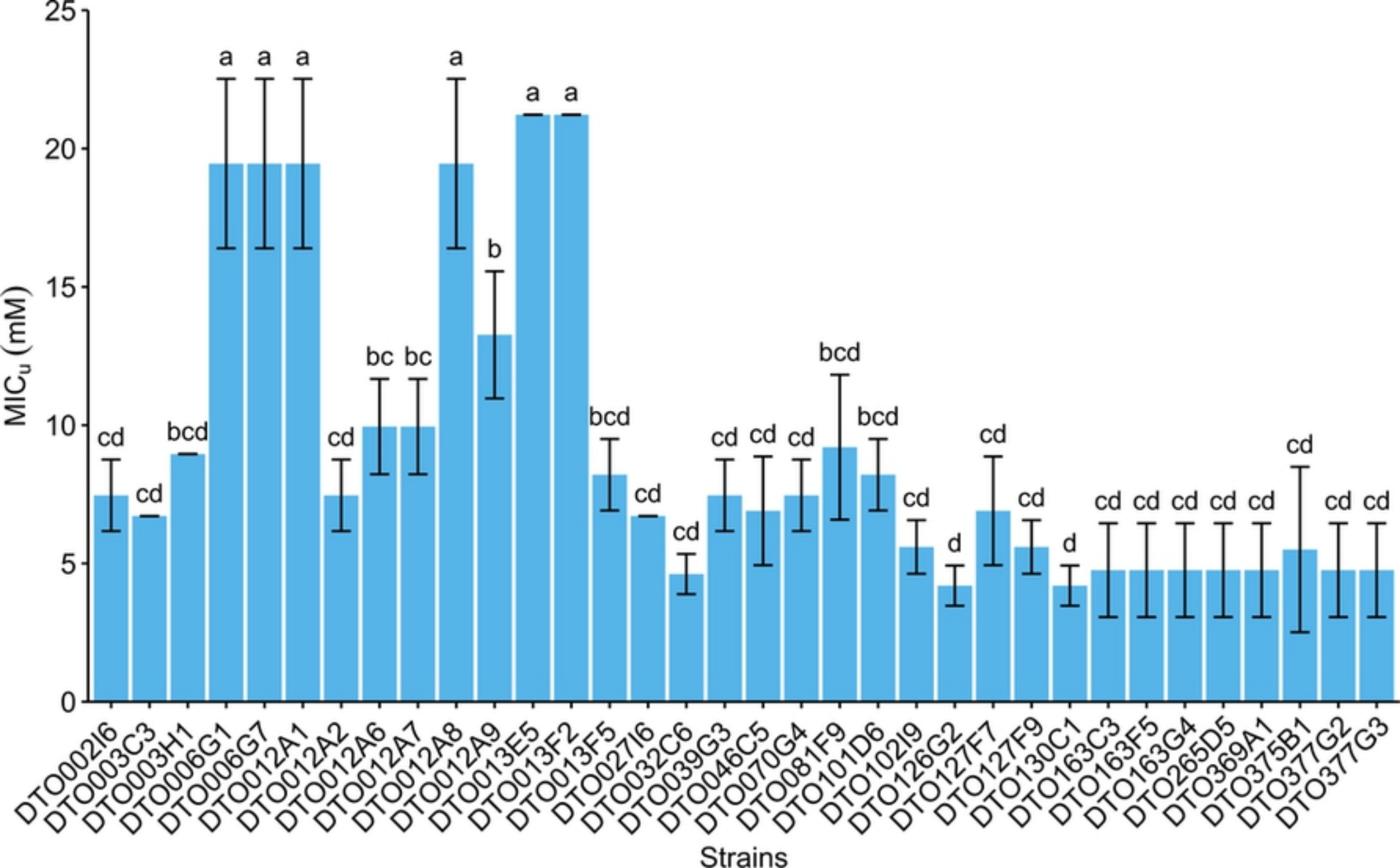
DTO377G2
DTO377G3


25
20
15
10
5
0
MIC$_u$ (mM)
a a a a a b a a bcd cd bc bc cd cd cd cd cd bcd cd cd cd cd d cd d cd cd cd cd cd cd cd cd
DTO002I6
DTO003C3
DTO003H1
DTO006G1
DTO006G7
DTO012A1
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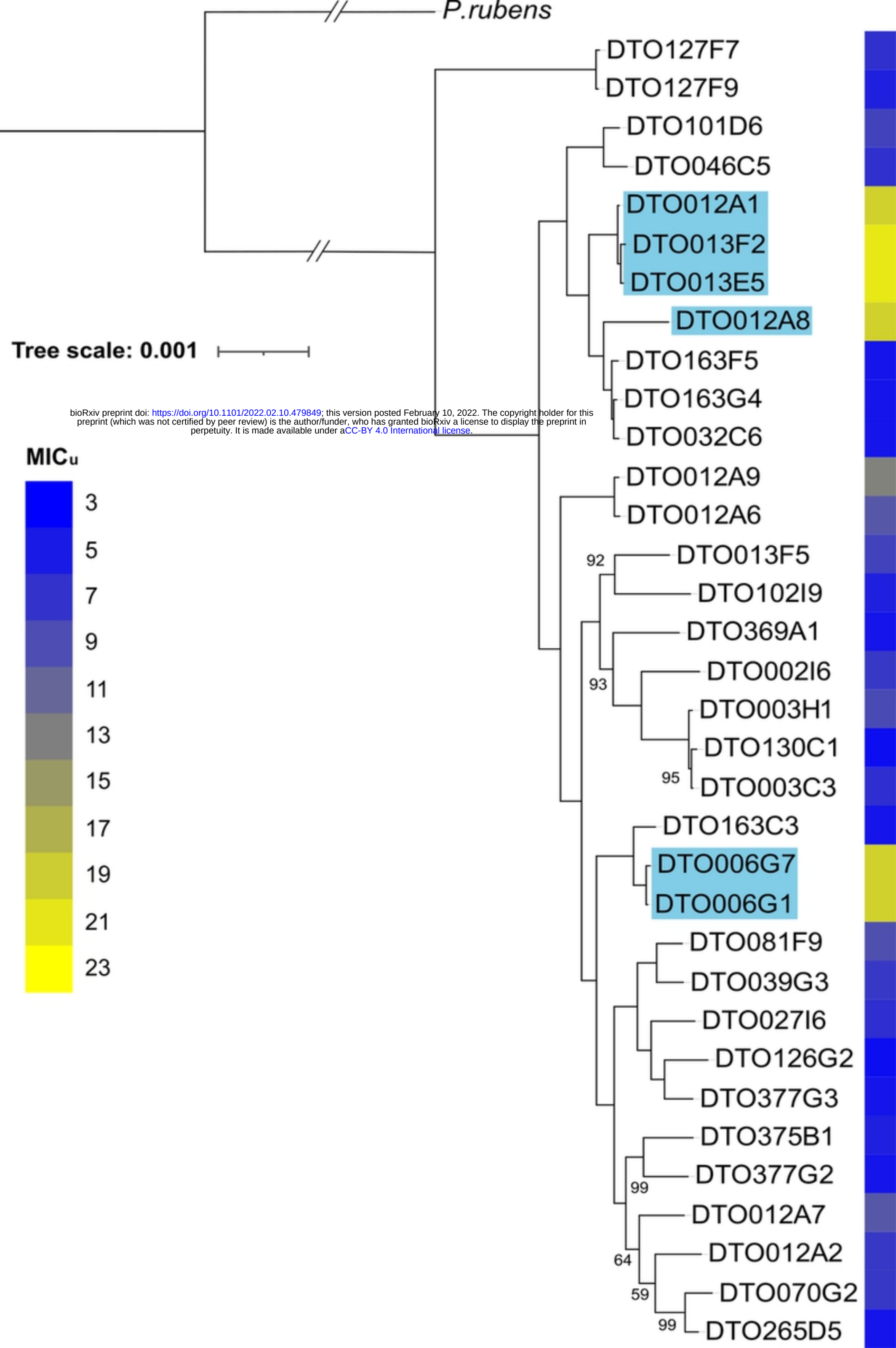
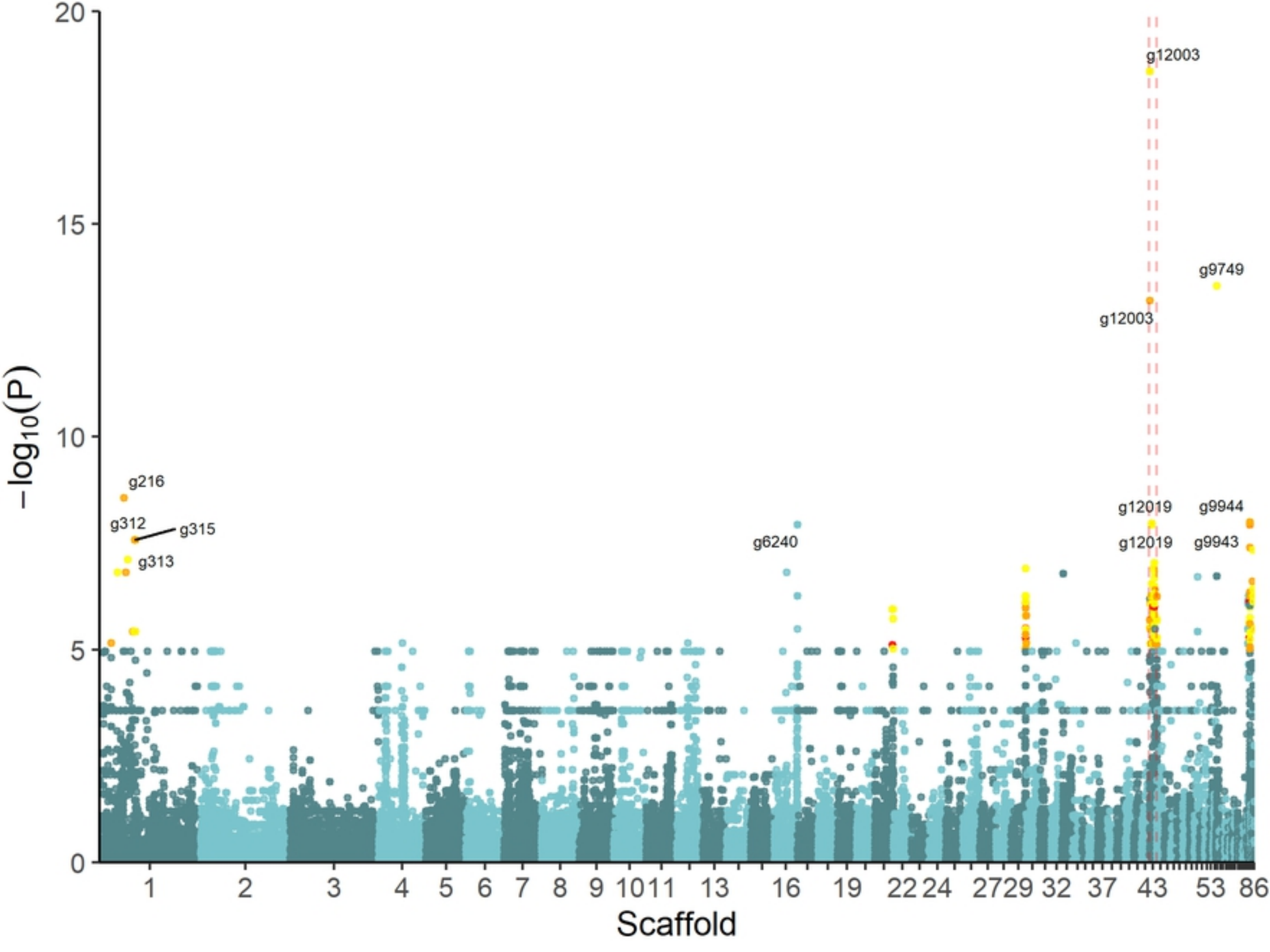
Strains




20
15
10
5
0
$-\log_{10}(P)$
g12003
g9749
g12003
g216
g312
g315
g313
g6240
g12019
g9944
g12019
g9943
1
2
3
4
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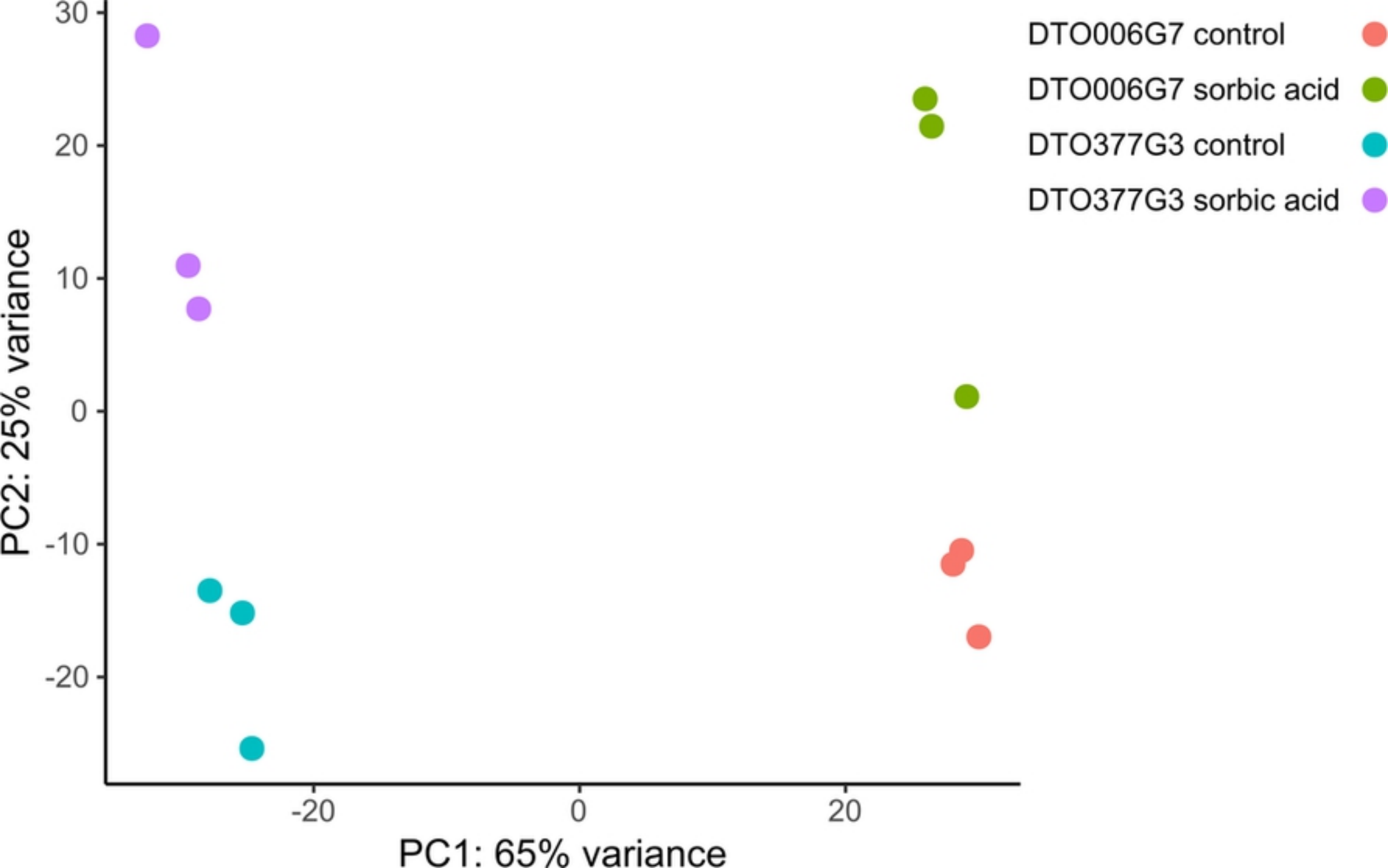
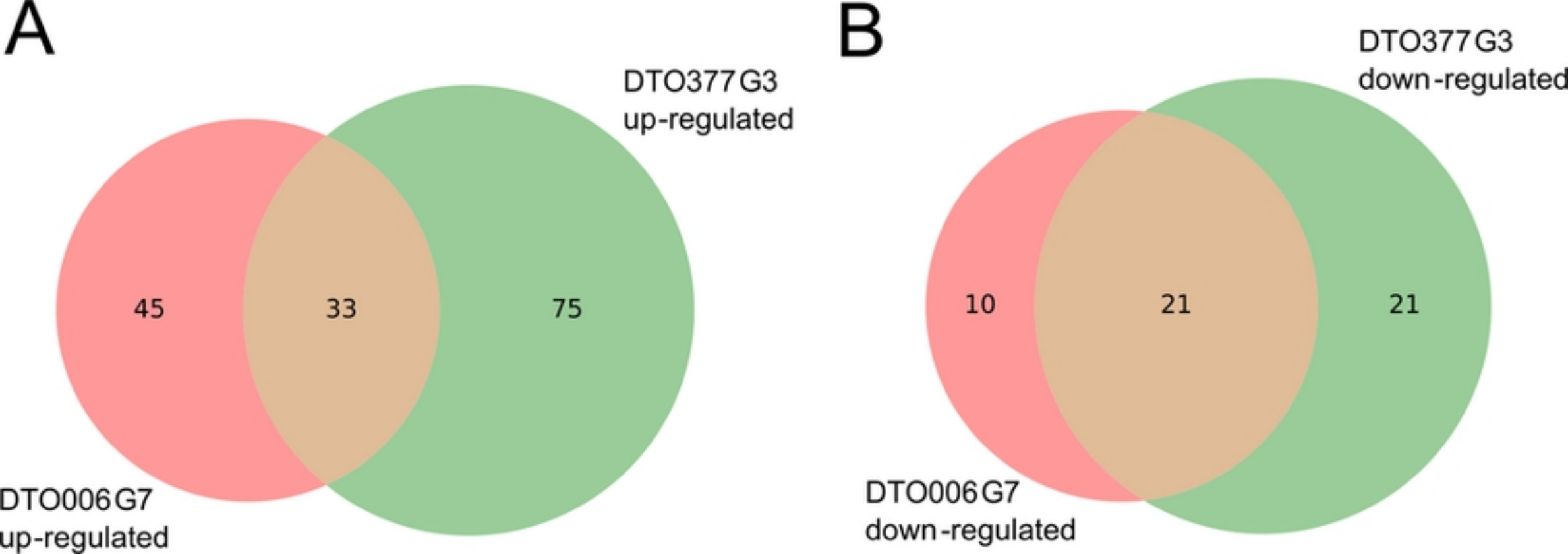
37
43
53
86
Scaffold


DTO006G7 control
DTO006G7 sorbic acid
DTO377G3 control
DTO377G3 sorbic acid
PC2: 25% variance
PC1: 65% variance
30
20
10
0
-10
-20
-20
0
20


A
DTO377 G3
up-regulated
45
33
75
DTO006 G7
up-regulated
B
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down-regulated
10
21
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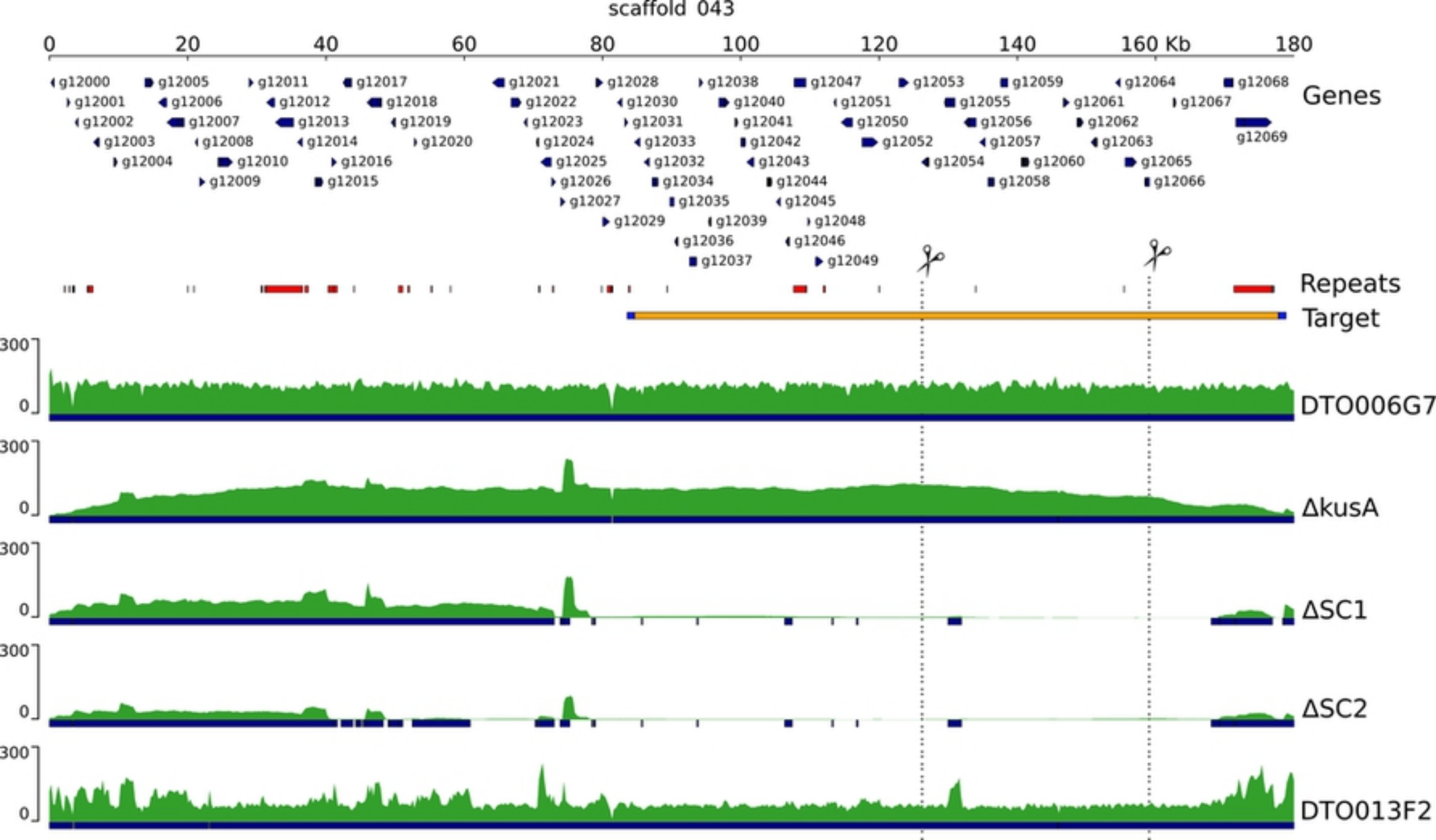
down-regulated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20
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100
120
140
160 Kb
180
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g12063
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g12066
g12067
g12068
g12069
Repeats
Target
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ΔkusA
ΔSC1
ΔSC2
DTO013F2