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## Large-scale genome mining allows identification of neutral polymorphisms and novel resistance mutations in genes involved in *Candida albicans* resistance to azoles and echinocandins

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1 **Large-Scale Genome Mining Allows Identifying Neutral Polymorphisms and**  
2 **Novel Resistance Mutations in Genes Involved in *Candida albicans* Resistance**  
3 **to Azoles and Echinocandins**

4  
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31

32 **Short running title:** Antifungal target gene polymorphisms in *Candida albicans*

33

## 34 **Synopsis**

35 **Background:** The genome of *Candida albicans* displays significant polymorphism. Point  
36 mutations in genes involved in resistance to antifungals may either confer phenotypic resistance  
37 or be devoid of phenotypic consequences.

38 **Objectives:** Catalog polymorphisms in azole- and echinocandin-resistance genes occurring in  
39 susceptible strains in order to rapidly pinpoint relevant mutations in resistant strains.

40 **Methods:** Genome sequences from 151 unrelated *C. albicans* strains susceptible to fluconazole  
41 and caspofungin were used to create a catalog of non-synonymous polymorphisms in genes  
42 involved in resistance to azoles (*ERG11*, *TAC1*, *MRR1*, *UPC2*) or echinocandins (*FKSI*). The  
43 potential of this catalog to reveal putative resistance mutations was tested in 10 azoles-resistant  
44 isolates, including 1 intermediate to caspofungin. Selected mutations were analyzed by  
45 mutagenesis experiments or mutational prediction effect.

46 **Results:** In the susceptible strains, we identified 126 amino-acid substitutions describing the  
47 catalog of phenotypically-neutral polymorphisms. By excluding these neutral substitutions, we  
48 identified 22 additional substitutions in the 10 resistant strains. Among these substitutions, 10  
49 had already been associated with resistance. The remaining 12 were in Tac1p (n=6), Upc2p  
50 (n=2) and Erg11p (n=4). Four out of the 6 homozygous substitutions from Tac1p (H263Y,  
51 A790V, H839Y and P971S) conferred increases in azoles MIC, while no effects were observed  
52 for those in Upc2p. Additionally, 2 homozygous substitutions (Y64H, P236S) had a predicted  
53 conformation effect on Erg11p.

54 **Conclusions:** By establishing a catalog of neutral polymorphisms occurring in genes involved in  
55 resistance to antifungal drugs, we provide a useful resource for rapid identification of mutations  
56 possibly responsible for phenotypic resistance in *C. albicans*.

57

## 58 **Introduction**

59 *Candida albicans* is the most frequent human fungal pathogen causing a variety of infections in  
60 both immunocompetent and immunocompromized individuals.<sup>1,2</sup> Azoles and echinocandins are  
61 the two main classes of antifungal agents currently used to treat severe *C. albicans* infections.<sup>3,4</sup>  
62 Emergence of azole- and echinocandin-resistant *C. albicans* isolates is a significant concern  
63 because the number of systemic antifungal drugs available to fight *C. albicans* infections is  
64 limited.<sup>5-9</sup> Echinocandin resistance can occur through point mutations within hot-spot regions of  
65 *FKSI* encoding the  $\beta$ -(1,3)-D-glucan synthase.<sup>5,10</sup> Azole resistance can occur through various  
66 mechanisms, such as alterations or overexpression of the drug target gene *ERG11* (encoding the  
67 14- $\alpha$ -lanosterol demethylase) or increased expression of genes encoding drug efflux transporters:  
68 *CDR1*, *CDR2*, or *MDR1*.<sup>11-14</sup> The expression of *CDR1/CDR2* or *MDR1* is regulated by the  
69 transcription factors Tac1p and Mrr1p, respectively. Gain-of-function (GOF) mutations in the  
70 corresponding *TAC1* and *MRR1* genes lead to overproduction of the pumps. Furthermore, up-  
71 regulation of *ERG11*, via GOF mutations in the transcriptional factor *UPC2*, has been described  
72 in resistant strains of *C. albicans*.<sup>15-17</sup> Finally, azole resistance can arise from large-scale genetic  
73 changes through genome rearrangements, including loss-of-heterozygosity events<sup>18-20</sup> as well as  
74 segmental or whole chromosome aneuploidies leading to copy number variations of relevant  
75 antifungal resistance genes.<sup>21-23</sup> The generation of such variants is facilitated by the high  
76 genomic plasticity of *C. albicans*, especially under stress conditions when exposed to, antifungal  
77 agents.<sup>24-28</sup>

78 The diploid genome of *C. albicans* displays a high level of natural heterozygosity with an  
79 average of 1 heterozygous position every 204 bp.<sup>29</sup> When genomes of *C. albicans* isolates are  
80 compared to the genome (haplotype A) of reference strain SC5314,<sup>30,31</sup> homozygous variants are  
81 observed every 235 to 1404 bp, depending on the genetic cluster from which isolates  
82 originated.<sup>32</sup> Therefore, genome resequencing of antifungal-resistant isolates does not allow to

83 rapidly distinguish the polymorphisms that underlie phenotypic resistance from those that may  
84 result from natural diversity. Here, we have made the hypothesis that mining the whole genome  
85 sequences from a large panel of epidemiologically unrelated antifungal-susceptible *C. albicans*  
86 strains <sup>29</sup> will allow establishing a comprehensive catalog of phenotypically-neutral non-  
87 synonymous polymorphisms in the key genes involved in resistance to azoles (*ERG11*, *TAC1*,  
88 *MRR1* and *UPC2*) and echinocandins (*FKSI*). This catalog could then be used to screen  
89 genomes of resistant isolates for mutations linked to antifungal resistance. We addressed this  
90 hypothesis through the analysis of 10 uncharacterized drug-resistant isolates and show that their  
91 resistance can be rapidly associated to well-known as well as novel resistance mutations.

92

## 93 **Material and methods**

### 94 **Strains collection**

95 Two sets of *C. albicans* clinical strains were used in this study and are listed in supplementary  
96 **Tables S1 and S2**. The first one consisted of 151 strains susceptible to fluconazole and  
97 caspofungin, selected to represent the natural population diversity within *C. albicans*.<sup>29</sup> The  
98 second set consisted of 10 azoles-resistant isolates, including 1 intermediate to caspofungin. The  
99 antifungal susceptible *C. albicans* reference strain SC5314 was also used.<sup>30</sup>

100

### 101 **Culture media**

102 *C. albicans* isolates were sub-cultured from frozen stock in liquid or solid YPD media (1% Yeast  
103 extract, 2% Peptone, 2% Dextrose). Nourseothricin (300 µg/mL) was added to YPD agar plates  
104 to select strains containing the *SAT1* nourseothricin-resistance gene when required. *Escherichia*  
105 *coli* TOP10 (Life Technologies) or XL10-Gold cells (Agilent Technologies) were used as a host  
106 for plasmid constructions and propagation. *E. coli* cells were cultured on LB (Luria-Bertani)  
107 broth or plates, supplemented with ampicillin (100 mg/L) or kanamycin (50 mg/L) when  
108 required.

109

### 110 **Minimal Inhibitory Concentrations (MICs) determination**

111 Antifungal MICs of *C. albicans* isolates were determined at 24h by using the Etest® method,  
112 according to the manufacturer's recommendations and interpreted according to CLSI  
113 breakpoints.<sup>33</sup>

114

### 115 **Illumina sequencing and genome analysis**

116 Genomic DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen, Courtaboeuf,  
117 France), as described previously.<sup>28</sup> Libraries were prepared using the NEXTflex™ PCR-Free  
118 DNA Sequencing kit (Illumina), according to the manufacturer's recommendations. Sequencing  
119 was performed on a HiSeq2000 or HiSeq2500 platform generating 100 bp paired-end reads  
120 (roughly 100x coverage).

121 Sequences and genomic variations were analyzed as described previously<sup>29</sup> using BWA v0.7.7<sup>34</sup>  
122 and the *C. albicans* reference strain SC5314 (genome assembly 22 version A22-s06-m01-r01<sup>35</sup>).

123 The variant calling was performed with GATK v3.1<sup>36</sup> according to the best practices  
124 recommended annotation for hard filters. In each strain, the entire ORF sequences of 5 genes  
125 involved in azoles (*ERG11*, *TAC1*, *MRR1* and *UPC2*) and echinocandins (*FKSI*) resistance were  
126 compared to that of reference strain SC5314. Only non-synonymous mutations were considered.

127 At each position, homozygous and heterozygous substitution frequency was calculated from the  
128 set of the 151 *C. albicans* susceptible strains. Genotype results are listed in the supplementary

129 **Table S3.**

130

### 131 **Multilocus Sequence Typing**

132 All strains were typed using Multi Locus Sequence Typing (MLST), as described.<sup>37</sup>

133

134 **Plasmid and mutant constructions**

135 To assess if the mutations are involved in phenotypic resistance to fluconazole, we have  
136 constructed, for each new mutation identified in *TAC1* and *UPC2*, a series of integrative  
137 plasmids that were introduced in *C. albicans* strains. The genotypes of the resulting strains are  
138 listed in the supplementary **Table S4**. Primers used for plasmids and strains constructions are  
139 listed in supplemental **Table S5**.

140 First, for strains harboring a single candidate mutation in *TAC1* (CEC723: 2369G>A  
141 corresponding to amino-acid substitution A790V - 2369G>A/A790V) or *UPC2* (CEC 4522:  
142 1960C>T/D654N and CEC718: 1970C>T/G657D) as well as SC5314 (wild type strain), the  
143 genomic regions harboring *TAC1* or *UPC2* including the promotor and terminator regions were  
144 PCR-amplified and cloned into the TOPO-BLUNT cloning vector (Invitrogen). Sequence of the  
145 amplified fragments was verified by Sanger sequencing. The resulting plasmids were digested by  
146 ad hoc restriction enzymes and the resulting fragments harboring a wild type or mutated *TAC1* or  
147 *UPC2* gene were cloned into the CIpSAT plasmid. *E. coli* TOP10 (Invitrogen) competent cells  
148 were used for cloning and maintaining the plasmid constructs.

149 Second, for strains harboring more than one candidate mutation in *TAC1*, we introduced each  
150 mutation independently by site-directed mutagenesis of the *TAC1* gene obtained from *C.*  
151 *albicans* strain SC5314. The plasmid harboring the wild type *TAC1* allele (CIpSAT-*P<sub>TAC1</sub>-TAC1*)  
152 was used as template for PCR amplification to introduce the following mutations  
153 (787C>A/H263Y, 2469A>C/N823K, 2515G>A/H839Y, 2573C>T/C858Y, 2911G>A/P971S).  
154 Primers were designed using the web-based QuikChange Primer Program available online  
155 ([www.agilent.com/genomics/qcpd](http://www.agilent.com/genomics/qcpd)). Reactions and cycling conditions were done according to the  
156 manufacturer's instructions with the QuikChange Lightning Site-Directed Mutagenesis kit  
157 (Agilent). *E. coli* XL10-Goold (Agilent) Ultracompetent cells were used for plasmids  
158 transformation. Complete fragments were verified by Sanger sequencing.

159 Two *C. albicans* strains (DSY4578 or DSY3984), deleted for *TAC1* and *UPC2* (Supporting  
160 Information **Table S4**) and susceptible to fluconazole, were used for transformation with the  
161 resulting CIPSAT-*P<sub>TAC1</sub>-TAC1* or CIPSAT-*P<sub>UPC2</sub>-UPC2* derivatives.

162 CIPSAT derivatives were integrated in the *C. albicans* genome at the *RPS1* locus using the  
163 lithium-acetate transformation protocol, as described.<sup>38</sup> Transformants were selected on YPD  
164 medium supplemented with nourseothricin (300 mg/L). Plasmid integration was checked by  
165 PCR with CIPSAT\_checkF and CIPSAT\_checkR primers (**Table S5**). *TAC1* or *UPC2* ORFs  
166 were sequenced to confirm the occurrence of each mutation.

167

### 168 **Prediction of mutation effect in Erg11p**

169 The impact of mutations in *ERG11* was predicted by using different software and the 5v5z entry  
170 <sup>39</sup> from the Protein Data Base (<https://www.rcsb.org/>) as a reference for Erg11p 3D structure.

171 The solvent accessibility of amino-acids within Erg11p and the prediction of protein  
172 thermodynamic stability ( $\Delta\Delta G$ ) and thermostability changes ( $\Delta T_m$ ) upon single-site mutations  
173 were evaluated using PoPMuSiCv3.1<sup>40</sup> and HoTMuSiCv0.5.<sup>41</sup> The global effect of mutations  
174 were evaluated using SNPmuSiC.<sup>42</sup> All output files were generated using MuSiCv3.0r3  
175 available online at <https://soft.dezyme.com/>. The amino-acid solvent accessibility ranged from 0  
176 (buried) to 100 (fully accessible). The mutation effect on protein stability was classified as  
177 destabilizing ( $\Delta\Delta G > 0$  and  $\Delta T_m < 0$ ) or stabilizing ( $\Delta\Delta G < 0$  and  $\Delta T_m > 0$ ). The predicted  
178 mutational effect was classified as deleterious or neutral.

179

### 180 **Statistical analysis**

181 Genetic polymorphism diversity was defined as the number of non-synonymous SNPs that could  
182 be identified from the antifungal-susceptible strains. This diversity was explored using  
183 accumulation curves showing the number of distinct SNPs as a function of the number of strains.

184 Using a bootstrapping method, the 151 susceptible strains ( $N_S$ ) were randomly permuted 10,000  
185 times and for each permutation the cumulative number of mutations was counted as a function of  
186 the increasing number of strains. The rate of new mutations (*i.e.* the number of new SNPs  
187 introduced in a pool of strains when adding one new strain) was derived from these resampled  
188 sets to assess the representativeness of susceptible strains from our experiment.

189 To assess the contribution of the 10 antifungal-resistant strains ( $N_R$ ) to genetic diversity, the  
190 above method was adapted to replace the 10 last susceptible strains from the random  
191 permutations with random permutations of the  $N_R$  strains. This resulted in two sets of  
192 bootstrapped distributions: the null with the  $N_S$  strains and the alternate with  $N_S - N_R$  susceptible  
193 strains and last  $N_R$  resistant strains. Finally, the number of new mutations identified in the last  
194  $N_R$  strains from the alternate distribution (that is the number of mutations from the pool of  
195 resistant strains that cannot be identified in susceptible strains) was compared to the distribution  
196 of the number of mutations in the null bootstrap using percentiles as credible intervals.

197

## 198 **Results**

### 199 **Constitution of a catalog of phenotypically-neutral non-synonymous polymorphisms in** 200 **genes involved in azole- and echinocandin-resistance.**

201 We took advantage of the availability of whole genome sequences of 151 susceptible *C. albicans*  
202 strains to extensively investigate the sequence variations of genes involved in resistance to  
203 azoles (*ERG11*, *TAC1*, *MRR1* and *UPC2*) and echinocandins (*FKS1*). Comparison of these  
204 whole genome sequences with that of SC5314 led to the identification of 126 distinct non-  
205 synonymous polymorphisms in these 5 genes (**Table 1**). As shown in **Figure 1**, we identified 38,  
206 20, 15, 33 and 20 amino-acid substitutions in Tac1p, Erg11p, Upc2p, Mrr1p and Fks1p,  
207 respectively. Sequencing statistics for the 151 susceptible *C. albicans* strains as well as their  
208 MICs distributions are summarized in the supplementary **Table S1 and Figure 2**. For each  
209 identified non-synonymous polymorphism, the genotypes obtained for all susceptible strains are  
210 provided in **Table S3**.

211 Among the 126 non-synonymous mutations, 41% (52/126) were found both at the heterozygous  
212 and homozygous state while 54% (69/126) were only found in the heterozygous state and 4%  
213 (5/126) in the homozygous state. For each of these 126 mutations, we could determine the  
214 homozygous and heterozygous mutation frequencies observed from the population of the 151  
215 susceptible strains (**Table 1**). As shown in **Table 1**, a low frequency of nucleotide mutation  
216 (<1%, corresponding to only one strain) was observed for 63 out of the 126 mutations (48  
217 heterozygous and 15 homozygous).

218 In order to assess whether the 126 non-synonymous mutations identified using the set of 151  
219 susceptible strains provided an exhaustive catalog of phenotypically-neutral polymorphisms in  
220 the 5 selected genes, we quantified the number of new mutations identified with the increasing  
221 number of strains analyzed (**Figure 3**). The number of newly identified mutations by addition of  
222 each new strain from the “set of susceptible strains” quickly dropped below 1 after accounting

223 for the first ~20 strains, reaching a plateau and thus confirming that natural genetic diversity is  
224 well captured by the chosen strain catalog (**Figure 3**).

225

226 **Assessment of the capacity to detect resistance mutations in resistant *C. albicans* isolates**  
227 **using the catalog of phenotypically-neutral non-synonymous polymorphisms as an**  
228 **exclusion set.**

229 A set of 10 clinical isolates exhibiting resistance to fluconazole and/or caspofungin were used to  
230 test the capacity of the strain catalog to facilitate the identification of resistance mutations in  
231 genes involved in antifungal resistance through exclusion of phenotypically-neutral  
232 polymorphisms. The characteristics of the resistant isolates used here and results of *in vitro*  
233 susceptibility testing are detailed in **Table 2** and in supplementary **Table S2**. All strains were  
234 resistant to fluconazole and itraconazole (MIC $\geq$ 8 mg/L and  $\geq$ 1 mg/L, respectively) with 7  
235 exhibiting high-MIC levels to fluconazole (MIC $>$ 256 mg/L).<sup>33</sup> One strain (CEC4524) displayed  
236 caspofungin MIC value of 0.5 mg/L and was classified as “intermediate”.<sup>33</sup>

237 Comparison of the whole genome sequences of the 10 resistant isolates (average sequencing  
238 depth of 123x, **Table S2**) to the genome sequence of the SC5314 reference strain led to the  
239 identification of 45 non-synonymous mutations across the 5 genes (**Table S3**). Among these  
240 mutations, 23 were present in the catalog of phenotypically-neutral polymorphisms (**Table 1**),  
241 leaving 22 amino-acid changes that could be responsible for antifungal resistance. These  
242 polymorphisms impact Erg11p (n=11), Tac1p (n=7), Upc2p (n=3) and Fks1p (n=1) and are listed  
243 in supplementary **Table S6**. Mrr1p did not exhibit novel substitution.

244 Using a bootstrapping procedure, replacing the last 10 susceptible strains by the 10 resistant ones  
245 led to a significant increase in the number of cumulative mutations, by comparison to that  
246 observed in the catalog of susceptible strains only (**Figure 4A**). As shown in **Figure 4B**,  
247 distribution of the number of novel mutations in the last 10 susceptible strains was significantly

248 lower than that observed for the 10 resistant strains, suggesting the appearance of additional  
249 mutations to those expected only from natural non-synonymous polymorphisms (**Figure 4B**;  
250  $p < 0.0001$ ).

251 Among the 22 substitutions predicted to be responsible for antifungal resistance based on their  
252 absence from the catalog of phenotypically-neutral polymorphisms, 10 had already been  
253 associated to azole or echinocandin resistance (**Table S6**). These include one mutation in Tac1p  
254 (A736T),<sup>43</sup> seven in Erg11p (T123I, Y132H, R157K, S405F, G448E, G450E, R467I),<sup>44–58</sup> one in  
255 Upc2p (G648D<sup>h</sup>; h=heterozygous state),<sup>59</sup> and one in Fks1p (R647G).<sup>5</sup> The strain distribution  
256 of these known mutations as well as the 12 remaining mutations selected by our approach is  
257 summarized in **Table 2**.

258 Each of the 10 fluconazole-resistant strains harbored at least one known mutation that could  
259 explain phenotypic resistance. However, the high level of resistance of some strains (CEC4504  
260 and CEC4503) could not be explained by the unique presence of one heterozygous or  
261 homozygous substitution in Erg11p, suggesting the presence of additional mutations involved in  
262 resistance. This hypothesis was supported by a new comparison of the mutation accumulation  
263 curves of the two sets of strains. We removed the 10 mutations already known to be involved in  
264 resistance so that differences between susceptible and resistant strains are only restricted to the  
265 newly-identified mutations (n=12). Again, the introduction of the 10 resistant strains caused an  
266 increase in the number of cumulative mutations (**Figures 4C**). The number of new mutations  
267 arising in the 10 resistant strains was still significantly different to the distribution of the number  
268 of new mutations arising in 10 susceptible strains (**Figure 4D**;  $p = 0.0014$ ). We predicted that at  
269 least 4 out the 12 new mutations were distinct from the occurrence of phenotypically-neutral  
270 polymorphisms.

271

272

273 **A subset of the newly identified mutations in *TAC1* and *UPC2* confer an increase azole**  
274 **MICs.**

275 To explore the possibility that the identified mutations contributed to the high level of resistance  
276 to fluconazole in our strains, we constructed mutant strains harboring each of the 6 homozygous  
277 mutations in *TAC1* and 2 in *UPC2* (see Materials and Methods). No change in fluconazole MIC  
278 was observed for the *UPC2* mutants (CEC5218 and CEC5220) by comparison to the *C. albicans*  
279 strain transformed with the *UPC2* wild-type allele (CEC5216) (**Figure 5B, Table S4**).  
280 Introduction of 4 out of the 6 *TAC1* mutations (CEC5222: 2369G>A/A790V; CEC5228:  
281 787G>A/H263Y; CEC5230: 2515G>A/H839Y, and CEC5232: 2911G>A/P971S) resulted in an  
282 increase of the fluconazole MIC (7.8-, 8-, 85- and 5.3-fold changes, respectively) and  
283 itraconazole MIC (7.7-, 8-, 16- and 2.7-fold changes, respectively) compared to the *C. albicans*  
284 strain transformed with the *TAC1* wild-type allele (CEC5221 or CEC5226) (**Table S4, S7 and**  
285 **Figure 5A**).

286

287 **Newly identified mutations impact Erg11p structure.**

288 In order to evaluate the putative effect of newly identified amino-acid substitutions in Erg11p,  
289 we explored the effect of each mutation using structural prediction with the newly described *C.*  
290 *albicans* CYP51/Erg11 structural model protein 5v5z as reference.<sup>39</sup> The impact of the 3 newly  
291 identified amino-acid substitutions (P30S<sup>h</sup>, Y64H, P236S) identified in Erg11p was evaluated  
292 and compared with the results obtained for mutations already known to be involved in  
293 phenotypic fluconazole resistance. The PoPMuSiCv3.1 and HoTMuSiCv0.5 software were used  
294 to predict  $\Delta\Delta G$ ,  $\Delta T_m$  and solvent accessibility of amino-acids and the SNPMuSiC software was  
295 used to predict the overall effect of mutations. The heterozygous substitution D9V<sup>h</sup> could not be  
296 evaluated due to absence of the starting positions information in the protein 5v5z entry.

297 The heterozygous substitution P30S<sup>h</sup> was predicted to have a neutral impact by SNPMuSiC and a

298 putative protein stabilizing effect ( $\Delta\Delta G = -0.27$  and  $\Delta T_m = 0.35$ ) using HoTMuSiC and  
299 PoPMuSiC. These results were not in favor of a mutation involved in resistance. However, the 2  
300 homozygous substitutions (Y64H and P236S) showed a low level of amino-acid accessibility <  
301 20% (5.85 and 17.94) and an assumed protein destabilizing effect ( $\Delta\Delta G = 2.03$  and  $1.41$ ;  $\Delta T_m = -$   
302  $2.48$  and  $-1.6$  for Y64H and P236S, respectively). These results were corroborated using the  
303 SNPMuSiC software that predicted a deleterious effect for both mutations. For comparison, the  
304 mean values obtained for substitutions already known to be involved in fluconazole phenotypic  
305 resistance and found in this study (T123I, Y132H, R157K, S405F, G448E, G450E, R467I) were  
306  $\Delta\Delta G = 1.0$  and  $\Delta T_m = -1.3$  with an average amino-acid accessibility level of 14.37%.

307

## 308 **Discussion**

309 In this study, we have taken advantage of a collection of 151 whole genomes of *C. albicans*  
310 strains<sup>29</sup> susceptible to fluconazole and caspofungin in order to establish a comprehensive  
311 catalog of phenotypically-neutral polymorphisms in the five main genes involved in azoles  
312 (*ERG11*, *TAC1*, *UPC2* and *MRR1*) and echinocandins (*FKSI*) resistance in *C. albicans*. This  
313 catalog includes 126 distinct non-synonymous substitutions and depicts with high confidence the  
314 global polymorphisms existing in these genes within the natural population of *C. albicans*.  
315 Indeed, this set of 151 *C. albicans* strains is representative of the global diversity of the *C.*  
316 *albicans* population as these strains are distributed across the known genetic clusters and have  
317 been isolated from various cases of infections (invasive or superficial) or during commensalism,  
318 but also from various geographical areas.<sup>29</sup> Furthermore, these strains were selected for their  
319 susceptibility to antifungals drugs. The distributions of their MICs to fluconazole and to  
320 caspofungin were similar to the epidemiological cut-off (ECOFF) described for *C. albicans*  
321 species (<https://mic.eucast.org>). Finally, the observation that sequencing of additional susceptible

322 isolates rarely leads to the identification of novel substitutions (**Figure 3**) strongly reinforced the  
323 comprehensiveness of the established catalog of 126 natural non-synonymous substitutions.

324 We demonstrated the usefulness of the catalog to select putative resistance mutations among the  
325 polymorphisms identified in genes involved in antifungal resistance for 10 azole- or  
326 caspofungin-resistant strains. Indeed, using this catalog as a prediction guide, 23 non-  
327 synonymous mutations identified in resistant isolates could be excluded from further analysis as  
328 since they were also present in susceptible strains. By contrast, 22 non-synonymous mutations  
329 were classified as putative resistance mutations, among which 10 were already involved in azole  
330 or caspofungin resistance. Four newly described mutations in *TAC1* were experimentally  
331 confirmed as contributing to azole resistance while two were predicted to enable azole resistance  
332 by their significant impact on Erg11 protein structure. In contrast, we could not confirm that the  
333 mutations identified in *UPC2* confer fluconazole resistance, suggesting that the strains harboring  
334 these mutations have other resistance mechanisms.<sup>60,61</sup> Despite these limitations, our approach  
335 was highly efficient to highlight novel resistant mutations. Other groups have conducted  
336 descriptions of phenotypically-neutral polymorphisms or mutations involved in azole resistance.  
337 However, these descriptions were often restricted to a single gene, or on a smaller number of  
338 clinical strains.<sup>62-65</sup> For example, in the study performed by Spettel *et al.*, the amino-acid  
339 substitution S937L in Tac1p, identified as a potentially causal resistance mutation, could have  
340 been ruled out using the neutral polymorphisms catalog.<sup>62</sup>

341 In our study, two amino-acid substitutions identified in Erg11p (Y64H, P236S) were predicted  
342 with high confidence as impacting Erg11p protein structure. Interestingly, these positions were  
343 already predicted to be vital for various antifungal interactions with the active site of Erg11p  
344 from *C. albicans* or *Saccharomyces cerevisiae*.<sup>66-68</sup> This suggests a likely role of these  
345 substitutions in increasing azole resistance in our strains. Obviously, significant protein  
346 structural changes in the Erg11p active site domain will occur if the amino-acids responsible for

347 key molecular interactions (with its substrate or inhibitors) are substituted.<sup>66</sup> However, the  
348 specific contributions of individual *ERG11* mutations to azole resistance in *C. albicans* have not  
349 been widely explored and very few mutations were demonstrated to be directly associated with  
350 resistance.<sup>69</sup> The impact of a non-synonymous mutation on azole resistance could be  
351 demonstrated by heterologous expression of the *ERG11* allele in different models, such as  
352 *Saccharomyces cerevisiae*<sup>44,45</sup> or, recently, in genetically engineered strain of *Candida*  
353 *lusitaniae*.<sup>70</sup> The CRISPR-Cas9 system could also be used.<sup>71</sup> These systems could be used to  
354 formally confirm the involvement of the Y64H and P236S mutations in fluconazole resistance.  
355 In conclusion, we have established a nearly comprehensive catalog that includes the  
356 phenotypically-neutral non-synonymous polymorphisms in genes involved in azole- and  
357 echinocandin-resistance in *C. albicans*. This catalog is now available to the community working  
358 with this pathogen. Interestingly, it could be used to evaluate the possible role of neutral-  
359 polymorphisms on MICs variability when resistance mutations were present in isolates. This  
360 catalog is a powerful tool to prioritize mutations putatively involved in antifungal resistance and  
361 to describe new mutations involved in azoles or caspofungin MIC increase in *C. albicans*.

362

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## 370 **Transparency declarations**

371 The authors declare no competing interests.

372 **Data availability**

373 Raw reads have been deposited at the NCBI Sequence Read Archive under BioProject ID  
374 PRJNA432884 and PRJNA563993.

375

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- 572

573 **Figure and table legends**

574 **Table 1: Characteristics of phenotypically-neutral non-synonymous polymorphisms in**  
575 **genes involved in resistance to azoles and echinocandins.** The catalog includes 126 non-  
576 synonymous phenotypically-neutral polymorphisms (ORF position and amino-acid  
577 correspondence) detected in the 5 genes involved in resistance to azoles (*ERG11*, *TAC1*, *MRR1*  
578 and *UPC2*) and echinocandins (*FKS1*) using genome sequences of 151 susceptible strains. For  
579 each strain, the genotype at each position is compared to that of the reference strain SC5314 and  
580 summarized by the homozygous and heterozygous substitution frequency. h=substitution found  
581 only at the heterozygous state, \*=indicate a stop codon.

582

583 **Table 2: Results of *in vitro* fluconazole, itraconazole, voriconazole and caspofungin**  
584 **susceptibility testing and amino-acid substitutions detected for the 10 resistant strains of *C.***  
585 ***albicans*.** Only amino-acid substitutions prioritized by our approach (n=22) as possibly being  
586 involved in antifungal resistance are shown. These include resistance mutations previously  
587 described in the literature and new amino-acid substitutions (boldface) in the protein Tac1p,  
588 Upc2p, Erg11p and Fks1p. New resistant mutations validated in this study are marked with an  
589 asterisk (\*). P=patient; h=heterozygous mutation found in a single allele; FLC=fluconazole;  
590 ITC=itraconazole; VOR=voriconazole; CAS=caspofungin.

591 *C. albicans* susceptible isolates to fluconazole, itraconazole, voriconazole and caspofungin were  
592 defined as MIC  $\leq$  2 mg/L, MIC  $\leq$  0.125 mg/L MIC  $\leq$  0.125 mg/L, MIC  $\leq$  0.25 mg/L,  
593 respectively.<sup>33</sup>

594

595

596 **Figure 1: Schematic mapping of the phenotypically-neutral amino-acid substitutions in**  
597 **Erg11p, Tac1p, Upc2, Mrr1p and Fks1p obtained from data of the 151 *C. albicans***  
598 **fluconazole and caspofungin susceptible clinical strains.**

599 This figure appears in colour in the online version of *JAC* and in black and white in the printed  
600 version of *JAC*.

601

602 **Figure 2: Distribution of fluconazole (A) and caspofungin (B) MICs for the 151 susceptible**  
603 **clinical strains of *C. albicans*, determined by the Etest® method.**

604

605 **Figure 3: Rate of new mutations detected in the set of 151 susceptible strains.** The solid line  
606 shows the mean number of new mutations that could be identified for each additional susceptible  
607 strain considered (bootstrapping method). The grey envelopes correspond to 95% and 90%  
608 credible intervals. Dashed line shows the threshold of 1 new mutation/strain.

609

610 **Figure 4: Contribution of resistant strains to genetic diversity of *C. albicans*.** Accumulation  
611 curves of mutations as a function of the random selection of clinical strains of *C. albicans*,  
612 accounting for all mutations present in resistant strains (A) or restricting those to undocumented  
613 mutations only (C). Solid lines present the mean accumulation number of mutations with  
614 increasing number of strains (black: random resampling of all susceptible strains ( $N_S=151$ );  
615 dashed line: random samples of 141 susceptible strains ( $N_S$ ) with final introduction of the 10  
616 resistant strains ( $N_R$ )). Grey envelopes correspond to 95% and 90% credible intervals.

617 Bar plots (B, D) show the bootstrapped, expected distribution of number of new mutations in the  
618 last 10 susceptible strains ( $N_S$ ), with black diamond identifying the observed value when  
619 considering resistant strains ( $N_R$ ) instead under scenarios used for panels A and C, respectively.

620

621 **Figure 5: Fluconazole MICs determination of the constructed mutant strains using the**  
622 **Etest® method. A. Mutants obtained for *TAC1*. B. Mutants obtained for *UPC2*.**

623 This figure appears in colour in the online version of *JAC* and in black and white in the printed  
624 version of *JAC*.

625

626

627 **Table 1:**

Gene	ORF position	SC5314 reference strain genotype	Nucleotide substitution state/frequency (%)				Amino-acid substitution
			Heterozygous		Homozygous		
<i>TAC1</i>	261	TT	TG	8.4	GG	0.65	K87N
<i>TAC1</i>	268	CC	CT	0.65			A90T <sup>h</sup>
<i>TAC1</i>	310	AC			AA/CC	48/2.6	F104V
<i>TAC1</i>	323	CC	CT	16.1	TT	3.2	S108N
<i>TAC1</i>	391	GG	GT	23.2	TT	1.3	L131I
<i>TAC1</i>	445	AA	AT	1.3			L149I <sup>h</sup>
<i>TAC1</i>	508	TT	TC	20.65	CC	3.2	M170V
<i>TAC1</i>	510	CC	CA	9.0	AA	0.65	M170I
<i>TAC1</i>	520	TT	TC	9.0	CC	0.65	N174D
<i>TAC1</i>	566	AA	AG	28.8	GG	9.2	F189S
<i>TAC1</i>	593	TT	TC	1.3			H198R <sup>h</sup>
<i>TAC1</i>	596	CT			TT/CC	66.05/0.65	S199N
<i>TAC1</i>	617	CT			TT/CC	61.9/2.0	R206H
<i>TAC1</i>	620	AG			GG/AA	61.4/2.0	V207A
<i>TAC1</i>	628	CC	CA	0.65			A210S <sup>h</sup>
<i>TAC1</i>	1121	GG	GA	0.65			T374I <sup>h</sup>
<i>TAC1</i>	1130	GG	GA	12.3			A377V <sup>h</sup>
<i>TAC1</i>	1187	TC			CC/TT	65.6/1.3	N396S
<i>TAC1</i>	1924	TT	TC	1.3			T642A <sup>h</sup>
<i>TAC1</i>	1994	AA	AG	0.65			F665S <sup>h</sup>
<i>TAC1</i>	2083	GG	GA	13.5	AA	1.3	L695F
<i>TAC1</i>	2123	TT	TG	0.65			K708T <sup>h</sup>
<i>TAC1</i>	2140	TT	TC	0.65			S714G <sup>h</sup>
<i>TAC1</i>	2276	AA	AG	0.65			L759S <sup>h</sup>
<i>TAC1</i>	2291	CC	CT	2.0			S764N <sup>h</sup>
<i>TAC1</i>	2298	TT	TA	1.3			K766N <sup>h</sup>
<i>TAC1</i>	2316	AT			AATT	43.8/5.2	N772K
<i>TAC1</i>	2326	CT			TT/CC	67.75/0.65	D776N
<i>TAC1</i>	2344	TT	TC	0.65			T782A <sup>h</sup>
<i>TAC1</i>	2485	CG			GG/CC	47.4/9.2	E829Q
<i>TAC1</i>	2495	AA	AG	0.65			F832S <sup>h</sup>
<i>TAC1</i>	2606	CC	CT	2.6			R869Q <sup>h</sup>
<i>TAC1</i>	2684	AA	AG	14.2			I895T
<i>TAC1</i>	2687	TT	TC	49.4	CC	4.5	N896S
<i>TAC1</i>	2723	CC	CT	1.3	TT	0.65	C908Y
<i>TAC1</i>	2804	GA			GG/AA	74.8/1.3	S935L

629 **Table 1:** (Continued)

Gene	ORF position	SC5314 reference strain genotype	Nucleotide substitution state/frequency (%)			Amino-acid substitution
			Heterozygous	Homozygous		
<i>TAC1</i>	2810	GG	GA	8.4		S937L <sup>h</sup>
<i>TAC1</i>	2822	AG			GG/AA 20.8/5.8	S941P
<i>ERG11</i>	49	GG	GA	0.65		L17F <sup>h</sup>
<i>ERG11</i>	125	GG	GA	0.65		S42L <sup>h</sup>
<i>ERG11</i>	348	AT			AATT 60/7.1	D116E
<i>ERG11</i>	383	TG			TT/GG 78.6/2.0	K128T
<i>ERG11</i>	386	CC	CG	2.0		G129A <sup>h</sup>
<i>ERG11</i>	433	AA	AT	0.65		F145I <sup>h</sup>
<i>ERG11</i>	459	AA	AC	0.65	CC 3.2	D153E
<i>ERG11</i>	475	CC	CT	0.65		V159I <sup>h</sup>
<i>ERG11</i>	488	CC	CT	0.65		R163K <sup>h</sup>
<i>ERG11</i>	632	CC			TT 0.65	R211K
<i>ERG11</i>	798	TT	TG	12.5	GG 29.7	E266D
<i>ERG11</i>	886	CC	CA	1.3		E296* <sup>h</sup>
<i>ERG11</i>	982	GG	GA	0.65	AA 0.65	H328Y
<i>ERG11</i>	1007	TT	TC	1.3		E336G <sup>h</sup>
<i>ERG11</i>	1008	TT	TG	0.65		E336D <sup>h</sup>
<i>ERG11</i>	1217	GG	GA	0.65		P406L <sup>h</sup>
<i>ERG11</i>	1309	CC	CT	10.9	TT 4.5	V437I
<i>ERG11</i>	1325	GG	GA	1.3		S442F <sup>h</sup>
<i>ERG11</i>	1447	TT	TC	0.65		I483V <sup>h</sup>
<i>ERG11</i>	1462	CC	CT	24.6	TT 2.6	V488I
<i>UPC2</i>	73	GG	GA	0.65		L25F <sup>h</sup>
<i>UPC2</i>	76	TT	TC	0.65	CC 0.65	K26E
<i>UPC2</i>	122	GG	GT	0.65		T41N <sup>h</sup>
<i>UPC2</i>	203	CC	CT	31.2	TT 2.6	R68K
<i>UPC2</i>	425	AA	AC	48.6	CC 12.2	I142S
<i>UPC2</i>	431	GG	GA	1.3		T144I <sup>h</sup>
<i>UPC2</i>	569	CC	CT	22.2	TT 0.65	S190N
<i>UPC2</i>	661	CC			GG 0.65	A221P
<i>UPC2</i>	683	CC	CT	30.9	TT 3.9	S228N
<i>UPC2</i>	749	TT	TC	0.65		N250S <sup>h</sup>
<i>UPC2</i>	817	TT	TC	5.7	CC 1.3	T273A
<i>UPC2</i>	961	CC	CT	1.3		G321S <sup>h</sup>
<i>UPC2</i>	1024	GG	GC	1.3		L342V <sup>h</sup>

631 **Table 1:** (Continued)

Gene	ORF position	SC5314 reference strain genotype	Nucleotide substitution state/frequency (%)				Amino-acid substitution
			Heterozygous	Homozygous			
<i>UPC2</i>	1519	CC	CT	1.3			V507I <sup>h</sup>
<i>UPC2</i>	1904	AA	AC	0.65			L635W <sup>h</sup>
<i>MRR1</i>	47	GG	GT	3.6	TT	1.3	S16I
<i>MRR1</i>	56	CC	CT	2.0	TT	18.7	P19L
<i>MRR1</i>	79	GG	GA	27.1	AA	3.9	V27I
<i>MRR1</i>	179	TT	TC	1.3	CC	0.65	V60A
<i>MRR1</i>	218	CC	CA	3.6	AA	1.3	T73N
<i>MRR1</i>	223	GG	GA	2.0	AA	16.0	G75R
<i>MRR1</i>	304	GG	GA	2.0			A102T <sup>h</sup>
<i>MRR1</i>	387	TT	TG	0.65			N129K <sup>h</sup>
<i>MRR1</i>	394	CC	CA	0.65			Q132K <sup>h</sup>
<i>MRR1</i>	511	CC	CT	0.65			S171L <sup>h</sup>
<i>MRR1</i>	512	TT	TC	37.2	CC	18.2	S171P
<i>MRR1</i>	539	CC	CT	0.65			P180L <sup>h</sup>
<i>MRR1</i>	626	AA	AC	0.65			Q209P <sup>h</sup>
<i>MRR1</i>	742	TT	TG	28.0	GG	3.9	L248V
<i>MRR1</i>	1022	TT	TA	32.3	AA	11.8	V341E
<i>MRR1</i>	1226	GG	GA	1.3			G409E <sup>h</sup>
<i>MRR1</i>	1432	AA	AT	1.3			I478L <sup>h</sup>
<i>MRR1</i>	1675	AA	AT	1.3			T559S <sup>h</sup>
<i>MRR1</i>	1774	CC	CT	30.7	TT	4.5	L592F
<i>MRR1</i>	1925	CC	CT	0.65			A642V <sup>h</sup>
<i>MRR1</i>	1951	GG	GA	2.0			D651N <sup>h</sup>
<i>MRR1</i>	2128	GG	GA	0.65			E710K <sup>h</sup>
<i>MRR1</i>	2497	CC	CT	1.3			R833* <sup>h</sup>
<i>MRR1</i>	2750	CC	CT	9.4	TT	1.3	T917M
<i>MRR1</i>	2756	CC	CT	2.0			A919V <sup>h</sup>
<i>MRR1</i>	2768	CC	CT	9.4	TT	1.3	T923I
<i>MRR1</i>	2811	CC	CG	0.65	GG	18.1	N938K
<i>MRR1</i>	2900	GG	GA	0.65			G967D <sup>h</sup>
<i>MRR1</i>	3058	GG	GC	30.7	CC	7.9	E1020Q
<i>MRR1</i>	3082	GG	GA	0.65			E1028K <sup>h</sup>
<i>MRR1</i>	3096	TT	TA	2.1	AA	18.8	F1032L
<i>MRR1</i>	3110	CC	CT	2.1	TT	17.2	S1037L
<i>MRR1</i>	3265	GG	GT	0.65			A1089S <sup>h</sup>

633 **Table 1:** (Continued)

Gene	ORF position	SC5314 reference strain genotype	Nucleotide substitution state/frequency (%)				Amino-acid substitution
			Heterozygous		Homozygous		
<i>FKS1</i>	71	CC	CT	7.1	TT	0.65	G24E
<i>FKS1</i>	109	CC			TT	0.65	G37S
<i>FKS1</i>	340	AA	AC	0.65			Y114D <sup>h</sup>
<i>FKS1</i>	400	CC			TT	0.65	G134S
<i>FKS1</i>	457	CC	CT	0.65			A153T <sup>h</sup>
<i>FKS1</i>	859	CC	CT	0.65			A287T <sup>h</sup>
<i>FKS1</i>	943	CC	CT	0.65			G315S <sup>h</sup>
<i>FKS1</i>	1679	TT	CT	0.65			Y560C <sup>h</sup>
<i>FKS1</i>	1891	AA	AG	0.65			F631L <sup>h</sup>
<i>FKS1</i>	2355	TT	TA	0.65			E785D <sup>h</sup>
<i>FKS1</i>	2356	TT	TC	0.65			I786V <sup>h</sup>
<i>FKS1</i>	2496	CC	CA	0.65			M832I <sup>h</sup>
<i>FKS1</i>	3274	CC	CA	0.65			A1092S <sup>h</sup>
<i>FKS1</i>	3430	CC	CT	0.65			D1144N <sup>h</sup>
<i>FKS1</i>	4834	AA			TT	1.3	L1612M
<i>FKS1</i>	4873	CC	CT	0.65			D1625N <sup>h</sup>
<i>FKS1</i>	5512	GC			CC/GG	45.5/9.1	P1838A
<i>FKS1</i>	5531	TT	TG	0.65			N1844T <sup>h</sup>
<i>FKS1</i>	5609	CC	CT	1.3			R1870K <sup>h</sup>
<i>FKS1</i>	5657	GC			CC/GG	39.4/24.5	T1886S

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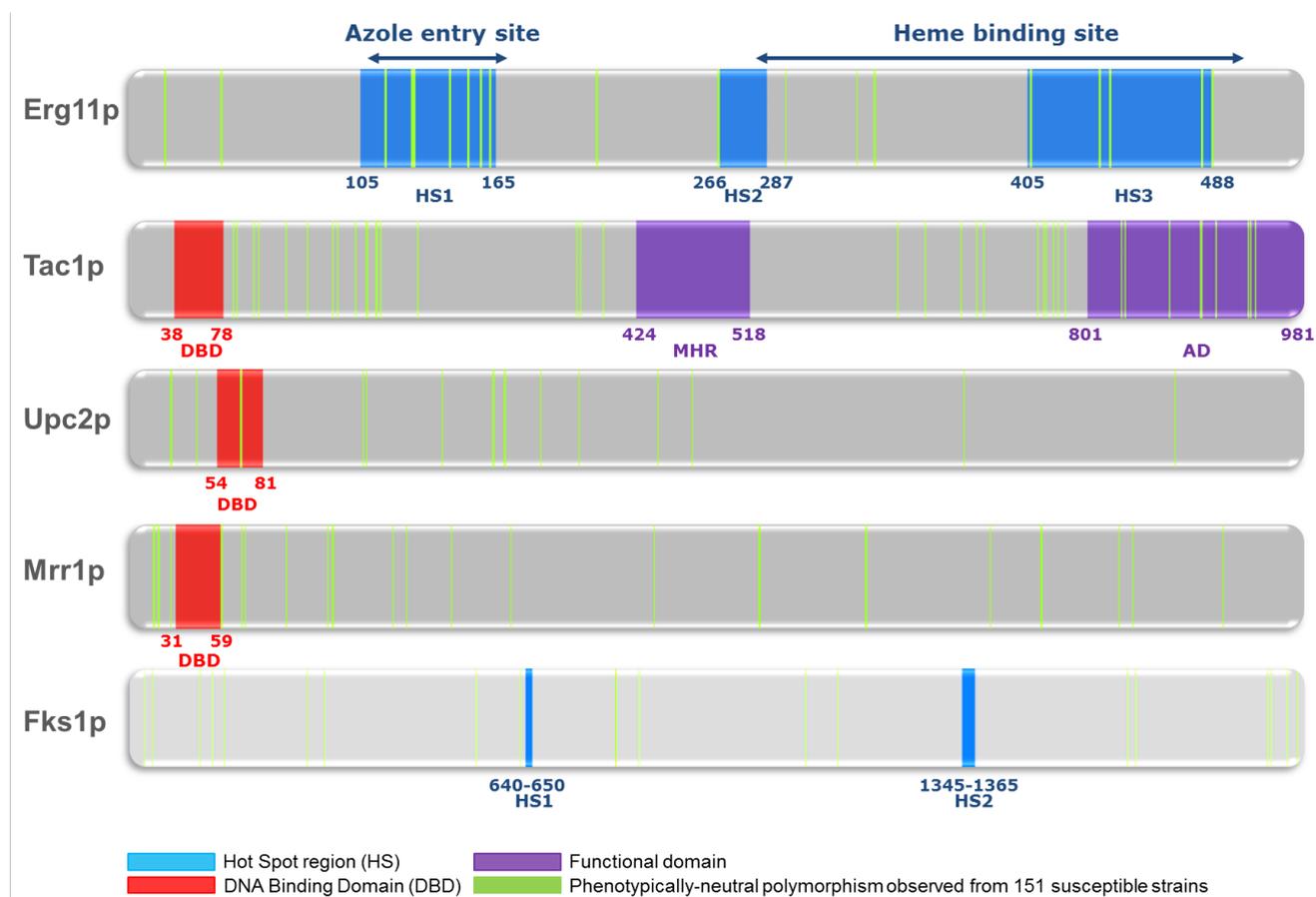
635

636 **Table 2:**

Strain	Origin	MIC (mg/L)				Amino-acid substitution			
		FLC	ITC	VOR	CAS	Erg11p	Tac1p	Upc2p	Fks1p
CEC718	P1	>256	>32	>32	0.047	Y132H, <b>P236S</b> , S405F	A736T	<b>G657D</b>	
CEC723	P2	16	1	0,094	0.064	<b>P30S<sup>h</sup></b> , S405F	<b>A790V*</b>		
CEC4504	P3	24	4	0,38	0.047	R157K <sup>h</sup>	<b>H263Y*</b> , <b>H839Y*</b> , <b>C858Y</b>		
CEC4503	P4	>256	>32	2	0.0125	<b>D9V<sup>h</sup></b> , R157K,	<b>H263Y*</b> , <b>H839Y*</b> , <b>C858Y</b>		
CEC4515	P5	>256	>32	>32	0.094	Y132H, G450E	<b>H263Y*</b> , <b>P971S*</b>	G648D <sup>h</sup>	
CEC4516	P5	>256	>32	>32	0.125	Y132H, G450E	<b>H263Y*</b> , <b>P971S*</b>		
CEC4521	P6	>256	12	4	0.064	Y132H, R467I	<b>H263Y*</b> , <b>P971S*</b>	<b>D654N<sup>h</sup></b>	
CEC4522	P6	>256	>32	12	0.19	Y132H, R467I	<b>H263Y*</b> , <b>P971S*</b>	<b>D654N</b>	
CEC4524	P6	>256	>32	3	0.5	<b>Y64H</b> , T123I, Y132H, R467I	<b>H263Y*</b> , <b>P971S*</b>	<b>D654N<sup>h</sup></b>	R647G
CEC4527	P7	8	3	0,125	0.047	G448E	A736T, <b>N823K</b> , <b>C858Y</b>		

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638 **Figure 1:**



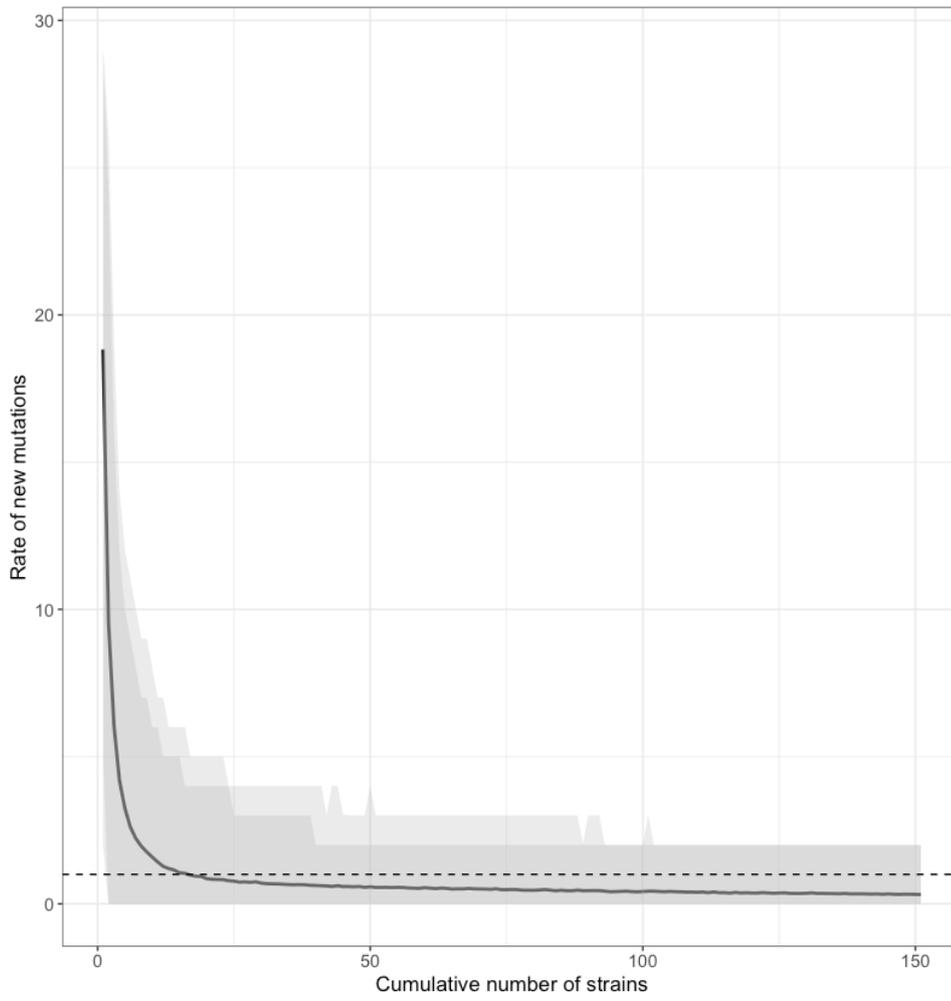
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640 **Figure 2:**

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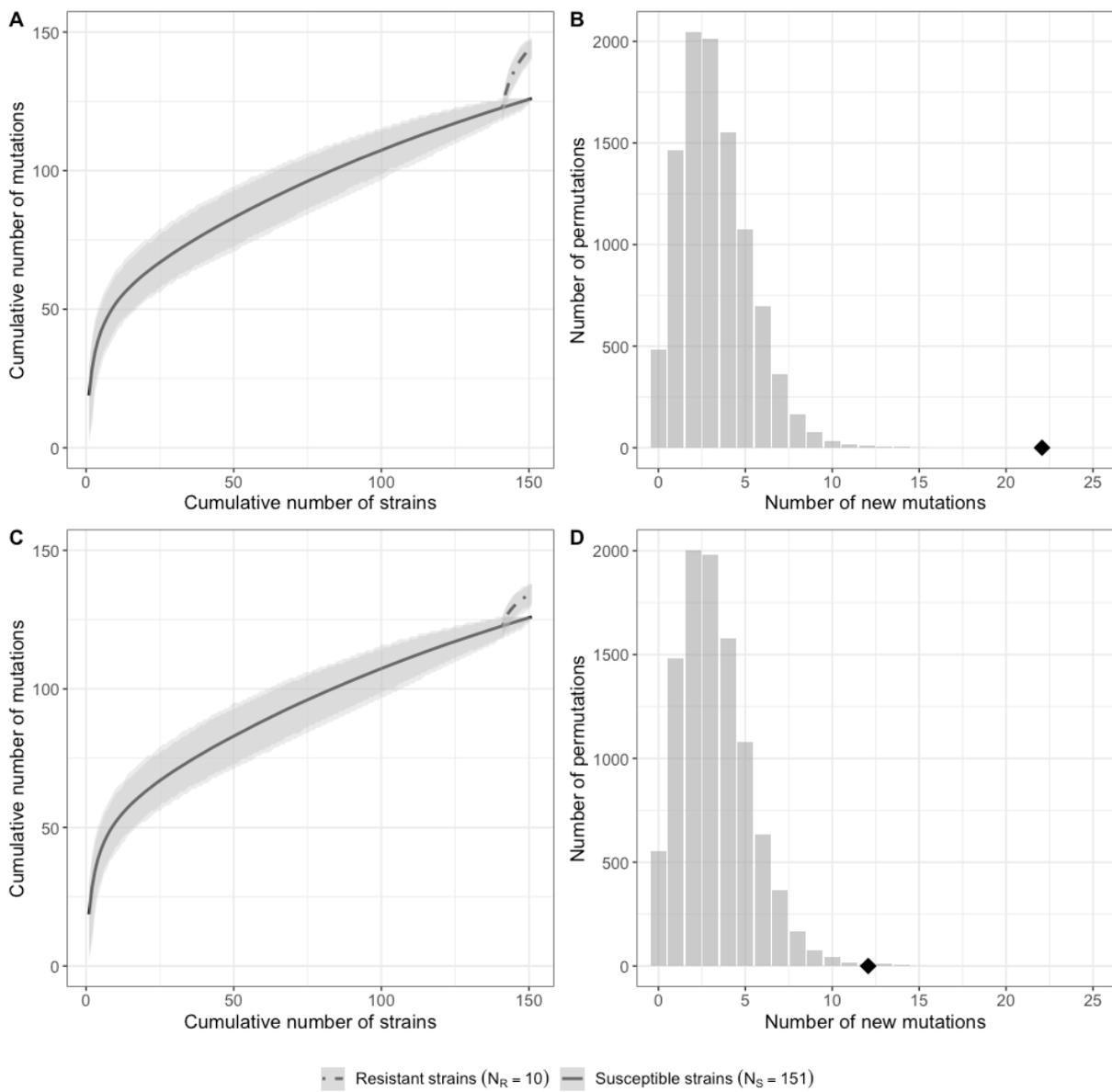
643 **Figure 3:**



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646 **Figure 4:**



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