

1 **Intimate genetic relationships and fungicide resistance**
2 **in multiple strains of human pathogenic fungus**
3 ***Aspergillus fumigatus* isolated from a plant bulb**

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22 Running title

23 Plant bulb-derived azole resistant *A. fumigatus*

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29 **Summary**

30 Fungal infections are increasingly dangerous because of
 31 environmentally-dispersed resistance to antifungal drugs. Azoles are commonly
 32 used antifungal drugs, but they are also used as fungicides in agriculture, which
 33 may enable enrichment of azole-resistant strains of the human pathogen
 34 *Aspergillus fumigatus* in the environment. Understanding of environmental
 35 dissemination and enrichment of genetic variation associated with azole
 36 resistance in *A. fumigatus* is required to suppress resistant strains. Here, we
 37 focused on eight strains of azole-resistant *A. fumigatus* isolated from a single
 38 tulip bulb for sale in Japan. This set includes strains with
 39 TR₃₄/L98H/T289A/I364V/G448S and TR₄₆/Y121F/T289A/S363P/I364V/G448S
 40 mutations in the *cyp51A* gene, which showed higher tolerance to several azoles
 41 than strains harboring TR₄₆/Y121F/T289A mutation. The strains were typed by
 42 microsatellite typing, single nucleotide polymorphism profiles, and mitochondrial
 43 and nuclear genome analyses. The strains grouped differently using each typing
 44 method, suggesting historical genetic recombination among the strains. Our
 45 data also revealed that some strains isolated from the tulip bulb showed
 46 tolerance to other classes of fungicide, such as QoI and carbendazim, followed
 47 by related amino acid alterations in the target proteins. Considering
 48 spatial-temporal factors, plant bulbs are an excellent environmental niche for
 49 fungal strains to encounter partners, and to obtain and spread
 50 resistance-associated mutations.

51

52 Introduction

53 Azoles are versatile compounds that show outstanding activity against a wide
 54 range of fungi, including plant and human pathogens. These compounds play an
 55 essential role in agricultural and clinical settings as fungicides and antifungal
 56 drugs (Fisher et al, 2018, Price et al, 2015). Their main mode of action is
 57 inhibition of the ergosterol biosynthesis pathway by inhibiting Cyp51, which
 58 functions as an 14-alpha-demethylase critical for the biosynthesis. Azole
 59 fungicides, known as demethylase inhibitors (DMIs), include triazole and
 60 imidazole compounds such as tebuconazole, propiconazole, triflumizole, and
 61 prochloraz. They are widely used to protect crops and fruits against pathogens
 62 by application during cultivation and postharvest preservation, as well as for
 63 seed disinfection. In medicine, azole drugs are essential options to combat
 64 dermatophytes and deep-seated fungal pathogens, such as *Trichophyton*
 65 *rubrum* and *Aspergillus fumigatus*, respectively. Azoles are the only class of
 66 compound used to control fungi in both agriculture and medicine.

67 *A. fumigatus* is a major causative agent of aspergillosis and ubiquitously
 68 present in the environment as a saprobe. A limited number of antifungals are
 69 approved for therapy of *A. fumigatus* infection; voriconazole (VRCZ) and
 70 itraconazole (ITCZ) are the first-line drugs for the treatment of pulmonary
 71 infection (Jenks and Hoenigl, 2018). However, this antifungal therapy is
 72 threatened by azole-resistant *A. fumigatus*, strains of which have been
 73 increasingly isolated since the beginning of this century (Howard et al, 2009).
 74 The resistance mechanisms to azole drugs that have been identified in *A.*
 75 *fumigatus* from clinical settings are mutations in Cyp51A, HMG-CoA reductase
 76 HMG1, and a subunit of CCAAT-binding complex HapE, and overexpression of
 77 *cdr1B*, which encodes an ABC transporter (Hagiwara et al, 2016a, Nywening et
 78 al, 2020, Hagiwara et al, 2018, Rybak et al, 2019, Camps et al, 2012,
 79 Hortschansky, et al, 2020, Fraczek et al, 2013). These azole resistance

mutations are thought to have emerged during therapy with prolonged azole treatment.

However, in addition to treatment-based resistance, environmentally derived resistance has been considered as a non-negligible source of azole drug resistance of *A. fumigatus* during the last decade (Berger et al, 2017, Lestrade et al, 2019). Typical resistant strains from the environment carry a tandem repeat (TR) and single-nucleotide polymorphisms (SNPs) in the promoter and coding regions of the *cyp51A* gene, respectively. The most prevalent variants are TR₃₄/L98H and TR₄₆/Y121F/T289A, which were isolated for the first time from patients in Europe in 1998 and North America in 2008, respectively (Jeanvoine et al, 2020). The mutants with TR₃₄ typically show high resistance to ITCZ, whereas the strains with TR₄₆ show VRCZ resistance, but some are pan-azole-resistant strains. These genotypes were later recovered from environments worldwide (Schoustra et al, 2019, Resendiz et al, 2018, Hagiwara, 2018). Diverse resistant mutants with tandem repeats in the Cyp51A-encoding gene have been reported (Table 1).

Table 1. Reported Cyp51A variants with tandem repeats in azole-resistant *A. fumigatus*

Cyp51A allele	Country	References
TR ₃₄ /L98H	Many places	-
TR ₄₆ /Y121F/T289A	Many places	-
TR ₅₃	Colombia	Alvarez-Moreno et al, 2017
TR ₃₄ /L98H/S302N	The Netherlands	Schoustra et al, 2019
TR ₃₄ /L98H/F495I	The Netherlands	Schoustra et al, 2019
TR ₃₄ /L98H/L343H	The Netherlands	Schoustra et al, 2019
TR ₃₄ /L98H/E356V	The Netherlands	Schoustra et al, 2019
TR ₃₄ /L98H/S297T/F495I	The Netherlands	Schoustra et al, 2019, Cao et al, 2020

TR ₃₄ /L98H/T289A/I364V/G448S	Japan	Nakano et al, 2020, this study
TR ₄₆ /Y121F/T289A/I364V	The Netherlands	Schoustra et al, 2019
TR ₄₆ /Y121F/M172I/T289A/G448S	The Netherlands, Japan, Iran	Zhang et al, 2017, Nakano et al, 2020, Ahangatkani et al, 2020, Fraaije et al, 2020
TR ₄₆ /Y121F/T289A/S363P/I364V/G448S	The Netherlands, Japan	Nakano et al, 2020, Fraaije et al, 2020, Zhang et al, 2021, this study
TR ₄₆ ³ /Y121F/M172I/T289A/G448S	The Netherlands, Japan	Zhang et al, 2017, Nakano et al, 2020
TR ₄₆ ⁴ /Y121F/M172I/T289A/G448S	The Netherlands	Zhang et al, 2021
TR ₉₂ /Y121F/M172I/T289A/G448S	The Netherlands	Zhang et al, 2021

99

100 Recently, a possible environmental hot spot for azole-resistant *A. fumigatus*
101 was proposed (Zhang et al, 2017). The TR-type mutants were prevalently
102 isolated from agricultural compost containing azole fungicide residues, whereas
103 azole-free compost was dominated by wild-type (WT) *A. fumigatus*. This view
104 was also supported in other studies (Schoustra et al, 2019, Zhang et al, 2021),
105 indicating that azole-resistant strains are enriched under the selective pressure
106 of environmental azoles. The work by Zhang et al. also suggested that sexual
107 reproduction plays an important role in developing and evolving new *cyp51A*
108 alleles for drug resistance in compost (Zhang et al, 2017). Taking into
109 consideration that TR-type drug-resistant *A. fumigatus* mutants show
110 cross-resistance to DMIs (Snelders et al., 2012), azole-containing environmental
111 niches may serve as evolutionary incubators through genetic recombination.

112 The propagation of azole-resistant *A. fumigatus* has been studied in an
113 epidemiological manner using microsatellite analysis by short tandem repeats
114 for *A. fumigatus* (STRAf), which is a widely accepted intraspecies typing method
115 with high-resolution discriminatory power (de Valk et al, 2005). TR-type mutant
116 strains were spread worldwide. Some isolates from multiple countries were
117 genetically closely related to each other and some had identical microsatellite

118 patterns (Pontes et al, 2020, Cao et al, 2020, Wang et al, 2018, Hagiwara et al,
119 2016b). Besides such international propagation, intranational clonal expansion
120 was also reported in several countries (Ahangarkani et al, 2020, Chowdhary et al,
121 2012). Recent population genomic studies revealed that the azole-resistant
122 strains are globally distributed. The isolates were divided into two broad clades,
123 and TR mutants belong to the populations in an uneven manner (Sewell et al,
124 2019). These data suggest that azole resistance primarily expanded by asexual
125 and sexual propagation from a limited number of ancestors with TR-type
126 mutation, rather than locally and independently emerging in each environment.

127 It was recently proposed that resistant *A. fumigatus* strains are transferred
128 internationally via imported plant bulbs (Dunne et al, 2017). Plant bulbs
129 produced in the Netherlands and sold in Ireland were contaminated with TR-type
130 *A. fumigatus* mutants. Similar cases were also reported by two independent
131 Japanese groups (Hagiwara 2020, Nakano et al, 2020); azole-resistant *A.*
132 *fumigatus* with diverse Cyp51A variants were isolated from plant bulbs that were
133 imported from the Netherlands and sold in Japanese gardening shops. These
134 studies suggest that the wide spread of azole-resistant *A. fumigatus* mutants is
135 attributable in part to trade in agricultural products including plant bulbs.

136 In the present study, to further understand genetic variations in plant
137 bulb-associated isolates, we focused on eight *A. fumigatus* strains that were
138 co-isolated from a single tulip bulb in a previous screening study (Hagiwara,
139 2020). Sensitivity to medical and agricultural azoles, as well as other classes of
140 fungicides, was compared between the strains. Whole genome comparison of
141 the eight strains showed several fragmental overlaps of their genomes,
142 suggesting genetic recombination had occurred between strains in the single
143 bulb. Our work indicates that plant bulbs are not only a vehicle for the pathogen
144 but also a place where the pathogen can evolve its drug resistance.

145

146 Results

147 Variation of Cyp51A mutation in strains from a single bulb

148 In a previous study, eight strains of *A. fumigatus* were isolated from a single tulip
149 bulb as different colonies (hereafter referred to as strains 3-1-A to 3-1-H)
150 (Hagiwara, 2020). Strain 3-1-H has no TR or SNPs in *cyp51A*, whereas TR₃₄ or
151 TR₄₆ occur in combination with various SNPs in the other seven strains (Table 2).
152 Strains 3-1-A, 3-1-E, 3-1-F, and 3-1-G have a typical variant, TR₄₆/Y121F/T289A.
153 Strain 3-1-D has mutations S363P, I364V, and G448S as well as
154 TR₄₆/Y121F/T289A. Strains 3-1-B and 3-1-C have TR₃₄/L98H and mutations
155 T289A, I364V, and G448S. Notably, TR₃₄/L98H and G448S are known to play a
156 role in azole resistance, and T289A is typically accompanied by TR₄₆ (Hagiwara
157 et al, 2016a). Thus, the Cyp51A of strains 3-1-B and 3-1-C showed complicated
158 sequence variation, including three mutations related to azole resistance.

159

Table 2. Cyp51A variation and microsatellite typing of the strains in this study

Strain ID	Cyp51A variation	2A	2B	2C	3A	3B	3C	4A	4B	4C
3-1-A	TR ₄₆ /Y121F, T289A	10	20	8	44	9	10	8	10	7
3-1-B	TR ₃₄ /L98H, T289A, I364V, G448S	23	10	9	35	9	6	8	10	18
3-1-C	TR ₃₄ /L98H, T289A, I364V, G448S	23	10	9	35	9	6	8	10	18
3-1-D	TR ₄₆ /Y121F, T289A, S363P, I364V, G448S	24	20	12	45	9	11	8	10	18
3-1-E	TR ₄₆ /Y121F, T289A	26	20	12	36	9	22	8	14	31
3-1-F	TR ₄₆ /Y121F, T289A	25	20	12	45	11	6	10	12	18
3-1-G	TR ₄₆ /Y121F, T289A	23	10	9	36	9	6	12	10	7
3-1-H	wt	23	19	15	33	11	7	13	9	5

160

161 Varied sensitivity to azoles in the strains from a single bulb

As previously reported, strains 3-1-A to G, which have TRs in *cyp51A*, showed VRCZ resistance (>32 µg/ml) in minimum inhibitory concentration tests (Hagiwara, 2020). To further understand the susceptibility to azole drugs, colony growth was evaluated on potato-dextrose-agar (PDA) containing 10 µg/ml of VRCZ (Fig. 1A). Strains 3-1-B, 3-1-C, and 3-1-D were more tolerant to VRCZ than the other strains. When grown on medium containing DMIs (triflumizole, imazalil, prochloraz, tebuconazole, epoxiconazole, or difenoconazole), strain 3-1-H, which harbors WT Cyp51A, showed the greatest growth inhibition among the strains. Strains 3-1-B, 3-1-C, and 3-1-D were less affected by the DMIs (except prochloraz) (Fig. 1B). On the basis of colony diameter measurement, strains 3-1-B, 3-1-C and 3-1-D showed higher tolerance to VRCZ and DMIs than strains 3-1-A, 3-1-E, 3-1-F, and 3-1-G (Fig. 1C). These results suggest that the combination of TR and G448S mutation increases resistance to azole compounds.

The expression levels of genes related to azole resistance were examined in the eight strains by quantitative real-time (qRT)-PCR. Compared with strain 3-1-H, which has the WT *cyp51A* gene, strains with a TR in the *cyp51A* gene showed higher expression of *cyp51A* (Fig. 2A). Overexpression of *cdr1B*, which encodes an ABC transporter, has been reported to confer azole resistance. Thus, the expression level of *cdr1B* was also determined in the eight strains by qRT-PCR. Strains 3-1-B and 3-1-C showed relatively high expression levels of *cdr1B* (Fig. 2B).

Microsatellite typing analysis of tulip bulb isolates

To investigate the genetic relationships between the eight strains co-isolated from a single tulip bulb, microsatellite analysis using STRAf was performed (Table 2). This analysis also included TR-type strains that were previously reported and isolated in different countries and strains isolated from plant bulbs

190 in Japan (Nakano et al, 2020, Hagiwara, 2020) (Fig. 3). Among the eight strains,
191 the STRAf patterns of 3-1-B and 3-1-C matched perfectly. Strain 3-1-D is closely
192 related to them, as this strain contains the same number of STRs in 4 of the 9
193 panels. Similarly, strain 3-1-D shares the same number of STRs as strain 3-1-F
194 in 4 of the 9 panels. These four strains grouped into the same clade. The other
195 strains were distantly positioned in the dendrogram. Interestingly, some strains
196 that were isolated from plant bulbs in the study by Nakano et al. (2020) showed
197 a close relationship with our strains. NGS-ER15 had an STR pattern similar to
198 that of our strains 3-1-B and 3-1-C (5 of the 9 panells), which is consistent with
199 these strains having the same Cyp51A allele (TR₃₄/L98H/T289A/I364V/G448S).
200 Strains NGS-ER6 and NGS-ER7 of Nakano et al. (2020) are closely related to
201 strains 3-3-A and 3-3-B that were isolated from a single another tulip bulb in our
202 previous study (Hagiwara, 2020). Note that these extraordinarily close relatives
203 were isolated from plant bulbs in different laboratories.

204

205 **Genome sequencing and comparison between strains**

206 To gain more insight into genetic differences or relatedness, genomes of the
207 eight strains (3-1-A to H) were sequenced using the Illumina platform. Complete
208 mitochondrial genomes were successfully obtained for the strains (31,749 to
209 31,770 base pairs [bp] long) (Table 3). A phylogenetic tree was constructed
210 using the mitochondrial genomes and those of other strains (IFM 61407, IFM
211 59365, and IFM 61578) that had been clinically isolated in Japan
212 (Takahashi-Nakaguchi et al, 2015) (Fig. 4A). This dendrogram indicated that the
213 eight strains isolated from the tulip bulb can be divided into three groups. Group
214 m1 contains strains 3-1-A, 3-1-D, and 3-1-G; strains 3-1-B, 3-1-C, 3-1-E, and
215 3-1-F are in Group m2. Strain 3-1-H was distantly positioned from both Group
216 m1 and m2. Differences in the length of the mitochondrial genome well reflect
217 the grouping, suggesting that strains within each group are very close relatives.

Table 3. Results of genome sequencing for strains isolated from a single tulip bulb

Strain ID	Total length of chromosomes [bp]	GC [%]	# of proteins	Mitochondrial genome [bp]	Mito Group	CSP type	Mating type
3-1-A	28,889,155	49.342	9,492	31,770	m1	t02	<i>mat1-1</i>
3-1-B	28,519,682	49.352	9,359	31,763	m2	t02	<i>mat1-1</i>
3-1-C	28,533,261	49.355	9,490	31,763	m2	t02	<i>mat1-1</i>
3-1-D	29,087,830	49.324	9,515	31,770	m1	t02	<i>mat1-1</i>
3-1-E	28,703,796	49.398	9,464	31,763	m2	t02	<i>mat1-1</i>
3-1-F	28,808,510	49.492	9,537	31,763	m2	t02	<i>mat1-2</i>

218 In the microsatellite typing analysis described above, strains 3-1-B, 3-1-C, 3-1-D,
219 and 3-1-F were grouped into the same clade, but this was inconsistent with the
220 grouping based on mitochondrial genomes, in which strain 3-1-D was not in the
221 same group as strains 3-1-B, 3-1-C, and 3-1-F.

3-1-G	29,178,518	49.295	9,543	31,770	m1	t02	<i>mat1-1</i>
3-1-H	28,716,638	49.611	9,617	31,749	m3	t01	<i>mat1-1</i>

222 Nuclear genomes of the eight strains were compared with the reference
223 genome of *A. fumigatus* strain Af293 (retrieved from AspGD,
224 <http://www.aspgd.org/>) ; 92.2% to 93.6% of the Af293 genome was covered in
225 the eight strains, and 69,949 to 79,391 SNPs were detected the genomes of the
226 eight strains compared with the sequence of Af293 (Table 4). Phylogenetic
227 analysis of the eight strains and previously-sequenced strains was performed by
228 using concatenated sequences of the SNP positions (Takahashi-Nakaguchi et al,
229 2015) (Fig. 4B). Among the eight strains, 3-1-H was distantly positioned in the
230 dendrogram as an independent clone. The other seven strains showed
231 moderately close genetic-relatedness to each other based on comparison with
232 the apparently independent clinical strains. Strains 3-1-B and 3-1-C showed the
233 closest relationship, which was supported by the lowest number (6,241) of SNPs
234 between strains (Table 4). This is consistent with the results of microsatellite and
235 mitochondrial genome typing. Nevertheless, in the mitochondrial genome typing,
236 strain 3-1-E was in Group m2 with strains 3-1-B, 3-1-C, and 3-1-F; however,
237 strain 3-1-E was relatively distant from these three strains in phylogenetic
238 analysis based on the nuclear genome (Fig. 4C).

239 From the genome sequences, CSP typing was performed, which can typify
240 strains by sequence variation at a single locus (*csp*: Afu3g08990) (Klaassen et al,
241 2009). The results showed that seven strains (3-1-A to 3-1-G) carried an
242 identical type (t02), but strain 3-1-H strain had type t01. Sequence analysis for
243 mating type revealed that all but strain 3-1-F harbored *mat1-1*, whereas 3-1-F
244 carried *mat1-2* (Table 3).

245

246 **Comparison of genome-wide SNP frequency pattern**

247 Inconsistency in strain typing among the typing methods using the mitochondrial
248 and chromosomal genome sequences caused us to speculate that genetic
249 recombination had occurred between the strains isolated from the single tulip
250 bulb. To help test this hypothesis, the SNP frequency and distribution were
251 investigated and compared among the strains in a genome-wide manner (Fig.
252 S1). There were several regions where the patterns of SNP frequency markedly
253 differed among the strains (Fig.

Table 4. Summary of SNPs in strains isolated from a single tulip bulb.

Strain ID	% of covered positions *1	# of SNPs *1	3-1-A	3-1-B	3-1-C	3-1-D	3-1-E	3-1-F	3-1-G	3-1-H
3-1-A	92.7%	74,865								
3-1-B	92.2%	77,715	46,380							
3-1-C	92.2%	77,879	46,296	6,241						
3-1-D	93.3%	78,394	38,184	46,181	46,244					
3-1-E	92.6%	76,218	50,656	56,072	55,902	59,081				
3-1-F	93.0%	75,553	40,062	45,535	45,284	52,533	54,507			
3-1-G	93.0%	79,391	32,777	36,509	36,566	36,517	57,646	44,451		
3-1-H	93.6%	69,949	95,960	91,751	91,720	88,448	89,321	92,982	93,204	

*1 These are relative to the genome of reference strain *A. fumigatus* Af293.

Table 5. The number of orthologous genes in the strains isolated from a single tulip bulb compared with reference strain *A. fumigatus* Af293.

Chromosome	# of orthologous genes compared with strain Af293
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	Af293	3-1-A	3-1-B	3-1-C	3-1-D	3-1-E	3-1-F	3-1-G	3-1-H
chr1	1,642	1,369	1,355	1,377	1,371	1,369	1,365	1,370	1,370
chr2	1,640	1,433	1,406	1,425	1,425	1,411	1,436	1,427	1,433
chr3	1,395	1,163	1,163	1,169	1,179	1,152	1,173	1,179	1,189
chr4	1,253	1,089	1,075	1,093	1,090	1,098	1,096	1,085	1,095
chr5	1,367	1,151	1,156	1,160	1,153	1,153	1,162	1,156	1,153
chr6	1,249	1,068	1,067	1,074	1,058	1,069	1,073	1,070	1,080
chr7	651	490	478	476	483	489	493	494	489
chr8	628	502	496	507	497	505	506	491	513
Total	9,825	8,265	8,196	8,281	8,256	8,246	8,304	8,272	8,322

S1). For example, regions 5-A, 5-B, and 5-C on chromosome 5 were particularly characteristic (Fig. 5A). In region 5-A, strains 3-1-A, 3-1-E, and 3-1-H showed similar patterns of SNP frequency. In region 5-B, the pattern of strain 3-1-A was similar to that of strains 3-1-F and 3-1-H. In region 5-C, the pattern of strain 3-1-A was similar to that of strains 3-1-E, 3-1-G, and 3-1-H. These results indicate that strain 3-1-A shares parts of the sequence of chromosome 5 with strains 3-1-E, 3-1-F, 3-1-G, and 3-1-H. Such intergenomic variations were also found on other chromosomes (Fig. 5B, Fig. S1). These results showed a genome-wide mosaic pattern of SNP frequency, which is indicative of genetic recombination events in the strains.

Comparing genome-wide distribution of orthologous genes

To further investigate genome shuffling in the strains, we compared the patterns of orthologous among the strains isolated from the tulip bulb. First, the genes shared with the reference genome of *A. fumigatus* strain Af293 were investigated based on reciprocal blast hits (RBHs), which resulted in the isolates containing 8,196 to 8,322 orthologs of genes in strain Af293 (Table 5). The positions of the orthologs were generally evenly distributed in the strains,

272 although fewer orthologs were found on chromosome 7. Notably, different
273 patterns of ortholog content were displayed in some regions of the genomes of
274 the various strains (Fig. 6). I.e., some sets of strains have lost particular sets of
275 genes, and other sets of genes have been lost in other sets of strains. Hence,
276 the set of strains that shares an ortholog pattern is different at each locus (Fig. 6).
277 This suggests repeated genome shuffling among the strains.

278

279 **Varied tolerance to agricultural fungicides**

280 As these strains were derived from a horticultural product, they may have been
281 exposed to agricultural fungicides besides DMIs. Hence, the susceptibility of the
282 eight strains to QoI (pyraclostrobin), SDHI (boscalid), methyl benzimidazole
283 carbamate (carbendazim), and phenylpyrrole (fludioxonil) was evaluated on
284 PDA plates. There was no significant difference among the strains in
285 susceptibility to fludioxonil and boscalid (Fig. 7A&B). However, the colony of
286 strain 3-1-H was smaller than those of the other seven strains on the medium
287 containing pyraclostrobin or carbendazim. These results suggest that there is
288 varied tolerance to pyraclostrobin and carbendazim among the strains.

289 From the genome sequences, mutations that are possibly responsible for
290 tolerance to carbendazim and pyraclostrobin were searched in the target
291 molecules tubulin and cytochrome b that are encoded by *tubA* (Afu1g10910) and
292 *cytB* (AfuMt00001), respectively (Fig. 6C). The amino acid substitution F219Y
293 was found in TubA of strains 3-1-A to 3-1-G. This substitution has been reported
294 in several carbendazim-resistant strains of plant pathogenic fungi (Yarden and
295 Katan, 1993, Zhou et al, 2020). To investigate how the mutation is distributed in
296 human pathogenic *A. fumigatus* genomes, the SNP database in FungiDB was
297 explored. According to the dataset, 18% (14 of 77 strains) of *A. fumigatus*
298 contain F219Y in TubA. Notably, eight of the 77 strains were isolated from the
299 environment, and four of these possess the amino acid substitution.

In CytB, mutations V13I and G143A were found in strains 3-1-A to 3-1-G, and V119I was found in 3-1-H. G143A in CytB has been reported to confer the resistance to QoI in many plant pathogenic fungi (Samuel et al, 2011, Bolton et al, 2012), suggesting that this mutation in *A. fumigatus* is related to low sensitivity to QoI fungicide. As mitochondrial genome sequences are scarce in public databases, we investigated the sequence of the *cytb* gene in nine strains that were clinically isolated in a previous study and whose mitochondrial genome sequence is at least partly available (Takahashi-Nakaguchi et al, 2015). Among these nine strains, no G143A mutation was observed, whereas seven of the strains contain V119I (as also observed in our strain 3-1-H).

310

311 Discussion

The distribution of azole-resistant *A. fumigatus* in natural environments has drawn increasing attention in recent years, with special interest in where the resistant strains have emerged, inhabit, and have been translocated to. However, deep understanding is still lacking. In this work, to fill in gaps in knowledge, we focused on strains that were isolated from a single tulip bulb.

Sexual reproduction of *A. fumigatus* was demonstrated in laboratory conditions in 2009 (O'Gorman et al., 2009). After this discovery, researchers paid more attention to the pan-genome of clinical isolates of this pathogenic fungus. However, gene flow in the environment has been poorly studied. Population genetics study using linkage disequilibrium analysis for genetic markers supported the view that *A. fumigatus* reproduces asexually and sexually in natural habitats (Klaassen et al., 2012). However, although there is accumulating evidence for genetic recombination in nature, proving the occurrence of sexual reproduction is difficult unless one can directly collect cleistothecia and ascospores formed in the environment. Genetic recombination in sexual development is suggested to cause the emergence of TR mutation in

328 *cyp51A* gene through unequal crossover (Zhang et al., 2017). Thus, sexual
329 reproduction is considered both to spread mutations by fusion with other strains
330 and production of progeny, and to locally produce *de novo* TR mutations, which
331 could affect the prevalence of resistance to drugs and fungicides. Our data show
332 that seven of the eight isolates from a single tulip bulb contain TR mutations, and
333 the genetic variation between the TR-containing strains is low compared with
334 that between apparently independent strains. In addition, on the basis of
335 genome-wide distributions of SNPs and orthologous genes, genetic
336 recombination is likely to have occurred between the seven strains.

337 Co-isolation of the strains from a single bulb indicates that they have had
338 much opportunity to physically interact with each other inside or on the bulb. In
339 addition to the close spatial relationship of the fungal strains, they may interact
340 for a long time. In the conventional process of plant bulb production, bulbs are
341 multiplied from a parental bulb. This bulb multiplication is continued every year,
342 which presumably causes the sustained presence of the fungi on/inside the
343 bulbs. As described here, several strains were attached to a single bulb. These
344 strains might have encountered others and genetically mixed many times. Once
345 mutations giving rise to resistance to azoles emerged, the mutations could be
346 preferentially and stably retained in the microbial community inside the bulb.

347 Sequencing analysis of the eight strains produced complete mitochondrial
348 genomes and chromosomal genomes. The mitochondrial genome was of great
349 help in interpreting whether there had been sexual reproduction among the
350 strains. On the basis of mitochondrial genome sequences, the strains with TR
351 mutation can be classed into Groups m1 and m2 (Fig. 4A). The length and
352 sequences of the mitochondrial genomes are highly conserved in each group,
353 indicating that they are genetically close progenies. However, the chromosomal
354 genomes were diverse among the strains with TR mutation to the extent that
355 there were 32,000 to 59,000 SNPs, excepting strains 3-1-B and 3-1-C which had

approximately 6,200 SNPs (Table 4). The differences in grouping based on mitochondrial and chromosomal genomes strongly suggested a genetic recombination event. We therefore propose that the complete mitochondrial genome is valuable for gaining deeper insight into genetic relatedness among and between environmental and clinical isolates.

Strains with complicated *cyp51A* alleles have been reported in the literature and this paper (Table 1). For instance, we have isolated *A. fumigatus* strains with TR₃₄/L98H/T289A/I364V/G448S (3-1-B and 3-1-C) and TR₄₆/Y121F/T289A/S363P/I364V/G448S (3-1-D) mutations in the *cyp51A* gene. TR₃₄/L98H is a typical TR-type mutation conferring resistance to ITCZ and in some cases to VRCZ, whereas TR₄₆/Y121F/T289A confers resistance to VRCZ and in most cases to ITCZ (van Ingen et al, 2015, Buil et al, 2018). Amino acid substitution G448S contributes to resistance to VRCZ and occasionally to ITCZ (Belleste et al, 2010, Toyotome et al, 2016, Cao et al, 2020). Our finding that three strains (3-1-B, 3-1-C, and 3-1-D) showed a higher tolerance to VRCZ and some DMIs than strains with only TR₄₆/Y121F/T289A mutation is suggestive of elevation of tolerance to azole drugs by combining mutations. Importantly, strains with G448S mutation have been isolated not only from clinical samples but also from soil (Cao et al, 2020). We cannot rule out the possibility that the G448S mutation originally emerged and was retained in strains with TR₄₆/Y121F/T289A under the selective pressure of fungicides.

The *A. fumigatus* strains used in the present work were isolated from a tulip bulb by culturing at 45°C on plates containing medium supplemented with fluconazole to select fungi that were resistant to fluconazole (Hagiwara 2020). In total in that study, *A. fumigatus* was isolated from 50.8% of tulip bulbs (96/189), and strains isolated from 20.6% of the bulbs (39/189) had TR mutation. Because *A. fumigatus* is a saprophytic fungus that widely inhabits soil, compost, plant debris, wood chips, the air, and aquatic environments, it was not surprising that

384 half of the tulip bulbs were contaminated with *A. fumigatus*. However, we have
 385 no idea how the fungus resides on or inside the bulbs from a biological viewpoint.
 386 Because of the high frequency of *A. fumigatus* isolation from tulip bulbs, there
 387 might be certain mechanisms by which *A. fumigatus* colonizes and infects the
 388 plant tissue, enabling persistence across bulb progenies. Notably, some *A.*
 389 *fumigatus* strains were isolated from *Citrus macrocarpa*, *Myricaria laxiflora*,
 390 *Ligusticum wallichii*, and *Moringa oleifera* (Francisco et al, 2020, Qin et al, 2019,
 391 Li et al, 2020, Arora and Kaur, 2019) as an endophyte. In general, however, the
 392 view that *A. fumigatus* has an endophytic mode in its life cycle remains to be
 393 established. In consideration of the dynamic mobilization of *A. fumigatus* in the
 394 environment, its association with plants may be overlooked, and we should pay
 395 more attention to it.

396 Recently, several field studies were published in which the prevalence of
 397 azole-resistant *A. fumigatus* was investigated in association with fungicide use.
 398 Work by Zhou et al. (2021) demonstrated that the concentration of triazoles in
 399 the soil of greenhouses was not significantly correlated with azole susceptibility
 400 of isolates. In another study from Germany, a low frequency of azole-resistant
 401 isolates from crop fields was reported regardless of azole fungicide use (Barber
 402 et al, 2020). A study by Frasijs et al. (2020) also reported a low number of
 403 azole-resistant isolates in the soils of wheat-cropping fields subjected to
 404 fungicide treatment. The authors considered that arable crop production is low
 405 risk for development of azole resistance. Conversely, a large-scale survey
 406 across China was conducted, which showed that the residual level of azole
 407 fungicides in paddy soils positively correlated with the prevalence of
 408 azole-resistant *A. fumigatus* (Cao et al, 2021). Field research on azole-resistant
 409 *A. fumigatus* has started in many countries. More studies are required on the
 410 effects of fungicide use on the occurrence and spread of azole-resistant *A.*
 411 *fumigatus* in the environment, including agricultural and horticultural settings.

In plant bulbs, there may be other pathogenic and nonpathogenic fungi beside *A. fumigatus*. They are also exposed to fungicides when the bulbs are treated with fungicide. Repeated use of fungicides would facilitate the occurrence of resistance mutations in non-targeted fungi as well as in the target fungi of the pesticide. In the present study, we found that mutations in CytB and TubA that are related to resistance to Qol and carbendazim fungicides, respectively, were detected in *A. fumigatus* strains as an example of non-target fungi. These mutations might have been resulted from fungicide exposure during bulb production. Importantly, identical mutations of *A. fumigatus* were reported by Fraaije *et al.* (2020) and are found in database. These findings suggest that mutations related to resistance to antifungal agents are already present in the genomes of environmental fungi regardless of their pathogenicity. The boundary between acquired and natural resistance to antifungal compounds may become unclear in the near future.

Materials and Methods

Strains and culture conditions

Strains 3-1-A to 3-1-H used in this study were obtained in previous study and were isolated from a single tulip bulb (Hagiwara, 2020). For plate and liquid cultures, PDA and potato-dextrose broth (PDB) were used, respectively. For colony growth tests, 10^5 conidia of each strain were inoculated and incubated for 48 h at 37°C before taking pictures. Insusceptibility tests, 10 µg/ml VRCZ, imazalil, prochloraz, triflumizole, tebuconazole, epoxiconazole, and difenoconazole were respectively added to PDA. The control plate contained the equivalent volume of dimethylsulfoxide (DMSO). For measuring colony diameter, the culture time was 28 or 30 h. The data were obtained in triplicate, and the mean and standard deviation are presented. The fungicides fludioxonil,

carbendazim, boscalid, and pyraclostrobin were used at 0.2 µg/ml, 5 µg/ml, 2.5 µg/ml, and 10 µg/ml, respectively.

Quantitative real-time RT-PCR

Strains were cultured in PDB at 37°C for 18 h and harvested. The mycelia were frozen in liquid nitrogen, and total RNA was isolated using Sepasol Super G (Nacalai Tesque, Kyoto, Japan). cDNA was obtained by reverse transcription reaction using the total RNA sample and ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan).

Real-time RT-PCR was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Inc., Santa Clara, CA, USA) as described previously (Ninomiya et al, 2020). Relative expression ratios were calculated using the comparative cycle threshold (Ct) method. The actin-encoding gene was used as a normalization reference. Each sample was tested in triplicate, and the standard deviation is presented. The primer sets used were described in Hagiwara et al, 2017.

Microsatellite typing

Microsatellite typing was performed as described previously (Hagiwara et al, 2014). Briefly, nine microsatellite regions of approximately 400 bp were PCR amplified using purified genome DNA as a template and sequenced by the Sanger method. The repeat numbers of each locus were counted from the sequences. A dendrogram was constructed using Cluster 3.0 by hierarchical clustering with City-block distance for average linkage, and drawn using Treeview ver. 1.1.6r2 (de Hoon et al, 2004, Saldanha, 2004).

Genome sequencing

Whole-genome sequencing using next-generation methods was performed as described previously (Hagiwara et al., 2018). In brief, we extracted genomic DNA from overnight-cultured mycelia with NucleoSpin Plant II (Takara Bio, Shiga, Japan). For paired-end library preparation, an NEBNext Ultra DNA Library Prep Kit (New England BioLabs, MA, USA) and NEBNext Multiplex Oligos (New England BioLabs) were used in accordance with the manufacturer's instructions. A total of 11 strains including 3-1-A to 3-1-H, IFM 59365, IFM 61407, and IFM 61578 were sequenced. Paired-end sequencing (150-bp) on a HiSeq 4000 system (Illumina, San Diego, CA, USA) was carried out by GENEWIZ (South Plainfield, NJ, USA).

SNP detection

In addition to the abovementioned 11 strains, we used raw data for seven strains for comparison, which have been taken in a study by Takahashi-Nakaguchi et al (2015). Adapters and low-quality bases from Illumina reads were trimmed by fastp (ver. 0.20.1) (Chen et al., 2018). Filtered reads were aligned against the *A. fumigatus* strain Af293 reference genome using BWA (ver. 0.7.17-r1188) (Li and Durbin 2009). SNP detection was performed as described previously (Hagiwara et al., 2018). Briefly, SNPs were identified by using SAMtools (ver. 1.9) (Li et al., 2009) and filtered with >20-fold coverage, >30 mapping quality, and 75% consensus using in-house scripts (Suzuki et al., 2014; Tenaillon et al., 2012).

Phylogenetic tree construction

Among the strains that were sequenced, mitochondrial genomes of 12 strains were available and aligned by MAFFT (ver. 7.475) (Kato and Standley, 2013). A phylogenetic tree was constructed using multithreaded RAxML (ver. 8.2.12) (Stamatakis, 2014), the GTRCAT model, and 1,000 bootstrap replicates, and visualized by iTOL (Letunic and Bork 2019). For chromosomal genome

494 phylogenetic classification, 90,987 polymorphic loci were predicted from 18
495 strains and concatenated, then used for construction of a phylogenetic tree by
496 the methods described above.

497

498 *Genome assembly and gene prediction*

499 Mitochondrial genomes were assembled and annotated using GetOrganelle (ver.
500 1.6.4) (Jin et al., 2020) and MITOS2 (Bernt et al., 2013), respectively. To filter
501 the mitochondrial reads, trimmed reads were aligned against mitochondrial
502 genomes by BWA (ver. 0.7.17-r1188) (Li and Durbin, 2009), and the mapped
503 reads were filtered by SAMtools (ver. 1.9) (Li et al., 2009) and SeqKit (Shen et
504 al., 2016). Contigs were assembled by VelvetOptimiser (ver. 2.2.6) (Zerbino and
505 Birney 2008), followed by generation of a simulated mate-paired library using
506 wgsim (ver. 0.3.1-r13) (<https://github.com/lh3/wgsim>). The assembly of nuclear
507 genomes was carried out by ALLPATHS-LG (ver. R52488) (Gnerre et al., 2011).
508 The annotation of assembled nuclear genomes was performed by the
509 Funannotate pipeline (ver. 1.7.4) (<https://funannotate.readthedocs.io/en/latest/>)
510 as described previously (Takahashi et al., 2021). Following identification of
511 repeat sequences by RepeatModeler (ver. 1.0.11)
512 (<http://www.repeatmasker.org/RepeatModeler.html>) and RepeatMasker (ver.
513 4.0.7) (<https://www.repeatmasker.org>), Funannotate *ab initio* prediction was
514 performed with the option “--busco_seed_species=aspergillus_fumigatus” by
515 Augustus (ver. 3.3.3) (Stanke et al., 2006), GeneMark-ES (ver. 4.38)
516 (Ter-Hovhannisyan et al., 2008), GlimmerHMM (ver. 3.0.4) (Majoros et al., 2004),
517 and SNAP (ver. 2006-07-28) (Ian, 2004) using exon hints from the proteins of *A.*
518 *fumigatus* Af293 and *N. fischeri* NRRL 181 downloaded from the *Aspergillus*
519 Genome Database (<http://www.aspgd.org/>) (Cerqueira et al., 2014). The
520 completeness of draft genomes and predicted proteins was evaluated by

521 BUSCO (ver. 4.0.6) (Seppey et al., 2019) with the database eurotiales_odb10.

522 Most tools were obtained through Bioconda (Grüning et al., 2018).

523

524 Detection of orthologous genes

525 Orthologous relationships with *A. fumigatus* Af293 were determined by RBH with
526 criteria BLASTp (ver. 2.9.0+) coverage >80% and identity >80% (Camacho et al.,
527 2009).

528

529 Visualization of genome-wide distribution of SNPs and orthologous genes

530 SNP frequency in each 1-kb window was calculated and plotted in 250-bp steps
531 using Python (Van Rossum and Drake 2009) and R (R Core Team 2019) scripts.
532 The orthologous genes of *A. fumigatus* Af293 in each strain were visualized by R
533 script.

534

535 Data availability

536 The genome sequencing data are deposited to DDBJ as DRA011961.

537 BioSample accession(s): SAMD00322244-SAMD00322251.

538

539 **Author contributions:** HT and DH designed the research; HT, SO, YK, SU, and
540 DH performed experiments; HT contributed new materials/tools; HT and DH
541 analyzed data; and HT and DH wrote the manuscript.

542

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551

552 **Figure legends**

553 **Fig. 1.** Colony growth of *Aspergillus fumigatus* strains isolated from a single tulip
554 bulb on potato-dextrose-agar (PDA) containing azoles. (A) Growth on PDA
555 containing voriconazole (VRCZ). Each strain was inoculated on PDA with
556 dimethylsulfoxide (DMSO) as a control or 10 µg/ml VRCZ, and was incubated for
557 48 h. (B) Growth on PDA containing demethylase inhibitors (DMIs). Each strain
558 was inoculated on PDA with DMSO as a control or 10 µg/ml DMI, and was
559 incubated for 48 h. (C) Colony diameter on PDA containing VRCZ or DMIs. Each
560 strain was inoculated on PDA with DMSO as a control or 10 µg/ml azole, and
561 incubated for 28 h. Error bars represent standard deviations based on three
562 independent replicates.

563

564 **Fig. 2.** Gene expression analysis by quantitative real-time (qRT)-PCR.
565 Expression levels of *cyp51A* (A) and *cdr1B* (B) were determined in the eight
566 strains isolated from a single tulip bulb. The strains were cultured in
567 potato-dextrose broth for 18 h. The *actin* gene was used as an internal control.
568 Error bars represent standard deviations based on three independent replicates.

569

570 **Fig. 3.** Microsatellite-typing analysis of *A. fumigatus* strains with tandem repeat
571 (TR) mutations. The dendrogram was constructed using short tandem repeat for
572 *A. fumigatus* (STRAf) patterns of the strains. The nine STR panels are shown.
573 The strains listed refer to the literature (Chen et al, 2019, Hagiwara et al, 2016b,
574 Nakano et al, 2020, Hagiwara, 2020). The names of strains isolated from plant
575 bulbs in this study or other study are highlighted in pale blue or yellow,

576 respectively. Cyp51A alleles with TR₃₄ are indicated in red, and those with TR₄₆
577 in blue.

578

579 **Fig. 4.** Phylogenetic trees constructed using mitochondrial (A) and nuclear (B)
580 genomes. The trees were constructed using genomes of strains isolated from a
581 single tulip bulb (3-1-A to 3-1-H) or clinically isolated in a previous study
582 (Takahashi-Nakaguchi et al, 2016), as well as *A. fumigatus* reference strain
583 Af293.

584

585 **Fig. 5.** Differences in patterns of single nucleotide polymorphism (SNP)
586 frequency among the strains isolated from a single tulip bulb. (A) The SNP
587 presence patterns are compared in certain regions on chromosome 5 (5-A, 5-B,
588 and 5-C). (B) The patterns in each region could typically be divided into two
589 groups, which are indicated by yellow or blue panels. Ten genomic loci are
590 shown and compared among the eight strains.

591

592 **Fig. 6.** Visualization of genome-wide orthologous gene content in the strains
593 isolated from a single tulip bulb. Orthologous genes were searched against the
594 reference genome of *A. fumigatus* strain Af293. The presence of an ortholog is
595 indicated by colored ribbons for each strain (3-1-A to 3-1-H). Some regions are
596 enlarged to enable easier comparison of the patterns of gene content.

597

598 **Fig. 7.** Colony growth of *A. fumigatus* isolated from a single tulip bulb on PDA
599 containing fungicide. (A) Growth on PDA containing fungicide. Each strain was
600 inoculated onto PDA with DMSO as a control, or QoI (pyraclostrobin; 10 µg/ml),
601 SDHI (boscalid; 2.5 µg/ml), methyl benzimidazole carbamate (carbendazim; 5
602 µg/ml), or phenylpyrrole (fludioxonil; 0.2 µg/ml), and incubated for 48 h. (B)
603 Colony diameter on PDA containing fungicide. Each strain was incubated for 30

h. Error bars represent standard deviations based on three independent replicates. (C) Amino acid substitutions detected in CytB and TubA of *A. fumigatus* strains isolated from a tulip bulb.

Supporting Information

Figure S1. Genome-wide SNP frequency compared among the eight strains (3-1-A to 3-1-H). The 10 regions where the pattern is characteristically distinct among the strains are marked by red boxes.

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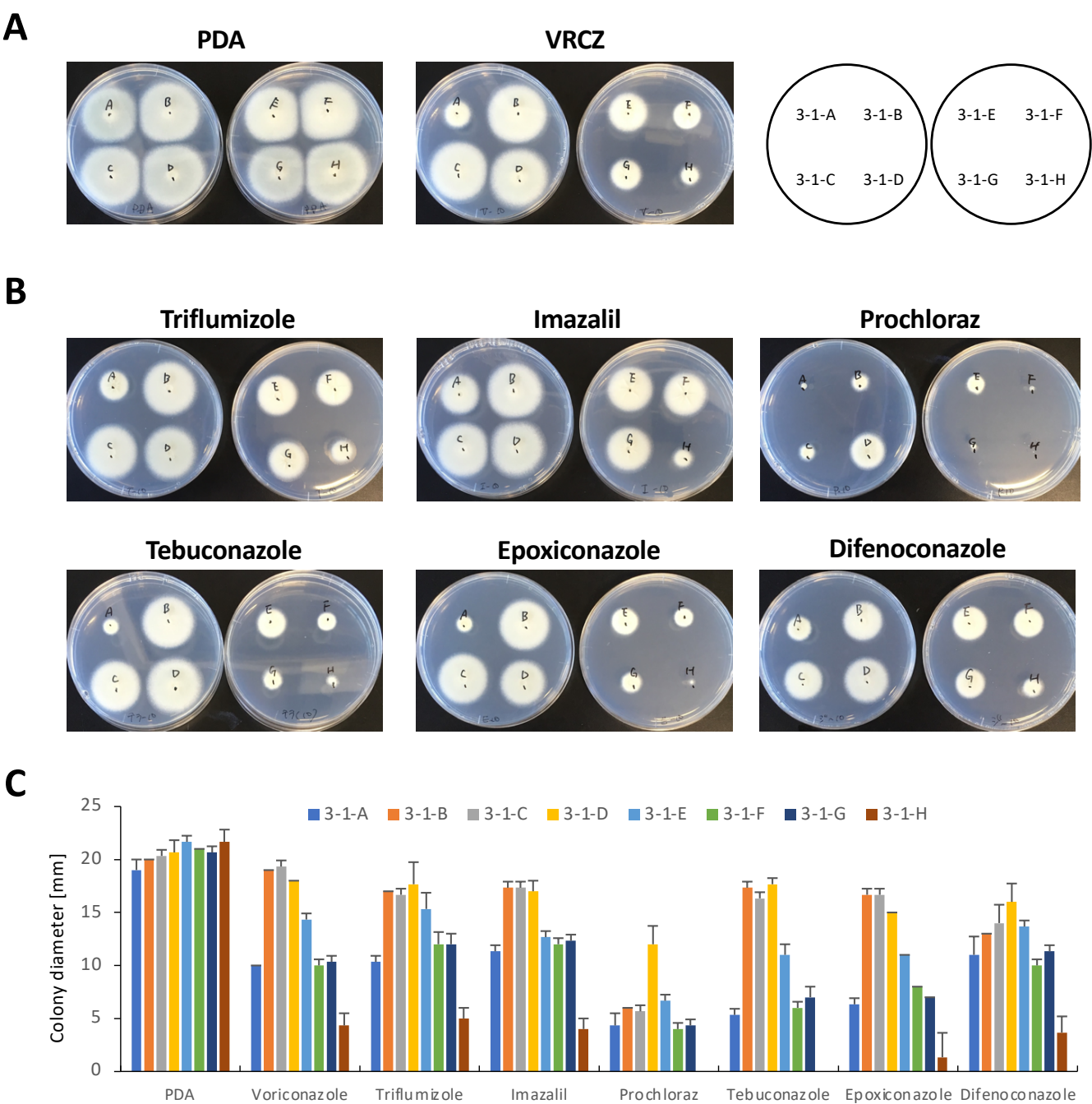


Fig. 1

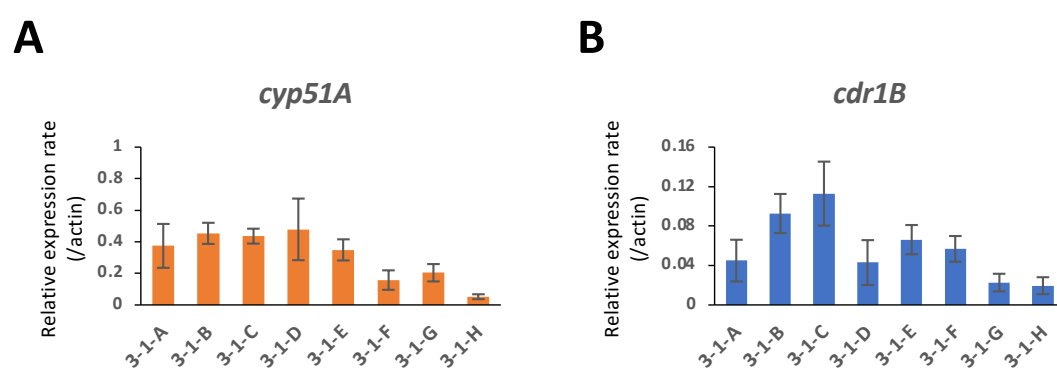


Fig. 2

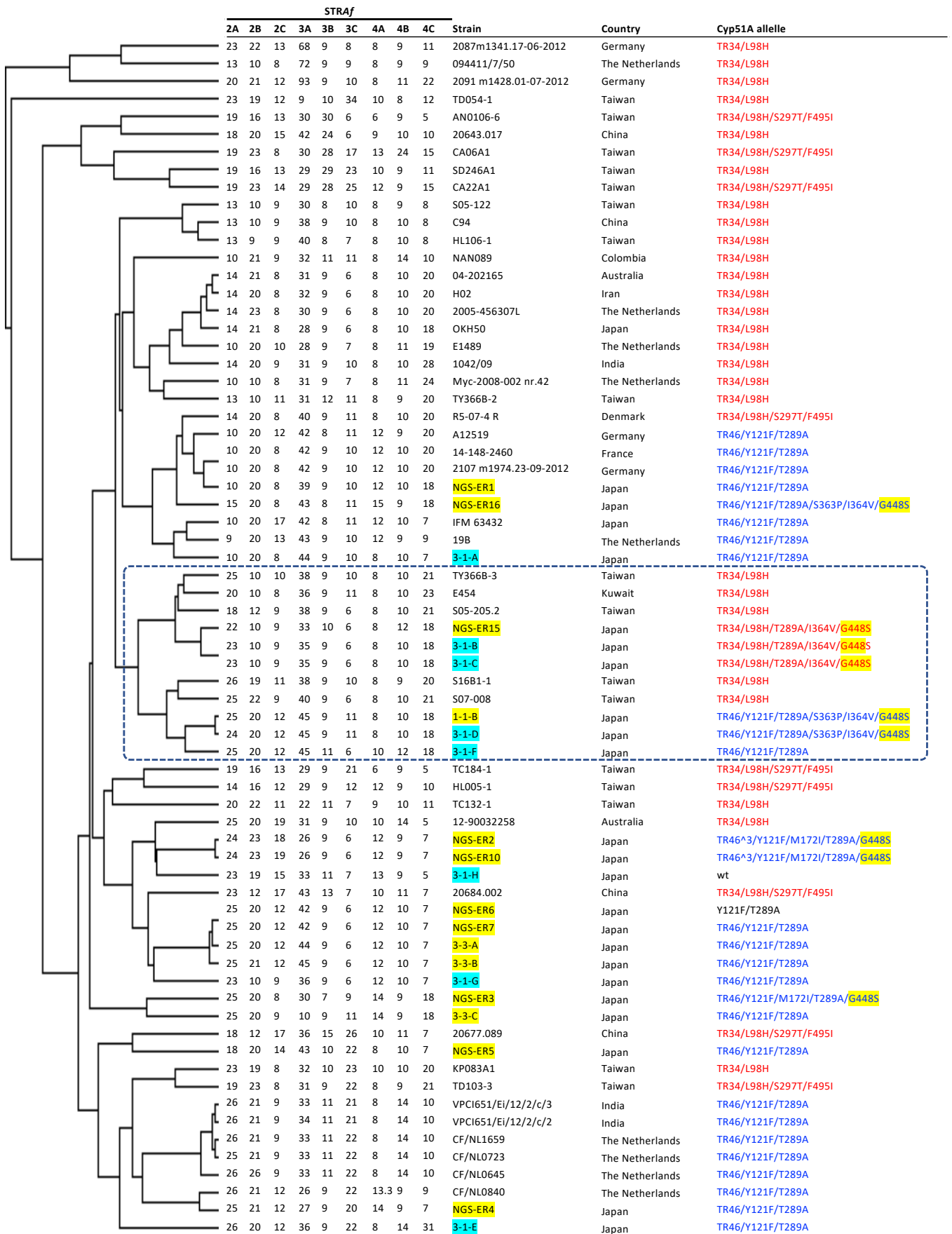


Fig. 3

A



B

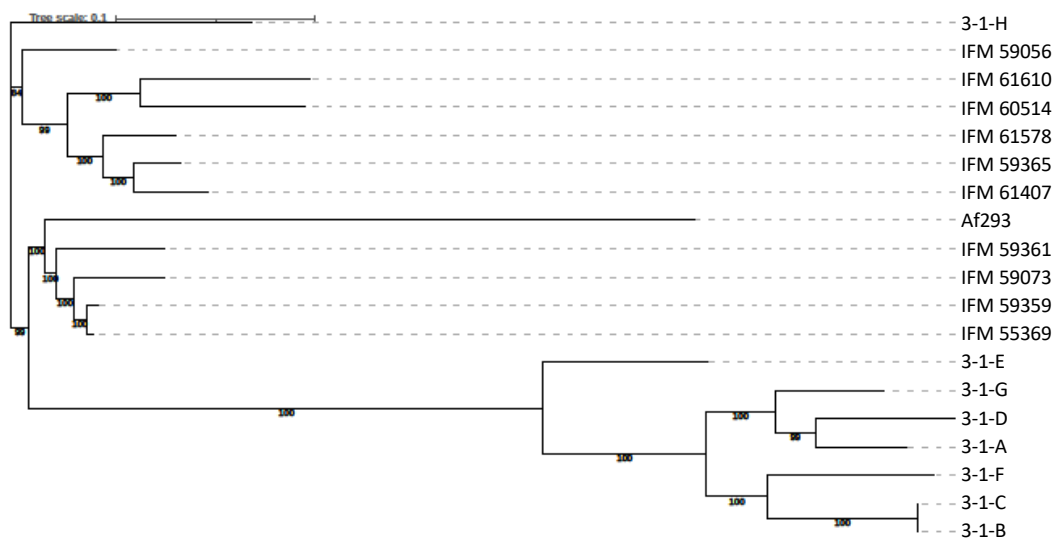


Fig. 4

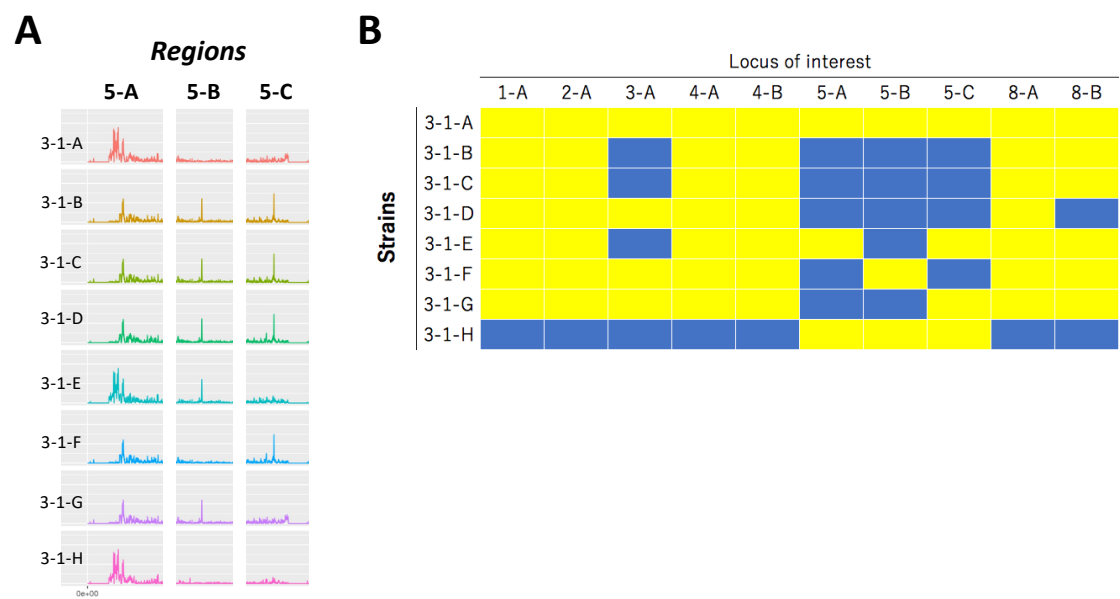


Fig. 5

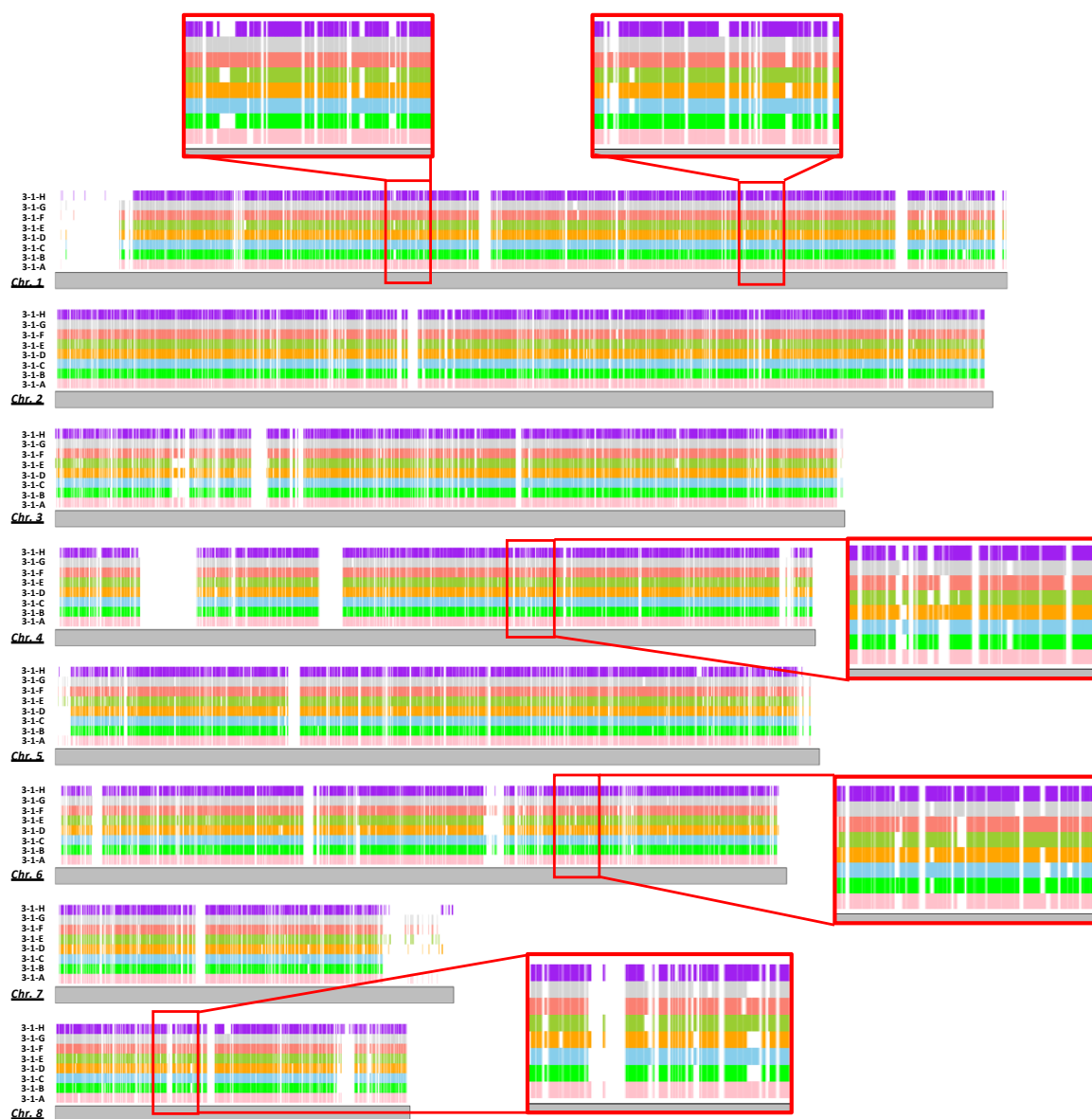
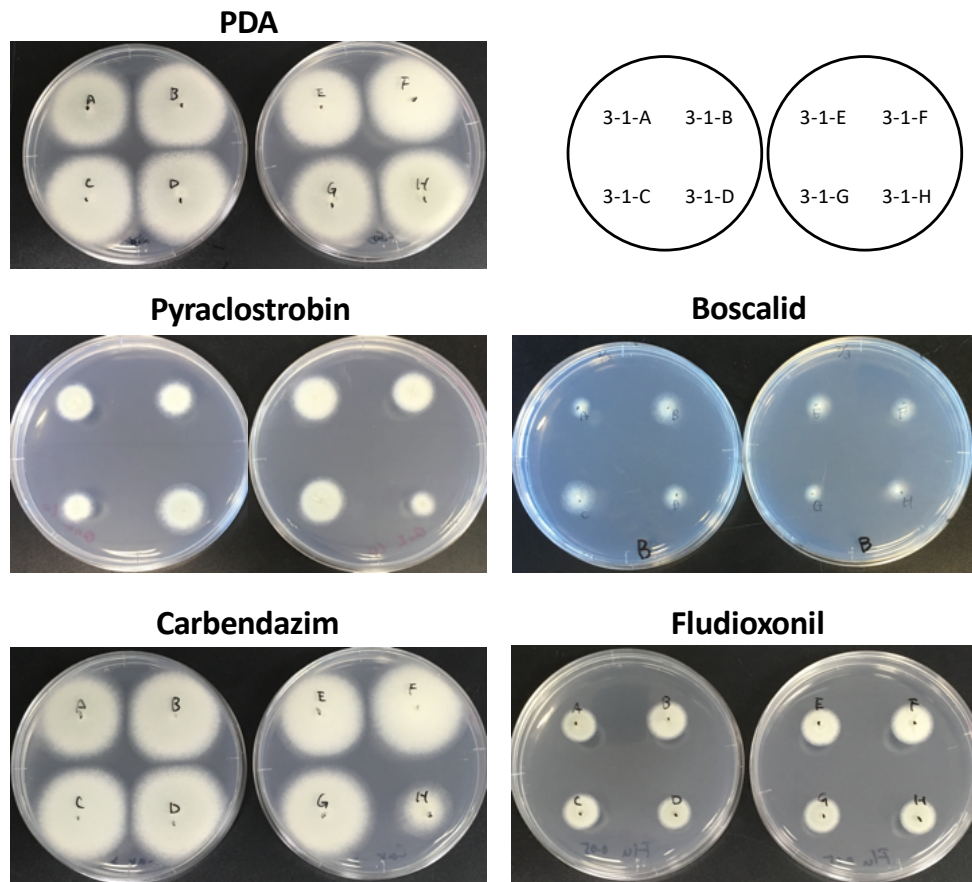
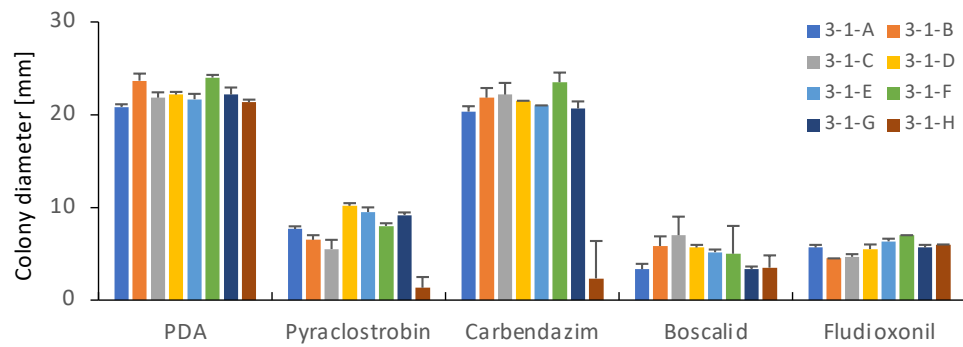


Fig. 6

A



B



C

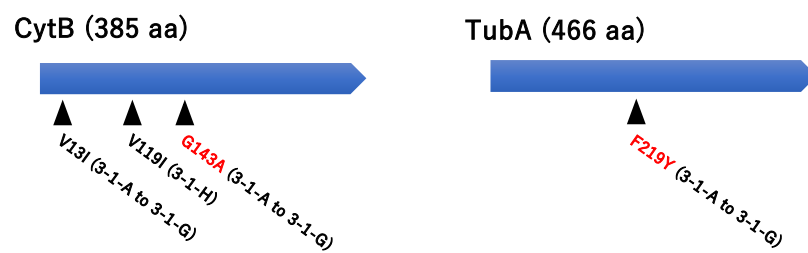


Fig. 7