Title: Multi-resistance to non-azole fungicides in *Aspergillus fumigatus* TR_{34}/L98H azole resistant isolates.

Running title: *Aspergillus fumigatus* resistance to non-azole fungicides.

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ABSTRACT

Drug resistance is a worldwide problem affecting all pathogens. The human fungal pathogen *Aspergillus fumigatus* coexists in the environment with other fungi targeted by crop protection compounds being unintentionally exposed to the selective pressure of multiple antifungal classes leading to the selection of resistant strains. *A. fumigatus* azole resistant isolates are emerging in both the clinical and environmental setting. Since their approval, azole drugs have dominated the clinical treatment for aspergillosis infections, and the agriculture fungicide market. However, other antifungal classes are used for crop protection including benzimidazoles (MBC), strobilurins (QoIs) and succinate dehydrogenase inhibitors (SDHIs). Mutations responsible for resistance to these fungicides have been widely researched in plant pathogens, but it has not been explored in *A. fumigatus*. In this work, the genetic basis underlying resistance to MBCs, QoIs and SDHIs were studied in azole susceptible and resistant *A. fumigatus* strains. E198A/Q and F200Y mutations in the β-tubulin conferred resistance to MBCs, G143A and F129L substitutions in the Cytochrome b to QoIs and H270R/Y mutations in SdhB to SDHIs. Characterization of the susceptibility to azoles showed a correlation between strains resistant to these fungicides and the ones with TR-based azole resistance mechanisms. Whole genome sequencing analysis showed a genetic relationship among fungicide multi resistant strains, which grouped together into subclusters that only included strains carrying the TR-based azole resistance mechanisms, indicating a common ancestor/evolution pattern and confirming the environmental origin of this type of azole resistant *A. fumigatus.*

Key words: *Aspergillus fumigatus*, fungicide classes, resistance origin and development, fungicides cross-resistance.

INTRODUCTION

Aspergillus fumigatus is an opportunistic human fungal pathogen, with worldwide distribution, that can affect immunocompromised individuals causing a broad range of clinical manifestations encompassed under the name of aspergillosis (1, 2). The most serious clinical manifestation of aspergillosis, invasive pulmonary aspergillosis (IPA), represents a major cause of morbidity and mortality, largely due to the difficulty of the diagnosis and late initiation of antifungal therapy (3). The first-line treatment and prophylaxis for aspergillosis relies on the employment of an antifungal class called azoles (4), that target the enzyme 14-α sterol demethylase (Cyp51) involved in the ergosterol biosynthesis (5).

Drugs targeting the 14-α sterol demethylase enzyme, also called demethylation inhibitors (DMIs), are not only used in the clinical setting but also in the environment, preventing crop damage by plant pathogens (6). *A. fumigatus* azole resistance is acquired through selective pressure that can develop in two different scenarios: a clinical route generated during long periods of exposure to azole treatments (7); or an environmental origin due to the extended use of demethylation inhibitor drugs (DMIs) in agriculture (8). In both scenarios strains with different resistance mechanisms and different azole susceptibility profiles can be selected, although in both cases the acquired resistance to azole antifungals is based on mutations in the gene *cyp51A* (5). Currently, the most frequent azole resistant mechanisms are based on tandem repeat (TR) insertions in the promoter with mutations in the coding sequence of the gene *cyp51A* (TR_{34}/L98H and less frequently TR_{46}/Y121F/T289A and TR_{53}) (5). Strains harboring these resistance mechanisms can infect azole-naïve immunocompromised patients seriously compromising their treatment options (9, 10). Although the number of cases of azole resistant *A. fumigatus* recovered from clinical samples is still limited, azole resistance

mechanisms continue to spread worldwide threatening the effectiveness of this important antifungal class in aspergillosis treatment (11).

A. fumigatus can grow in a wide variety of ecological niches and play a role in recycling organic matter from the soil (1). In the environment, *A. fumigatus* is exposed to a strong antifungal selective pressure not only to DMI fungicides but also to other classes of fungicides used for crop protection. Different classes of antifungals are currently commercialized, each of them targeting different mold species and with diverse mechanisms of action (12). DMIs, targeting the fungal enzyme Cyp51, have dominated the agricultural market since their approval in the 1970s (13), but other drugs such as methyl-benzimidazole carbamates (MBCs), quinolon oxidation inhibitors (QoIs) and succinate dehydrogenase inhibitors (SDHIs) are widely employed as well (14).

The continuous exposure of fungal species, including *A. fumigatus*, to chemical compounds in the fields favors the emergence of resistant strains to different classes of fungicides. DMIs, MBCs, QoIs and SDHIs are all site-specific inhibitors and resistance to them arises due to point mutations of the target site, which can only happen if the activity of the protein is not significantly affected and the fitness of the strain is not compromised (14-16). Reports of resistance to DMIs in *A. fumigatus* associated to their use in agriculture are being acknowledged (6, 8, 13); however, only one study has recently reported this phenomenon regarding other classes of antifungal compounds in environmental strains of *A. fumigatus* (17).

In 1966 the fungicide class SDHIs was added to the market (12, 18, 19). This fungicide class acts blocking the Krebs cycle by binding to the ubiquinone-binding site of the mitochondrial complex II, disrupting the electron transport chain (12, 20). The complex II is a heterotetramer and point mutations in the *sdhB* subunit have been associated with development of resistance to SDHIs in several fungal pathogens (18-20). MBCs, introduced in the market in the 1970s, are heterocyclic

compounds that target the protein encoded by the β-tubulin gene (*benA*) (21-23). Point mutations associated with resistance to MBCs in the coding sequence of *benA* have been acknowledged in more than 100 fungal species (24-26), thus, MBCs are currently considered fungicides at high-risk of developing resistance (27). QoIs or strobilurins, launched in the market in 1996, target the cytochrome bc1 enzyme complex (complex III) in the mitochondrial electron transport chain (15, 28). Resistance mechanisms against QoIs have been described in other fungal species as point mutations in the mitochondrial gene cytochrome b (*cytB*) (29-30).

To date, the effects on resistance development produced by these antifungal drugs have been studied in several plant pathogens but, to our knowledge, they have only recently started to be studied in the human fungal pathogen *A. fumigatus* (17). We hypothesized if *A. fumigatus* DMIs resistance is related to resistance to other antifungals frequently used for crop protection. In this work, the susceptibility of a large collection of *A. fumigatus* clinical strains, including DMI susceptible and resistant strains, was tested against compounds of the three antifungal classes MBCs, SDHIs and QoIs. Molecular characterization was performed by sequencing the three antifungal targets *benA*, *cytB* and *sdhB.* In addition, we use WGS analysis to compare the genetic relationship of multifungicide resistant *A. fumigatus* strains from different geographical origins. A phylogenetic tree showing the relationship among *A. fumigatus* strains resistant to the four fungicide classes was constructed.

RESULTS

Antifungal susceptibility to methyl-benzimidazole carbamates (MBCs) targeting the *benA* gene and sequence analysis of *A. fumigatus benA* gene.

The *A. fumigatus* susceptibility to benomyl (BNY) and carbendazim (CBZ) was tested in 60 strains (Table 1). The MIC concentration ranges to consider BNY and CBZ susceptibility were established in base to the MIC values obtained with *A. fumigatus* wild type reference strains used in the mycology laboratory. Using these ranges, two thirds of the *A. fumigatus* strains included in this study could be considered BNY and CBZ susceptible. However, 23 strains had MIC values over 32 mg/L to both BNY and CBZ, and therefore were considered MBC resistant (Table 1).

PCR amplification and sequencing of the *benA* gene from 138 *A. fumigatus* strains showed two different amino acid substitutions: the substitution of a glutamic acid (E) for an alanine (A) or a glutamine (Q) in position 198 of the amino acid sequence and the change of a phenylalanine (F) for a tyrosine (Y) in position 200 of the protein. The E198A/Q substitution was harbored by four and two strains respectively, and the F200Y mutation was present in sixteen isolates. Strains harboring these substitutions showed resistance to MBCs in the antifungal susceptibility testing (AFST) (Table 1).

Antifungal susceptibility to Qo inhibitors (QoIs) and sequence analysis of *A. fumigatus* gene *cytB.*

Results of the AFST to QoIs azoxystrobin (AZB) and pyraclostrobin (PYB) are shown in Table 1. MIC ranges for AZB and PYB susceptibility were established based on the MICs obtained for the *A. fumigatus* wild type reference strains. Based in those ranges we can consider most of our strains susceptible to AZB except for nine strains that showed MICs over 32 mg/L and, therefore, were considered AZB resistant. All AZB resistant strains but one were also PYB resistant (Table 1).

PCR amplification and sequencing of the gene *cytB* in the *A. fumigatus* collection of 138 strains revealed several polymorphisms responsible for synonymous mutations. The I119V polymorphism, in which an isoleucine (I) is substituted for valine (V) in position 119 of the protein, was present in QoI susceptible and resistant strains so its implication in resistance to QoIs was discarded. Two polymorphisms were found in the strains that showed resistance to both QoIs; the change of a glycine (G) for an alanine (A) in position 143 (G143A) present in eight strains and the substitution of a phenylalanine (F) for a leucine (I) in position 129 (F129L) in one strain. It was this strain harbouring the F129L substitution which was resistant to AZB but not to PYB.

Antifungal susceptibility to succinate dehydrogenase inhibitors (SDHIs) and sequence analysis of *A. fumigatus* gene *sdhB*.

AFST was performed against boscalid (BCL) and floupyram (FLP) drugs. MIC ranges for susceptibility testing were compared to the MIC values obtained from the *A. fumigatus* reference strains to consider susceptibility. Based on these criteria, six strains were resistant to SDHIs with MICs over 32 mg/L (Table 1).

PCR amplification and sequencing of the *sdhB* gene in 138 *A. fumigatus* strains revealed three amino acid modifications. R51G substitution was present in only one strain and demonstrated not to have any impact in the susceptibility to BCL or FLP. An amino acid substitution in position 270 of histidine (H) for an arginine (R) or a tyrosine (Y) (H270R/Y) was present in four and two strains, respectively, which seems to be associated with resistance only to BCL but not to FLP.

In addition, all *A. fumigatus* strains with mutations at *benA*, *cytB* or *sdhB* had mutations at Cyp51A (TR_{34}/L98H or TR_{46}/F121Y/T289A) and were also multi-resistant to clinical azole drugs as well as to environmental DMIs (8).

Polymorphisms of *benA*, *cytB* and *sdhB* genes in *A. fumigatus* strains analyzed by whole genome sequencing.

We extended the search for mutations in *benA, cytB* and *sdhB* genes in a larger collection of 205 *A. fumigatus* genomes including 163 whole genome sequences that had been previously sequenced in our laboratory or downloaded from public databases (31) and 42 strains that were received later (Table 2).

Genomes analysis revealed that *benA* is a highly conserved gene and the same two non-synonymous mutations previously detected were found (E198A/Q and F200Y). In total, E198A substitution was present in 5 strains (2.5%), E198Q was found in 3 strains (1.5%) and F200Y mutation was present in 29 strains (14%) (Table 2). Both amino acid substitutions were found in azole resistant *A. fumigatus* strains harbouring the resistance mechanisms TR_{34}/L98H or TR_{46}/Y121F/T289A, except for one Japanese azole susceptible strain that harbored a F200Y mutation in *benA*.

Three non-synonymous mutations were found in the *cytB* gene. The I119V mutation found in 36.6% of the strains was present in azole susceptible as well as in azole resistant strains. The mutation F129L was present only in one azole resistant strain (0.5%) and the G143A was identified in eight azole resistant strains (3.9%) (Table 2). Both substitutions were always found in azole resistant *A. fumigatus* strains harboring the resistance mechanisms TR_{34}/L98H or TR_{46}/Y121F/T289A.

The *sdhB* gene presented a lower frequency (4%) of non-synonymous mutations. Of the total genomes included in this analysis, the R51G mutation was only found in one azole resistant strain (0.5%), the H270Y substitution was detected in three strains (1.5%) and in four strains the substitution was H270R (2%) (Table 2). All the amino acid substitutions were found only in azole-

resistant *A. fumigatus* strains harboring the resistance mechanisms TR_{34}/L98H or TR_{46}/Y121F/T289A.

Phylogenic tree representation of a collection of 163 *A. fumigatus* genomes.

The collection of 163 *A. fumigatus* strains included in the whole-genome sequencing (31) were clustered attending to their genetic proximity. A figure representing the relatedness among strains, indicating their susceptibility to azoles and the existence of mutations in *cyp51A* (separating single point mutations from tandem repeat insertions), *benA, cytB* and *sdhB* genes is shown (Figure 1 and Table S2).

The *A. fumigatus* strains were divided into four clear clusters. Cluster I included azole susceptible strains and azole resistant strains with single point mutations in the coding sequence of *cyp51A*. Cluster II included azole susceptible strains and azole resistant strains with resistance mechanisms based on tandem repeat insertions in the promoter of *cyp51A* as well as strains with single point mutations. However, strains harboring tandem repeat insertions were grouped together into three well-supported sub-clusters within cluster II (sub-clusters II.1, II.2 and II.3) in which only TR-based Cyp51A mutations strains were included. In cluster III, a set of azole susceptible strains with five *cyp51A* modifications (F46Y, M172V, N248T, D255E, E427K) were grouped together, while cluster IV included azole susceptible strains with three *cyp51A* modifications (F46Y, M172V, E427K); neither of them had mutations in any of the three *benA, cytB* or *sdhB* genes.

All the strains with mutations in the genes *benA, cytB* and *sdhB* except for two, belonged to cluster II and all of them but one also harbored a TR insertion in the promoter of *cyp51A*. In addition, all of the strains with mutations in *benA, cytB* and *sdhB* genes were grouped inside sub-clusters II.1 and II.3 along with tandem repeat strains. As mentioned, only two strains with a tandem repeat insertion in

195 *cyp51A* and mutations in the genes *benA, cytB* and *sdhB* were detected in cluster I
196 (TR_{46}/Y121F/T289A and wild-type Cyp51A) and no other strains with mutations in these three genes
197 were found outside sub-clusters II.1 and II.3, neither in azole susceptible nor azole resistant strains.
198 All the strains included in sub-clusters II.1 and II.3 had a substitution in *benA* except one (F16216).
199 Substitutions in *cytB* and *sdhB* were less frequent (Figure 1).

200 **DISCUSSION**

201 *Aspergillus fumigatus* is an opportunistic human pathogen that coexists in its natural environment
202 with plant pathogenic molds and in consequence is exposed to a strong selective pressure from
203 different fungicide classes. The hypothesis that the continuous exposure of *A. fumigatus* to DMI
204 antifungals in the environment favors the development of resistant strains to clinical azoles, is being
205 acknowledged by several authors (5). However, only one study has recently reported resistance to
206 other classes of fungicides in *A. fumigatus* from environmental origin (17). In the present study, a
207 collection of *A. fumigatus* strains, including azole susceptible and azole resistant isolates, was tested
208 against three fungicide classes: MBCs, QoIs and SDHIs, including 38.4% of strains resistant to DMIs
209 (8).

210 Eighteen percent of the *A. fumigatus* strains included in our study had mutations in the *benA* gene
211 and were resistant to MBCs. The amino acid substitutions E198A, E198Q and F200Y found in β-
212 tubulin have been previously described in several plant pathogenic fungi such as *Venturia inaequalis,*
213 *Botrytis cinerea* or *Podosphaera xanthii* with very high prevalence percentages that can reach up to
214 90% (15, 26, 32, 33). All of them showed higher prevalence of resistance than the observed in *A.*
215 *fumigatus.* However, we have to consider that the *A. fumigatus* azole resistant strains in this study
216 were mainly obtained from the clinical setting. An environmental search looking specifically for *A.*

217 *fumigatus* resistant to any of the fungicide classes will probably provide higher percentages of
218 resistance.

219 Resistance to QoIs was present in 4.4% of the strains harboring two different mutations at the *cytB*
220 gene (G143A and F129L). The amino acid substitution G143A in the Cytochrome b protein has been
221 one of the most described mutations in plant pathogenic isolates resistant to QoIs to date including
222 *Mycena galopoda, Erysiphe necator* or *V. inaequalis* (34, 35). Resistance frequencies to QoIs are
223 also quite high in plant pathogens with percentages that can go up to 80-90% (15). Furthermore, the
224 F129L mutation has also been detected in resistant isolates from several fungal species including
225 *Cercospora beticola, Pythium aphanidermatum, Pyriculaira grisea* and the genus *Pyrenophora* (30,
226 35, 36). According to the results of the susceptibility testing carried out in this study, the F129L
227 mutation is responsible for resistance to strobilurin but not to pyraclostrobin, which correlates with
228 previous studies (30, 35, 36).

229 The SdhB substitutions H270R/Y were found in 3.5% of the *A. fumigatus* isolates. This mutation
230 confers resistance to boscalid but not to fluopyram as previously described in other plant pathogens
231 (15). The location of the SdhB amino acid in position 270 in *A. fumigatus* changes depending on the
232 fungal species under study. In *B. cinerea* the corresponding H272Y/R substitution has also been
233 related with resistance to boscalid but not to fluopyram (20). *A. fumigatus* resistant rates to SDHIs, as
234 with other fungicides, are considerably lower than in other plant pathogens (15). To our knowledge,
235 the R51G substitution has not been described in the literature before. In fact, the susceptibility results
236 obtained with boscalid and fluopyram do not lead us to think that the R51G substitution has any
237 relevance in conferring resistance to SDHIs in *A. fumigatus*.

238 QoIs and SDHIs are fungicides that block the electronic transport chain by targeting mitochondrial
239 genes, which are less exposed to DNA repair mechanisms than nuclear DNA (16), favoring the rapid

emergence of resistance mechanisms against mitochondrial target drugs. However, when mitochondrial genes are implicated, the level of resistance to a drug not only depends on the nature of the amino acid substitution itself, but also in the heteroplasticity of the cell, which is defined by the number of mitochondria inside the cell that harbor the mutated allele (37-39). In the case of the G143A mutation, the risk of strobilurin resistance is determined by the percentage of A143 alleles in the fungal population and not only the presence of this substitution (40).

In mammals, mitochondrial DNA does not have introns (41); however, in fungi, the existence of introns in the mitochondrial DNA depends on the species (37). In some species such as *Puccinia* spp*., Alternaria alternata* or *Saccharomyces cerevisiae*, an intron in the *cytB* gene in a position right after the G143 amino acid has been described (30). As a consequence, the A143 allele is not possible in these species since its base position is located at the intron boundary, which is crucial for the correct intron splicing (30). In the case of *A. fumigatus*, we ruled out the existence of an intron at this position (results not shown). Furthermore, we have shown that CytB with an A143 allele is encoding a functional protein since resistance to azoxystrobin and pyraclostrobyn was demonstrated in isolates with this mutation.

Selection of resistant isolates depends on two factors, the selection pressure of the fungicide and the fitness cost associated to the change of the protein functionality. In general, mutated strains tend to have a fitness disadvantage in the absence of antifungal drugs. Previous studies about the relationship between resistance and fitness cost in fungal species are contradictory, as in some species like *Phakopsora pachyrhizi* the G143A mutation in CytB is correlated with a fitness penalty while in others like *C. beticola* or *B. cinerea* it is not (20, 36, 42). Similarly, in *Pyrenophora teres* the presence of F129L substitution in CytB did not correlate with a fitness cost (35). The same has been described for mutations in the *sdhB* gene; depending on the *sdhB* amino acid substitution and the

fungal species implicated, results of the fitness studies show contradictory results (20). In *A. fumigatus*, adaptive mutations might favor the survival of the mutated strains in the environment for generations without any fitness cost associated. The rapid dispersal of the azole-resistant *A. fumigatus* strains with the TR_{34}/L98H genotype in Asia also supports the hypothesis that in natural environments these strains have comparable or even higher fitness than that of wild-type strains (43). Nevertheless, this hypothesis needs further consideration and an in-depth study.

The WGS analysis of *A. fumigatus* from very diverse geographical origins (Europe, Japan, Canada and India), isolated 20 years apart, showed four differentiated clusters including azole susceptible and azole resistant strains (Figure 1). Clusters I and II could be further divided into well-defined sub-clusters. A remarkable finding was that all genomes harboring the TR_{34}/L98H Cyp51A alleles were grouped inside cluster II and more specifically, they were included in three sub-clusters within cluster II (clusters II.1, II.2 and II.3) that only harbored strains with TR insertions in *cyp51A*. This particular clustering of TR strains was independent of their geographical origin, isolation year or their clinical or environmental origin; and it suggests that the selective phenomenon occurring among these isolates does not imply a lack of fitness, but further experiments are necessary to clarify this important subject. Our *A. fumigatus* WGS collection only has two genomes belonging to strains with TR_{46}/Y121F/T289A Cyp51A azole resistance mechanism, one located in cluster I and the other in cluster II together with TR_{34}/L98H strains. The absence of more strains with TR_{46}/Y121F/T289A makes it difficult to draw conclusions about this specific resistance mechanism, although the presence of mutations in all *benA, cytB* or *sdhB* genes strongly supports a similar evolution to the TR_{34}/L98H strains. However, until we have more of these strains whole genome sequenced, we cannot reach any further conclusions.

285 A remarkable finding was that, with the exception of two strains included in cluster I, strains harboring
286 mutations in the genes *benA, cytB* and *sdhB,* resistant to MBCs, QoIs and SDHIs respectively,
287 grouped within two sub-clusters of cluster II (sub-clusters II.1 and II.3) composed of TR_{34}/L98H
288 Cyp51A strains. Sub-clusters II.1 and II.3 contained 26 and 6 strains, respectively. Among those 32
289 azole resistant isolates, 31 were also resistant to MBCs, 13 were resistant to QoIs and 6 were
290 resistant to SDHIs. Only two strains included in sub-cluster II.1 were resistant to all four antifungals
291 tested, harboring mutations in all the fungicide target genes (Figure 1). Multifungicide resistance is a
292 well-known phenomenon in some plant pathogen species (14, 27) with some fungal species showing
293 resistance to up to six fungicide classes (44). The *A. fumigatus* isolates included in this work showed
294 25% resistance to more than one antifungal class, and some particular isolates even had a
295 resistance profile to four different fungicidal classes. This percentage is quite high considering that *A.*
296 *fumigatus* is not the target pathogen of these fungicides and that the origin of these strains was
297 mainly clinical and these drugs are never used in the clinical setting. A recent study performed in the
298 UK looking for environmental hotspots of *A. fumigatus* azole resistance, reported similar results in
299 multi-fungicide resistance as our study. Some of the mutations found - F200Y in *benA*, G143A in *cytB*
300 and H270Y in *sdhB* - were also found in combination with TR-based Cyp51A resistance mechanisms
301 (17). The results of this study on environmental strains and our results obtained from clinical isolates
302 would confirm the environmental origin of strains carrying this type of azole resistance mechanisms.

303 In plant pathogens the application of single-site specific fungicides, including rotations and mixtures
304 for crop protection, seems to be favoring the selection of multi drug resistant isolates (45). The
305 development of multifungicide resistance in fungi is believed to be via independent rounds of
306 selection (46). This is supported by the accumulation of mutations in the target genes of respective
307 fungicides causing resistance to them (47). Although some fungi showed a predisposition to selection

for resistance in isolates that were already resistant to an unrelated fungicide (45, 47); this phenomenon implies that fungi might not be selecting only for resistance, but also for an increased genetic plasticity that enables accelerated resistance development (46). Many of these non-azole fungicides have activity against *A. fumigatus* and although they are not employed to target *A. fumigatus*, selection of resistant strains seems to mirror what is happening with most of the plant pathogens. The multi-resistant profile of the strains within sub-clusters II.1 and II.3 (Figure 1) carrying several mutations can suggest a common origin based on selection due to multiple environmental fungicide exposure. Future studies aimed to understand correlations between mutator genotypes and multifungicide-resistant phenotypes are needed. The genetic relationship among the multiresistant strains supports the idea that these *A. fumig*atus strains have a common background and may have a common ancestor reflecting biological adaptations to the selective pressure of fungicide applications. Whether this *A. fumigatus* genetic background implies an improved fitness for the strain, or not, needs to be studied and clarified.

In conclusion, the application of fungicides for crop protections seems to be favoring the selection of *A. fumigatus* multi drug resistant isolates. Whichever the mechanism for resistance selection is applied, this finding would confirm the environmental origin of the TR-based azole resistance mechanisms since the antifungals here tested (MBCs, QoIs and SDHIs) are not used in clinical settings. The current evidence supporting environmental resistance selection is a very important finding that will help to design and change fungicide application in the environment and medical applications in order to retain the drug resistance spread. If an improved strain fitness is associated to fungicide mutiresistance this finding could have severe consequences in the future, in both the clinical and agricultural use of azoles and other antifungals.

MATERIALS AND METHODS

A. fumigatus strain collection and DNA extraction

A total of 100 Spanish unrelated azole resistant *A. fumigatus* strains with known azole-resistance mechanisms were included in this work, as well as 38 azole susceptible strains. The *A. fumigatus* reference strains AF293 and CBS144.89 (CEA10), that have been whole genome sequenced, were used as control strains. In addition, other reference stains such as *A. fumigatus* ATCC204305 (reference strain for EUCAST susceptibility testing) and strains CM237 and ATCC46645, frequently used in *A. fumigatus* laboratories, were also included. Apart from the 138 strains available in our laboratory, other genomes downloaded from databases were used to perform this study. A supplementary figure explaining how the isolates were selected and used is included (Figure S1).

Conidia from each strain were cultured in 3 ml of GYEP broth (0.3% yeast extract, 1% peptone; Difco, Soria Melguizo, Madrid, Spain) with 2% glucose (Sigma-Aldrich Química, Madrid, Spain) and grown overnight at 37°C, after which mycelium mats were harvested and genomic DNA was extracted as described previously (48). All isolates were identified at the species level by PCR amplification and sequencing of ITS1-5.8S-ITS2 regions and a portion of the β-tubulin gene (49).

Characterization of azole resistance molecular mechanisms in a collection of *A. fumigatus* strains

To study the mechanisms associated with azole resistance the full coding sequence of the *cyp51A* gene, including its promoter sequence, was amplified using the PCR conditions described before (50). A DNA 1-kb molecular ladder (Promega, Spain) was used for all electrophoresis analyses and both strands were sequenced with the Big-Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA) following manufacturer's instructions. To exclude the possibility that any

353 change identified in the sequences was due to PCR-induced errors, each isolate was independently
354 analyzed twice. All *cyp51A* DNA sequences were edited and assembled using Lasergene software
355 package (DNAStar Inc., Madison, WI, USA) and the *A. fumigatus* reference strain CBS144.89 (NCBI
356 accession number AFUB_063960) for comparison.

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359 **PCR and sequencing conditions for *benA, cytB* and *sdhB* gene amplification**

360 The determination of gene modifications associated with fungicide resistance, the full coding
361 sequences of the genes *benA*, *cytB* and *sdhB* were amplified and sequenced. PCR reaction mixtures
362 contained 0.5 µM of each primer, 0.2 µM of deoxynucleoside triphosphate (Roche, Madrid, Spain), 5
363 µL of PCR 10x buffer, 2 mM of $MgCl_2$, DMSO 5.2%, 2.5 U of Taq DNA polymerase (Applied
364 Biosystems, California, USA), and 100–200 ng of DNA in a final volume of 50 µL. The samples were
365 amplified in a GeneAmp PCR System 9700 (Applied Biosystems, California, USA). The parameters
366 used were 1 cycle of 5 min at 94°C and then 35 cycles of 30 s at 94°C, 45 s at 60°C for *benA*, 56°C
367 for *cytB*, 58°C for *sdhB*, and 2 min at 72°C, followed by a 1 final cycle of 5 min at 72°C. PCR
368 products were analyzed and sequenced as described above. Primers used to amplify and sequence
369 the genes included in this study are listened in Table S2.

370 **Clinical antifungal drugs susceptibility testing**

371 Antifungal susceptibility testing (AFST) was performed following the European Committee on
372 Antimicrobial Susceptibility Testing (EUCAST) broth microdilution reference method 9.3.1 (51). A
373 representation of 60 *A. fumigatus* isolates among the collection of 138 strains was tested including a
374 group of 14 wild type strains (wild type for all genes under study, *cyp51A*, *benA*, *cytB* and *sdhB*) and

all the strains that harbored mutations in any of the genes of interest (Figure S1). Antifungal drugs used were amphotericin B (Sigma-Aldrich Química, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer SA, Madrid, Spain), posaconazole (Schering-Plough Research Institute, Kenilworth, NJ) and isavuconazole (BasileaPharmaceutica, Basel, Switzerland). The final concentrations tested ranged from 0.03 to 16 mg/L for amphotericin B and 0.015 to 8 mg/L for the four azoles tested. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. Minimal inhibitory concentrations (MICs) were visually read after 24 and 48 hours of incubation at 37°C in a humid atmosphere. MICs were performed at least twice for each isolate (biological duplicates). Clinical breakpoints for interpreting AFST results established by EUCAST (52) were used for classifying the *A. fumigatus* strains as azole susceptible or resistant.

Environmental fungicides susceptibility testing

AFST was also performed against six different fungicides used in agriculture following the EUCAST methodology as described before. The antifungals tested were: (i) two methyl benzimidazole carbamate compounds (MBC) targeting β-tubulin - benomyl and carbendazim - (ii) two Qo inhibitors (QoI) - azoxystrobin and pyraclostrobin - targeting the cytochrome b of mitochondrial complex III and (iii) two succinate dehydrogenase inhibitors (SDHIs) - boscalid and fluopyram - targeting succinate deshydrogenase. All antifungal compounds were purchased at Sigma-Aldrich, Química, Madrid, Spain.

All drugs were dissolved in DMSO and auto sterilized for 30 minutes at room temperature, as stated in the EUCAST protocol for clinical azoles (52). The final concentrations tested ranged from 0.06 to 32 mg/L. Visual reading at 24 hours was used to determine the minimal inhibitory concentrations (MICs) or minimum effective concentrations (MECs). As breakpoints for interpreting AFST results to

environmental fungicides have not been established yet, isolates were considered susceptible or resistant based on the MIC/MEC shown by the group of *A. fumigatus* WT reference strains. AFST was performed at least twice for each isolate (biological duplicates). MIC was recorded for benomyl and carbendazim whereas MEC (Minimal Effective Concentration) was used for the other four fungicides. MIC is defined as the lowest concentration of drug that yields no growth and MEC is the lowest concentration of drug that results in macroscopic changes of filamentous growth to micro colonies or granular growth when compared with growth control wells (53).

AFST had been previously performed against a set of demethylation inhibitor drugs (DMIs) for the collection of 138 *A. fumigatus* strains (8) following the same procedures described before for clinical and environmental fungicides.

Aspergillus fumigatus whole genome sequencing alignment

A search for variants or mutations in *benA*, *cytB* and *sdhB* genes was performed in a collection of 163 *A. fumigatus* whole genome sequences that had been previously sequenced in our laboratory using Nextera® XT Library Prep Kit (Illumina Inc., San Diego, CA, USA) as described before or obtained from public databases (Figure S1) (31). All data used in this analysis included information about clinical antifungal susceptibility and azole resistance mechanisms of the strains (Table S1).

The Illumina reads were trimmed using Trimmomatic (version 0.32) (54). The sequencing adapters and sequences with low quality scores on 30 ends (Phred score [Q], <20) were trimmed. Raw Illumina WGS reads were quality checked performing a quality control with FastQC (version 0.11.3; Babraham Institute). Data sets were analysed against the *A. fumigatus* reference genome A1163 (GenBank accession number ABDB00000000.1) using WGS-outbreaker v1.0 (Instituto de Salud Carlos III, Madrid, Spain) (https://github.com/BU-ISCIII/WGS-Outbreaker) with default parameters.

The pipeline comprised all steps needed for single nucleotide variant (SNV) analysis using whole genome sequencing data. Mapping against genome reference was performed with bwa mem (version 0.7.12-r1039) (55), duplicated reads removed using Picard (version 1.140) (http://broadinstitute.github.io/picard), and the bedtools coverage v2.26 program (56) was used to perform further quality controls. Hereafter, in order to identify genetic variations among strains, SNV detection (variant calling) and SNV matrix generation were performed using GATK version 3.8.0 (57) with best practices parameters. ENSEMBL variant effect predictor script (version 88) was used for variant annotation. The whole genome sequencing project has been deposited in NCBI SRA (project accession number SRP151231).

Phylogenetic analysis and determination of modifications in genes of interest

Final step of WGS-Outbreaker pipeline comprised Maximum-likelihood trees construction using RaxML software (version 8.2.9) (58) with GTRCAT model and 100 bootstrap replicates. Phylogenetic tree was visualized and annotation performed using ggtree R package (59). In order to see if the population structure could be based on particular genomic modifications, some genes that have already been described as important in *A. fumigatus* biology were analysed in depth in each of the *A. fumigatus* population clusters formed from the SNV comparisons. The genes analysed were the *cyp51A* gene including its promoter (AFUB_063960), the *benA* gene (AFUB_010330), the *cytB* gene (AfuMt00001), and the *sdhB* gene (AFUB_057960). The modifications found in these genes were used to determine the fungicides susceptibility phenotype based on the resistance mechanisms.

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Tables

Table 1: Amino acid substitutions in Cyp51A, β-tubulin, Cytochrome B and SdhB and MIC or MEC ranges to agricultural antifungal drugs in a collection of 60 *A. fumigatus* strains including azole-resistant and azole-susceptible isolates. β-tubulin (β-tub), cytochrome B (CytB), succinate dehydrogenase inhibitor (SDHB), benzimidazoles (MBCs), strobilurins (QoIs), succinate dehydrogenase inhibitors (SDHIs), benomyl (BNY), carbendazim (CBZ), azoxystrobin (AZB), pyraclostrobyn (PYB), boscalid (BCL) and fluopyram (FLP).

# of isolates	Amino acid substitutions				MIC/MEC ranges to agricultural antifungal drugs (mg/L)					
					MBCs		QoIs		SDHIs	
	Cyp51A	β-tub	CytB	SDHB	BNY	CBZ	AZB	PYB	BCL	FLP
Azole-susceptible strains with no mutations (14)										
14	WT	WT	WT	WT	2	0.25-1	0.25-2	0.125-1	0.25-2	1-8
Azole-resistant strains with no mutations (21)										
4	R-Non Cyp51A	WT	WT	WT	2	0.25	0.25-0.5	0.125-1	0.5-2	0.5-2
9	Point mutations*	WT	WT	WT	2-4	0.25-1	0.125-2	<0.064-2	0.5-4	1-8
8	TR_{34}/L98H	WT	WT	WT	2	0.25	0.125-1	0.25-0.5	0.5-2	1-4
Azole-resistant strains with mutations in β-tub (12)										
4	TR_{34}/L98H	E198A	WT	WT	>32	>32	1-2	0.5-4	0.25-1	2
2	TR_{34}/L98H	E198Q	WT	WT	>32	>32	0.125-2	<0.064-0.5	0.125-2	0.25-8
6	TR_{34}/L98H	F200Y	WT	WT	>32	>32	0.5-2	0.5-1	0.25-1	0.25-4
Azole-resistant strains with mutations in CytB (2)										
2	TR_{34}/L98H	WT	G143A	WT	2	0.25-1	>32	>32	1	1-2
Azole-resistant strains with mutations in β-tub and CytB (4)										
1	TR_{34}/L98H	F200Y	F129L	WT	>32	>32	>32	2	1	2
2	TR_{34}/L98H	F200Y	G143A	WT	>32	>32	>32	>32	0.25-0.5	0.5-2
1	TR_{34}/L98H	E198A	G143A	WT	>32	>32	>32	>32	0.5	2
Azole-resistant strains with mutations in β-tub and SDHB (4)										
1	TR_{34}/L98H	F200Y	WT	R51G	>32	>32	0.5-4	0.5-1	0.5-2	1-2
3	TR_{34}/L98H	F200Y	WT	H270R	>32	>32	0.5-1	0.25-0.5	>32	1-8
Azole-resistant strains with mutations in β-tub, CytB and SDHB (3)										
1	TR_{46}/F121Y/T289A	F200Y	G143A	H270R	>32	>32	>32	>32	>32	1-2
2	TR_{46}/F121Y/T289A	F200Y	G143A	H270Y	>32	>32	>32	>32	>32	0.25-8

* Point mutations include isolates with mutations G54, M220 and G448.

617 **Table 2:** Analysis of the *benA, cytB* and *shdB* polymorphisms and amino acid substitutions found in
618 our set of 205 *A. fumigatus* azole susceptible and azole resistant strains. Only polymorphisms
619 involving non-synonymous mutations are shown. Azl: azole, S: susceptible, R: resistant.
620

Gene	Nucleotide position (cDNA)	Codon	Amino acid change	Azl S	Azl R	Percentage (%)
benA	a593c	gAg/gCg	E198A	0	5	2.5
	g592c	Gag/Cag	E198Q	0	3	1.5
	t599a	tTc/tAc	F200Y	1	29	14
cytB	a355g	Ata/Gta	I119V	18	57	36.6
	c387a	ttC/ttA	F129L	0	1	0.5
	g428c	gGt/gCt	G143A	0	8	3.9
sdhB	a151g	Agg/Ggg	R51G	0	1	0.5
	c808t	Cac/Tac	H270Y	0	3	1.5
	a809g	cAc/cGc	H270R	0	4	2

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Figure legends

Figure 1. Phylogenic tree representation of a whole genome alignment of a collection of 163 *A. fumigatus* genomes clustered attending to their genetic proximity. SP: Spain, PT: Portugal, CN: Canada, UK: United Kingdom, IT: Italy, JP: Japan, NT: The Netherlands, DN: Denmark, IN: India, FR: France. Azole resistance is marked in red, strains harboring azole resistance mechanisms based on tandem-repeat insertions in the promoter of the *cyp51A* gene are marked in blue and azole resistance mechanisms based on point mutations in the *cyp51A* gene are marked in pink. Mutation in the three fungicide targets are also colour coded: *benA* (green), *cytB* (orange) and *sdhB* (yellow).

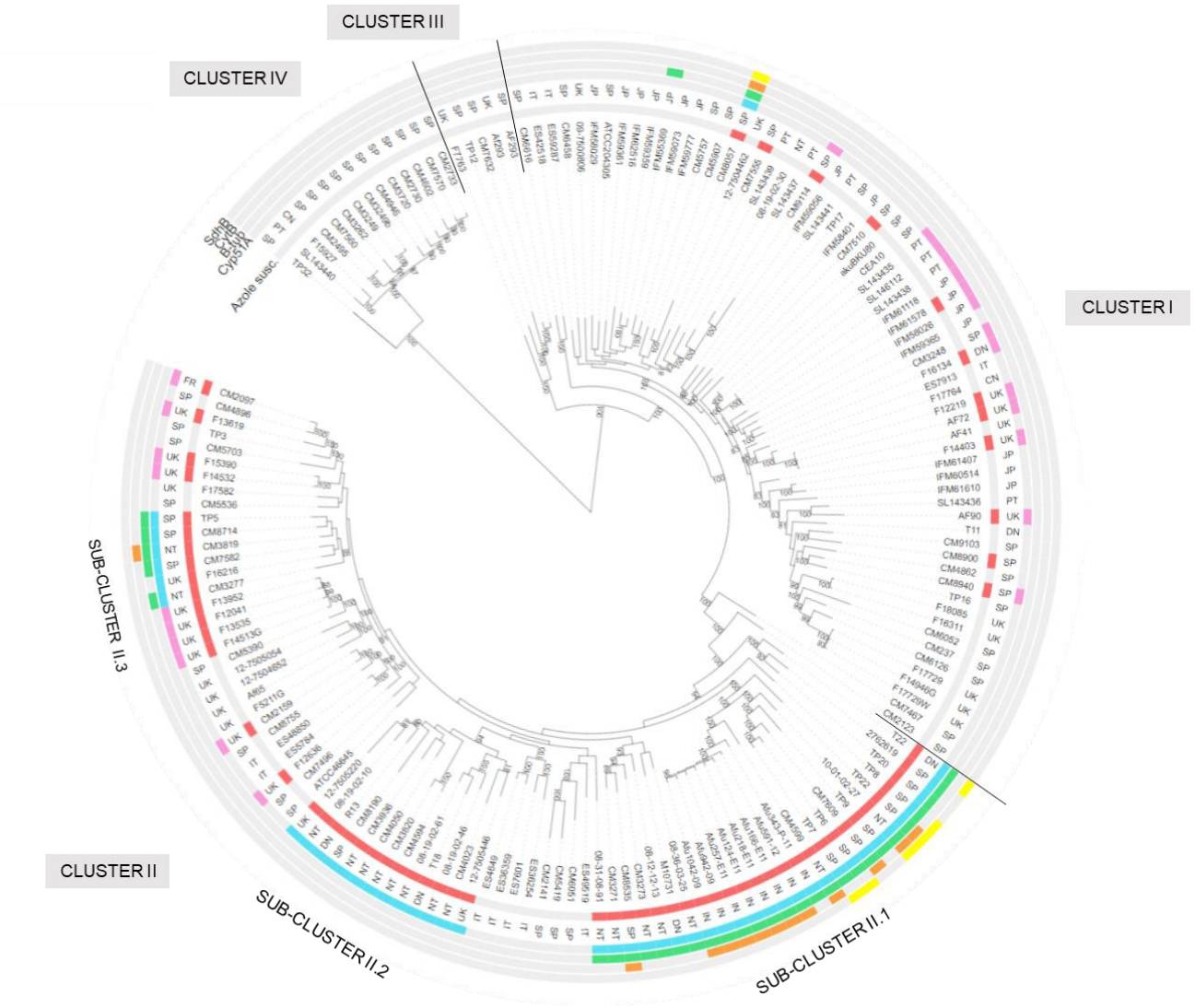

Figure 1. Phylogenic tree representation of a whole genome alignment of a collection of 163 *A. fumigatus* genomes clustered attending to their genetic proximity. SP: Spain, PT: Portugal, CN: Canada, UK: United Kingdom, IT: Italy, JP: Japan, NT: The Netherlands, DN: Denmark, IN: India, FR: France. Azole resistance is marked in red, strains harboring azole resistance mechanisms based on tandem-repeat insertions in the promoter of the *cyp51A* gene are marked in blue and azole resistance mechanisms based on point mutations in the *cyp51A* gene are marked in pink. Mutation in the three fungicide targets are also colour coded: *benA* (green), *cytB* (orange) and *sdhB* (yellow).