Examining Signatures of Natural Selection in Antifungal Resistance Genes Across Aspergillus Fungi

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Certain Aspergillus fungi cause aspergillosis, a set of diseases that typically affect immunocompromised individuals. Most cases of aspergillosis are caused by Aspergillus fumigatus, which infects millions of people annually. Some closely related so-called cryptic species, such as Aspergillus lentulus, can also cause aspergillosis, albeit at lower frequencies, and they are also clinically relevant. Few antifungal drugs are currently available for treating aspergillosis and there is increasing worldwide concern about the presence of antifungal drug resistance in Aspergillus species. Furthermore, isolates from both A. fumigatus and other Aspergillus pathogens exhibit substantial heterogeneity in their antifungal drug resistance profiles. To gain insights into the evolution of antifungal drug resistance genes in Aspergillus, we investigated signatures of positive selection in 41 genes known to be involved in drug resistance across 42 susceptible and resistant isolates from 12 Aspergillus section Fumigati species. Using codon-based site models of sequence evolution, we identified ten genes that contain 43 sites with signatures of ancient positive selection across our set of species. None of the sites that have experienced positive selection overlap with sites previously reported to be involved in drug resistance. These results identify sites that likely experienced ancient positive selection in Aspergillus genes involved in resistance to antifungal drugs and suggest that historical selective pressures on these genes likely differ from any current selective pressures imposed by antifungal drugs.

Keywords: section Fumigati, antifungal drug resistance, positive selection, cryptic species, azole, Aspergillus fumigatus, echinocandin

INTRODUCTION

Aspergillus fumigatus is an important fungal pathogen that causes aspergillosis, a spectrum of diseases that includes aspergilloma, allergic bronchopulmonary aspergillosis, and invasive pulmonary aspergillosis (Latgé and Chamilos, 2019). A few other Aspergillus species, including closely related species that also belong to section Fumigati, are known to be pathogenic (Steenwyk et al., 2019; Rokas et al., 2020). Some of these pathogenic so-called cryptic species, such as A. lentulus (Balajee et al., 2005) and A. fumigatiaffinis (Hong et al., 2005), are morphologically similar and indistinguishable from each other and from A. fumigatus by classical clinical microbiology methods (Alastruey-Izquierdo et al., 2014).
The first-line drugs against aspergillosis are the azoles, with echinocandins or amphotericin B as alternative or complementary treatments (Denning and Bromley, 2015; Novak et al., 2020). Azoles such as itraconazole, voriconazole, and posaconazole are fungicidal to Aspergillus spp. and target the ergosterol biosynthesis pathway by inhibiting lanosterol 14α-demethylase (cyp51A), resulting in accumulation of 14-methylated sterols in the cell membrane. Altered membrane fluidity followed by disruption decreases the activity of membrane-bound enzymes, which in turn inhibits cell growth and proliferation (Pérez-Cantero et al., 2020). Echinocandins are fungistatic to Aspergillus spp. and act by inhibiting β-1,3-glucan synthase, encoded by fks1 gene, the enzyme responsible for the synthesis of β-(1,3)-D-glucan, a major component of the fungal cell wall. Echinocandins disrupt the tips of hyphal walls, increasing the internal osmotic pressure and causing cell death (Nishiyama et al., 2005; Enoch et al., 2014).

Antifungal resistance is a worldwide concern, both in the clinic and in the field (Hagiwara et al., 2016; Verweij et al., 2016; Garcia-Rubio et al., 2017; Perlin et al., 2017; Fisher et al., 2018; Wassano et al., 2020). In A. fumigatus, resistance was first reported in 1997; since then, azole-resistant isolates have been reported in several different countries and are associated with therapy failure and increased mortality rates in immunocompromised patients (Wei et al., 2015). Additionally, closely related pathogenic species have been shown to differ from A. fumigatus in their drug susceptibility to amphotericin B and azoles (Alastruey-Izquierdo et al., 2014). For example, most A. lentulus isolates exhibit an increased resistance to several antifungal drugs (e.g., itraconazole, voriconazole, caspofungin, and amphotericin B) compared to A. fumigatus (Swilaiman et al., 2013).

Several molecular mechanisms are thought to be involved in antifungal drug resistance (Resendiz Sharpe et al., 2018; Chen et al., 2020b; Pérez-Cantero et al., 2020). Azole resistance mainly stems from mutations in the drug target gene, cyp51A, involving amino acid substitutions as well as tandem repeats (TRs) in its promoter region. In A. fumigatus, changes in the Cyp51A protein sequence that correlate withazole resistance include amino acid substitutions, such as in positions G54, G138, M220, and G448, or combinations of substitutions with TRs in the promoter region, such as TR34/L98H and TR46/Y121F/T289A (Wei et al., 2015; Beardsley et al., 2018). Changes in cyp51A expression have also been reported in several cases of drug-resistant Aspergillus species (Osherov et al., 2001). A mutation in cyp51B, a paralog of cyp51A, has also been linked to azole resistance in A. fumigatus (Gonzalez-Jimenez et al., 2020). In Aspergillus flavus, mutations in cyp51C, a third cyp51 paralog, have also been associated with resistance (Sharma et al., 2018).

Several other non-cyp51 genes have been associated with azole resistance (Chen et al., 2020b; Pérez-Cantero et al., 2020). For example, genes involved in transport, including the ATP-binding cassette (ABC) and the Major Facilitator Superfamily (MFS) (Slaven et al., 2002; Nascimento et al., 2003; da Silva Ferreira et al., 2004; Fraczek et al., 2013; Paul et al., 2013; Meneau et al., 2016; Chen et al., 2020a), ergosterol biosynthesis (e.g., hmg1, the HMG-CoA reductase enzyme that participates in the regulation of sterol synthesis in eukaryotes) (Rybak et al., 2019; Araí et al., 2021), stress response (e.g., calcium signaling pathway) (Chen et al., 2020a), mitochondrial processes (Wei et al., 2017; Li et al., 2020), and regulatory genes (Willger et al., 2008; Blosser and Cramer, 2012; Song et al., 2016, 2017; Hagiwara et al., 2017; Furukawa et al., 2020; Hortschansky et al., 2020) are implicated in azole resistance. Beyond azoles, echinocandin resistance is associated with mutations in the fks1 gene in both Candida yeasts (Desnos-Ollivier et al., 2008; Garcia-Effron et al., 2008) and in A. fumigatus (Jiménez-Ortigosa et al., 2017; e Silva et al., 2020). Molecular mechanisms of resistance to amphotericin B have been hypothesized to be linked to mutations in genes involved in sterol biosynthesis that cause changes in the cell membrane composition, oxidative stress response, and cell wall (mainly the 1,3-α-glucan portion). However, these mechanisms are understudied among fungal pathogens from section Fumigati (Carulos et al., 2020).

Whether other pathogenic Aspergillus species differ from A. fumigatus in their resistance profiles to antifungals is a question of great interest (Alastruey-Izquierdo et al., 2014). Isolates of A. fumigatus are phenotypically heterogeneous (Fuller et al., 2016; Kowalski et al., 2016, 2019; Keller, 2017; Ries et al., 2019; Steenwyk et al., 2020a,b), and we recently showed that this heterogeneity extends to antifungal drug resistance among clinical isolates in A. fumigatus and closely related cryptic species, A. lentulus and A. fumigati affinis (Dos Santos et al., 2020b), needing examination of multiple isolates per species. Previous genomic examinations of azole resistance solely focused on A. fumigatus (Abdolrasouli et al., 2015; Garcia-Rubio et al., 2018). Recently, Parent-Michaud et al. (2020) explored mechanisms of antifungal resistance in the pathogenic species A. thermomutatus and A. turcicus, in particular cyp51A and efflux-pump-mediated drug resistance, analyzing orthologs of the ABC transporters previously associated with azole resistance, such as cdr1B, AfuMDR1-4, and atrF. Despite these advances, studies that examine the evolution of drug resistance genes across several species of section Fumigati are lacking.

To address this gap of knowledge, we examined signatures of ancient positive selection in genes known to be involved in drug resistance across 42 isolates from 12 Aspergillus species in section Fumigati. To account for heterogeneity in antifungal susceptibility among isolates of closely related species, we were particularly interested in verifying whether resistant isolates were available for all clades of section Fumigati sequenced so far, and in identifying sites that have experienced positive selection across isolates of different species. We identified resistant and susceptible isolates across all the Fumigati clades. Among sites under positive selection, none were previously reported to be associated with antifungal resistance, suggesting that the selective pressure imposed by drugs today is not the same as the ancient selective pressure in the same genes.
Data Collection and Ortholog Identification

We selected genomes of 42 isolates from 12 species in Aspergillus section Fumigati for analysis and used Aspergillus clavatus (section Clavati) as an outgroup (Supplementary Table 1). We followed a recent classification of Aspergillus (Houbraken et al., 2020) when referring to specific series or sections. Augustus 3.1.1 (Stanke et al., 2004) was used for gene prediction in cases of genomes that lacked a publicly available gene annotation. To identify gene orthogroups, we used DIAMOND (Buchfink et al., 2015) in an all-versus-all protein search of all proteomes, followed by OrthoFinder v.2.3.3 (Emms and Kelly, 2019) to group proteins into orthogroups.

Antifungal Susceptibility Testing and Classification of Fungal Isolates

Antifungal susceptibility testing (AST) was conducted for four Aspergillus isolates and these results were merged with those from a previous publication (Dos Santos et al., 2020a). We used the EUCAST (European Committee for Antimicrobial Susceptibility Testing) reference microdilution method version 9.3.2 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9.3.2_Mould_testing_definitive_revised_2020.pdf) (Arendrup et al., 2015), in which isolates are grown on plates with increasing drug concentrations and the first concentration in which growth is inhibited (MIC) is recorded. For the four isolates of A. hiratsukae and A. felis recently sequenced by our group (Dos Santos et al., 2020a), we tested their susceptibility to four antifungal drug classes as previously described (Dos Santos et al., 2020b).

For isolates and species that were not in our collection and did not undergo AST, we searched the literature for reports of drug resistance (Supplementary Table 2). Whenever it was not EUCAST, the classification that the paper used was taken. When the EUCAST method was employed, isolates were classified based on the breakpoint values established for A. fumigatus and according to the following guide: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/AFST_BP_v10.0.200204_updatd_links_200924.pdf.

Identification of Gene Markers and Phylogenetic Inference

To reconstruct the phylogeny of our isolates in section Fumigati (including the outgroup species, A. clavatus), we retrieved the sequences of the beta-tubulin (bтаn), calmodulin (CaM), actin (act), and the RNA polymerase II second-largest subunit (RPB2) gene markers. Each individual gene was aligned with MAFFT v.7.397 option L-INS-i (Katoh and Standley, 2013). Nucleotide sequences were then threaded onto the protein alignment using pal2nal (Suyama et al., 2006). Alignments were visually inspected with Jalview v.2.10.3 (Waterhouse et al., 2009).

Selecting Genes Involved in Antifungal Drug Resistance

We analyzed all genes previously studied in the context of antifungal drug resistance, in particular those connected to the azole class of drugs, that were present in single-copy or fewer across all isolates in our orthogroup analyses (Supplementary Tables 3, 4). Genes where more than four isolates (10%) lacked an ortholog were excluded. To avoid inconsistencies in how proteins were annotated in each genome, the corresponding coding sequences for all proteins of interest were recovered from NCBI using NCBI efetch v.13.8 (options--format fasta_cds_na--db protein). All coding sequences were converted to amino acids using the transeq function in EMBOSS v.6.6.0.0 (Rice et al., 2000), and aligned with MAFFT v.7.397 option L-INS-i (Katoh and Standley, 2013). Nucleotide sequences were then threaded onto the protein alignment using pal2nal (Suyama et al., 2006). Alignments were visually inspected with Jalview v.2.10.3 (Waterhouse et al., 2009).

Analyzing Sites for Evidence of Positive Selection

The evolutionary rate ratio of dN/dS (also known as ‘ω’), assesses the rate of non-synonymous substitutions to the rate of synonymous substitutions in DNA codon sequence alignments. Values of ω > 1 are suggestive of positive selection, whereas ω = 1 and ω < 1 are suggestive of neutral evolution and negative selection, respectively. To determine dN and dS values, we used codeml from PAML v.4.9i (Yang, 2007) with the following parameters: runmode = 0, seqtype = 1, CodonFreq = 2, ndata = 1, clock = 0, model = 0, icode = 0, fix_omega = 0, omega = .4, and cleandata = 1. We used two “site models” of codon evolution, the M7 model (beta model) that considers one ω across all isolates, plus 10 site classes with ω ≤ 1, and the M8 model (beta model and ω model) that considers 11 classes, 10 with ω ≤ 1 and one additional class with ω > 1. We also tested models M1a (two classes: 0 ≤ ω < 1 and ω = 1) and M2a (three classes: 0 ≤ ω < 1, ω = 1, and ω > 1). To test whether positive selection occurred in a given gene, the log-likelihood values (lnL) from the two models were used in a likelihood ratio test (LRT), with M7 being the null model and M8 the alternative model (the same test was applied to M1a vs. M2a). The LRT value for each gene was compared to a χ2 value (Jeffares et al., 2015). For genes that rejected the null hypothesis, the Bayes Empirical Bayes (BEB) analysis (Yang et al., 2005) was used to detect positively selected sites.

Given the extensive use of the A. fumigatus A1163 strain in laboratory studies, sequences in this organism were used as the reference to describe sites under positive selection in section Fumigati. A Python script was developed to recover the sites in the reference sequence (checkPositionsAfterGaps.py) for each site under positive selection. In cases where a gene was missing for one or more isolates, treehouse (Steenwyk and Rokas, 2019) was used to prune taxa from the species tree (Figure 1) to match the taxa in the gene tree. Given
the importance of the alignment quality in evolutionary studies (Sackton, 2020), we employed GBlocks for codons with default parameter settings (Castresana, 2000) to identify poorly aligned regions.

RESULTS

Isolates of Section Fumigati Species Exhibit Heterogeneity in Their Drug Resistance Profiles

To investigate the evolution of genes involved in antifungal resistance in members of Aspergillus section Fumigati, we studied the genomes of 42 isolates from 12 species from Europe (24), Asia (9), America (7), Oceania (1), and unknown (1). Most isolates correspond to clinical isolates, but some were environmental soil samples (Supplementary Tables 1, 2).

Using four phylogenetic gene markers (benA, CaM, RPB2, and act), we reconstructed the evolutionary history of these 42 section Fumigati isolates, using A. clavatus as the outgroup (Figure 1). Our phylogeny is consistent with previous studies (Houbraken et al., 2020; Dos Santos et al., 2020a,b) that assigned isolates of species into four series (or lineages within section Fumigati): Fumigati (A. novofumigatus, A. fischeri, A. oerlinghausenensis, A. fumigatus, A. fumigatiaffinis, and A. lentulus), Unilateralis (A. turcosus and A. hiratsukae), Viridinutantes (A. udagawae, A. viridinutans, and A. felis), and Thermomutati (A. thermomutatus). A high consistency in the phylogenetic reconstruction is important since it is required for our analyses of ancient selection.

To gain insights into the relationship between genome evolution and drug resistance, we searched the literature for antifungal susceptibility tests (ASTs) on these isolates (Supplementary Table 1: Figure 1). The most common AST uses the European Committee on Antimicrobial Susceptibility Testing (EUCAST) protocol, which we used to classify isolates as susceptible or resistant based on A. fumigatus breakpoints. However, for some isolates, the Clinical Laboratory Standards Institute (CLSI) and the YeastOne panel procedures were used for some of the isolates included (Tamiya et al., 2015; Lyskova et al., 2018). In these cases, we relied on the conclusions and criteria established by the original authors (Fedorova et al., 2008; Tamiya et al., 2015; Houbraken et al., 2016; Kusuya et al., 2016; Lyskova et al., 2018; Parent-Michaud et al., 2019a,b; Talbot et al., 2019; Dos Santos et al., 2020b) in order to describe these strains as susceptible or resistant.

Using these antifungal resistance data and the reconstructed phylogeny, we were able to identify members of all four series in section Fumigati that are resistant to azoles (Figure 1). We previously phenotyped several clinical isolates of A. fumigatus, A. fumigatiaffinis, and A. lentulus, and showed that most A. fumigatus were susceptible to both azoles and to amphotericin B (AMB). We also have previously noticed that A. fumigatiaffinis and A. lentulus are overall more resistant to AMB relative to A. fumigatus (Dos Santos et al., 2020b). Here, we expanded our analysis to other cryptic species of section Fumigati. In the series Viridinutantes, we obtained the AST for A. felis CNM-CM5623, which showed resistance to ICZ and V CZ, but was susceptible to AMB. We also phenotyped two A. hiratsukae (series Unilateralis) isolates that were recently sequenced by our group (Dos Santos et al., 2020a). Interestingly, the CNM-CM6106 isolate was resistant to azoles and to AMB, whereas CNM-CM5793 was susceptible to AMB and to some but not all azoles. In the same series, we identified isolates of A. turcosus with sequenced genomes that varied with respect to azole resistance. Finally, the only sequenced isolate of A. thermomutatus (series Thermomutati) is known to be azole resistant but its resistance to AMB is currently unknown. A summary of the drug resistance profiles, where known, of our isolates is shown in Figure 1 and the full data are shown in Supplementary Tables 1, 2.

A Few Sites in Genes Involved in Resistance to Azoles and Echinocandins Have Signatures of Ancient Positive Selection

We next aimed to identify sites that experienced ancient positive selection and whether these sites overlap with known sites involved in antifungal resistance (Supplementary Tables 3, 4). To avoid complications associated with inferring evolutionary events across multiple paralogs (Jeffares et al., 2015), we only analyzed those genes associated with drug resistance that were present in single copy across either all or most of the 42 isolates from section Fumigati with available genomes. From the 41 genes in our initial list (Supplementary Table 4), 10 were removed because eight (AFUB_047000, AFUB_016810, AFUB_013880, AFUB_038670, AFUB_062080, AFUB_099400, AFUB_036760, AFUB_045980) had paralogs in at least one isolate and two (AFUB_092980, AFUB_078550) were absent from many isolates. Fourteen genes were single-copy in all studied isolates, and 17 were single-copy with orthologs missing in a few isolates resulting in 31 genes that were used in downstream analyses.

Among the 31 genes, we individually calculated the average value of ω (the evolutionary rate ratio of dN/dS, which assesses the rate of non-synonymous substitutions to the rate of synonymous substitutions) across sites (Supplementary Table 4). The mean of average ω values across genes was low (0.11; min: 0.06; max: 0.37). To identify genes with sites under positive selection, we used the log likelihood estimates (lnL) generated by different site models (M8 vs. M7) in likelihood ratio tests (LRT). We also calculated the LRT statistics for comparison of the simpler models M1a and M2a but found that most comparisons were not significant (Supplementary Table 4); given that the M7 and M8 models are more sensitive, we focused on the results from the M8 vs. M7 comparisons. Eleven of the 31 genes rejected the null hypothesis (≥ 9.21; 2 d.f. and 0.01 sign. level) (Table 1), suggesting these genes have undergone positive selection (ω > 1). Among the eleven genes, we identified a damage resistance protein, transporters, regulators, and metabolic enzymes that had all been previously reported in literature as involved in azole resistance.
FIGURE 1 | Phylogenetic reconstruction of strains in section Fumigati with sequenced genomes and the different susceptibility levels to different antifungals (the outgroup is not shown). We observed that the four sequenced Series (Fumigati, Unilateralis, Viridinutantes, Thermomutati) have members that exhibit resistance to at least one azole (marked in red in the internal nodes). ITC, itraconazole; VCZ, voriconazole; “R”, resistant strain; “S”, susceptible strain; “U”, AST (EUCAST) information is not available. “Azole resistant” or “Azole susceptible” means that this general pattern was reported in the literature. *EUCAST has designated this an Area of Technical Uncertainty (ATU), that corresponds to an MIC value where the categorization is doubtful.
In addition, we found that the gene encoding a drug target for echinocandins, fks1, has sites under positive selection.

Among the genes for which the LRT rejected the null hypothesis, ten out of eleven had sites under positive selection (\( \omega > 1 \)) according to the Bayes Empirical Bayes (BEB) analysis (\( > 0.95% \)) (Figure 2). In some genes (fks1, ramA, erg4B, atmA, and atrA), positively selected sites were clustered at the terminal region of their protein products, whereas in the damage resistance protein (dapB) and in transporter genes (abcA, abcF, and tpo3) these sites were distributed over their entire length. Finally, mot1 only had one site under positive selection that was present in the DUF3535 domain of the protein.

To examine the impact of poor alignment quality on our inferences, we analyzed the location and alignment among sites under positive selection. Some of the sites clustered at terminal regions of some proteins (e.g., ramA and erg4B) appeared to be embedded in low quality alignment regions. Therefore, we employed GBlocks in the codon alignments to identify poorly aligned regions. In general, most alignment positions were maintained in all genes, with poor alignment regions including putative sites under positive selection identified in three genes: one position from atrA (856), one from fks1 (1,626) and five clustered positions were eliminated from ramA (508, 510, 516, 517, and 519) (Figure 2; Supplementary Table 5). This resulted in 36 sites across ten genes with robust signatures of positive selection. The variation observed in sites under positive selection is consistent with our phylogeny (Figure 1). For example, we did not observe many differences between (closely related) strains of A. fumigatus but did observe differences between A. fumigatus and other (more distantly related) species.

We next searched for evidence in the literature of sites that we identified as potentially being positively selected in these ten genes as being involved in antifungal resistance susceptibility (Supplementary Table 5). To our knowledge, literature-based evidence of point mutations among our eleven genes is only available for fks1 (Jiménez-Ortigosa et al., 2017; e Silva et al., 2020). Importantly, these sites are unchanged in all isolates with sequenced genomes included in our study. To our knowledge, none of the remaining ten genes with evidence of positive selection have been reported as having specific point mutations associated with antifungal susceptibility.

### Table 1: Genes with signatures of positive selection (the Likelihood Ratio Test rejected the null hypothesis).

| Gene | Category | Antifungal class | Justification of selection | Reference(s) |
|------|----------|------------------|----------------------------|--------------|
| abcA | Transporter | Azole | Overproduction of AbcA in A. fumigatus yielded increased azole resistance, S. cerevisiae expressing abcA were more resistant to fluconazole than the PDRS-deleted background strain. | Esquivel et al., 2020 |
| abcF | Transporter | Azole | Saccharomyces cerevisiae expressing abcf had the strongest efflux activities, had the broadest range of substrate specificity, and were more resistant to fluconazole. | Esquivel et al., 2020 |
| atrA | Kinase | Azole | atrA and atrA mutant populations grown under sub-inhibitory drug concentration resulted in voriconazole resistance and discrete alterations in cyp51A and/or the Cdr1B efflux transporter; these kinases are likely involved in genetic stability | Dos Reis et al., 2018, 2017 |
| erg4B | Reductase | Azole | erg4A and erg4B null mutant displays remarkable increased susceptibility to antifungal azoles (but not their isolated mutants) | Long et al., 2017 |
| fks1 | Drug target | Echinocandin | hot spot FKS1 mutation E671Q might be responsible for the reduced susceptibility | e Silva et al., 2020 |
| mdr3 | Transporter | Azole | Overexpression of Mdr3, Mdr4 or both drug efflux pump genes of A. fumigatus and/or selection of drug target site mutations are linked to high levels of triazole resistance | Nascimento et al., 2003 |
| mot1 | Transcriptional regulator/modulator | Azole | Involved in regulation of the negative cofactor 2 complex, which is a key regulator of drug resistance in A. fumigatus | Furukawa et al., 2020 |
| ramA | Enzyme | Azole | Loss of ramA led to a Cyp51A/B-independent increase in resistance to triazole antifungal drugs | Norton et al., 2017 |
| tpo3 | Transporter | Azole | tpo3 and dur3 played important roles in susceptibility to ITC via a potential mechanism (tpo3 and dur3 → polyamine homeostasis → ROS content → ITC susceptibility) | Chen et al., 2020, 2020a |
| dapB | Damage resistance protein | Azole | Overexpression of dapB and dapC causes dysfunction of erg5 and erg11, resulting in abnormal accumulation of sterol intermediates and further accentuating the sensitivity of ΔdapA strains to azoles | Song et al., 2016 |
DISCUSSION

Here, we identified signatures of ancient positive selection in genes known for their involvement in antifungal resistance across sequenced strains of section *Fumigati*. Sites previously reported to contribute to antifungal resistance did not overlap with sites under positive selection, suggesting that any current selective pressures imposed by antifungals may differ from historical signatures of selection on these genes.

*Aspergillus fumigatus* is an important human pathogen (Latgé and Chamilos, 2019). Importantly, there has been an increased report of antifungal resistance across several isolates in *A. fumigatus* and cryptic species, in particular against azoles that comprise the main antifungal class used in clinical treatment. Azole resistance in *A. fumigatus* and closely related species is due to mechanisms in genes of different categories, including mutations and changes in expression of *cyp51* genes, transporters (ABC and MFS transporters), and genes involved in stress response, biofilm formation and mitochondria function (Pérez-Cantero et al., 2020).

The recent increase in availability of genomes in all groups of organisms has facilitated the analyses of adaptive evolution and the study of genes across whole genomes (Jeffares et al., 2015; Sackton, 2020). In fungi, the availability of genomes of *Aspergillus* has allowed the community to carry out species-wide comparative genomic analyses (de Vries et al., 2017; Mead et al., 2021). In order to study possible biological interactions shaping the evolution of species that might have favored antifungal resistance in *Aspergillus* section *Fumigati*, we used genomes of all sequenced species of this section to study positive selection with codon-based models as implemented in PAML (Yang, 2007). Importantly, we were able to measure or find in the literature tests of susceptibility across all clades of sequenced isolates in this section and classify them as resistant or susceptible to antifungals.

Several studies have classified cryptic species into resistant or susceptible categories based on their response to the main antifungal classes (Perlin et al., 2017; Imbert et al., 2020), although Imbert et al., emphasized that denser sampling of isolates and species would provide a more realistic and accurate classification. Interestingly, in all taxonomic series present in our study we identified azole resistance in at least one isolate. Unfortunately, several isolates with sequenced genomes have not been assayed in an AST (including isolates of *A. udagawae* and *A. novofumigatus*), and we therefore could not assign them to different susceptibility categories. Moreover, sequencing of additional members of section *Fumigati*, including unrepresented series *Brevipes*, *Spathulati*, *Neoglabri*, and *Fenneliarum* (Houbraken et al., 2020)
would also increase the power of our analyses. Consistency of AST results across the different species was challenging, suggesting that future projects should rely on the same (e.g., EUCAST) or comparable AST methods.

We previously identified patterns of antifungal susceptibility among different isolates of pathogenic species in series *Fumigati* and suggested the potential for synergistic effects or trade-offs between different antifungals (Dos Santos et al., 2020b). Such patterns comprise important aspects to clinicians. Here, we showed that besides the clinical isolates phenotyped by our group (*A. fumigatus, A. fumigatiaffinis, A. lentulus, A. hiratsukae,* and *A. felis*) (Dos Santos et al., 2020a,b), there are no other reports of sequenced isolates that also have been tested for amphotericin B (AMB) using the EUCAST method. We previously observed an increased resistance to AMB in *A. fumigatiaffinis* relative to *A. fumigatus* (both in series *Fumigati*) (Dos Santos et al., 2020b), and these differences are highlighted in Figure 1. In the present study, however, we noticed that isolates susceptible to AMB were found among the recently sequenced species *A. hiratsukae* (series Unilateralis) and *A. felis* (series Viridimutantes) (Dos Santos et al., 2020a). Interestingly, in addition to isolates being resistant/susceptible to several drug classes, or being resistant to one class and not to others, we also observed cases of resistance to some azole antifungals but not to others, suggesting that isolates can develop a set of responses to drug that cannot be widely applied to all therapeutics.

Possible evolutionary mechanisms for the emergence of resistance in *A. fumigatus* in the clinic and the environment have been proposed (Bui et al., 2019; Schoustra et al., 2019). Schoustra et al. (2019) studied “hotspots,” environments that support growth, reproduction, and genetic variation of *A. fumigatus* and contain fungicides that facilitate emergence, amplification, and spread of resistance mutations. However, given the diversity of sources and the geographic distribution of strains across section *Fumigati,* in this study we were particularly interested in identifying sites that experienced ancient positive selection across the entire lineage instead of sites that may have more recently evolved to directly counteract current antifungals. To identify more ancient changes instead of the more recent ones studied by Schoustra et al. (2019), we employed site models, which allow the identification of sites that may have been subjected to repeated positive selection across the lineage (Sackton, 2020).

We identified several genes in the literature that have mutations previously linked to antifungal resistance (Supplementary Table 3). For example, several mutations in cyp51A are known for their involvement in antifungal resistance, such as G54, L98, G138, M220, G448, Y121, P216, F219, A284, Y431, G432, and G434 (Wei et al., 2015; Pérez-Cantero et al., 2020), as well as a mutation in cyp51B (G457S) (Gonzalez-Jimenez et al., 2020). Studying mitochondrial genes, (Li et al., 2020) identified sites important in antifungal resistance in the *cox10* gene, D234A and R243Q. A mutation associated with antifungal resistance (P88L) was also identified in the *hapE* gene, a conserved euukaryotic transcription factor (Hortschansky et al., 2020). Mutations (F262del, S305P, I142S, F290L) in the 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase-encoding gene, *hmg1,* have also been linked to antifungal resistance (Rybak et al., 2019; Gonzalez-Jimenez et al., 2020). However, none of these mutations were under positive selection in our results.

As stated previously, several studies have reported genes involved in antifungal resistance and susceptibility with a focus on specific mutations, the impact of knockouts, or overexpression of different genes. Previous works analyzed evolution of drug resistance in single species (Li et al., 2017). Recently, the evolution of antifungal resistance was studied in *Aspergillus fumigatus* (Rhodes et al., 2021). However, no previous work exploited the signatures of evolution in genes involved in antifungal resistance across all sequenced species in section *Fumigati.* We identified sites under positive selection in different regions of genes involved in azole resistance, including transporters, regulators, metabolic enzymes, and in *fks1,* the gene encoding the target of echinocandins. Interestingly, these sites were present in regions inside and outside conserved domains (Figure 1). For example, in the *abcA* (ABC transporter) gene, a site was found in the transmembrane domain (TMD) of the product. Mutations in ABC transporters are known to affect efflux and change the selectivity and susceptibility to substrates, including antifungals, in *Candida albicans* and *Saccharomyces cerevisiae* (Moreno et al., 2019). On the other hand, in the MFS *tpo3* transporter, previous experiments identified its role in importing polyamines that in turn seem to protect the cell from drug action in *A. fumigatus* (Chen et al., 2020a). Another example of a gene with sites under positive selection was *atmA,* a kinase known for involvement in DNA damage response, for which experiments performed with deletions combined with *atrA* (also with sites under positive selection) showed that null mutant *A. fumigatus* isolates had defective DNA repair and were azole-resistant (Dos Reis et al., 2018). Interestingly, sites identified in *atmA* were in the FATC domain, in which mutations are known to hamper the kinase activity (Awasthi et al., 2015).

Variation in these sites might have impacted how different *Aspergillus* species responded to selective pressures, which probably differs from the pressure imposed by antifungals today. Although sites under positive selection from this work are located in genes currently associated with antifungal resistance in *Aspergillus fumigatus,* it is indeed possible that they can be involved in increased survival of fungi in environments with selection pressures unrelated to those imposed by azole antifungals. Also of relevance was the fact that cyp51A, which is often associated with azole resistance, did not contain sites under positive selection while *fks1,* which is associated with echinocandin resistance, does contain sites that experienced ancient positive selection. Interestingly, unlike azoles (for which natural analogs are not known), echinocandin has structural analogs of natural origin (Denning, 2003) present in the environmental niches. Thus, the signatures of ancient positive selection observed in the *fks1* gene could reflect complex antagonistic interactions between *Aspergillus* fungi and their microbial competitors.

Further work will be able to address whether sites under positive selection in the studied genes confer advantages in
survival of isolates exposed to azoles or echinocandins. Moreover, given the heterogeneity observed across different Aspergillus strains (Dos Santos et al., 2020a), population genetic analyses with respect to evolution of drug resistance in species with various sequenced genomes (e.g., A. fumigatus) is also an important next step.

**DATA AVAILABILITY STATEMENT**

Publicly available datasets were analyzed in this study. This data can be found here: All genomes included in this study are available from the NCBI GenBank database. Assembly accession numbers are presented in Supplementary Table 1, including the reference when a link is available. The data and scripts used in this project are available on the Gitlab repository under https://gitlab.com/SantosRAC/Santosetal2021_evolutionGenesAntifungalsFumigati.

**AUTHOR CONTRIBUTIONS**

RS, JS, MM, AA-I, GHG, and AR designed the experiments. OR-M performed the experiments. RS ran bioinformatic analyses. RS, MM, JS, and AR wrote the manuscript. All authors revised the manuscript.

**REFERENCES**

Abdolrasouli, A., Rhodes, J., Beale, M. A., Hagen, F., Rogers, T. R., Chowdhary, A., et al. (2015). Genomic context of azole resistance mutations in aspergillus fumigatus determined using whole-genome sequencing. MBio 6:e00536. doi: 10.1128/mBio.00536-15

Alastruey-Izquierdo, A., Alcalaz-Fuoli, L., and Cuenca-Estrella, M. (2014). Antifungal susceptibility profile of cryptic species of Aspergillus. Mycopathologia 178, 427–433. doi: 10.1007/s11046-014-9775-z

Arai, T., Umeyama, T., Majima, H., Inukai, T., Watanabe, A., Miyazaki, Y., et al. (2021). Hmg1 mutations in Aspergillus fumigatus and their contribution to triazole susceptibility. Med. Mycol. Off. Publ. Int. Soc. Hum. Anim. Mycol. 21:573. doi: 10.1093/mmy/mya026

Arendrup, M. C., Guinea, J., and Cuenca-Estrella, M. (2015). Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. Eur. Comm. Antimicrob. 48, 1782–1786. doi: 10.1128/JCM.02316-09

Awasthi, P., Foiani, M., and Kumar, A. (2015). ATM and ATR signaling at a glance. J. Cell Sci. 128, 4255—4262. doi: 10.1242/jcs.169730

Balajee, S. A., Gribskov, J. L., Hanley, E., Nickle, D., and Marr, K. A. (2005). Aspergillus lentulus sp. nov., a new sibling species of A. fumigatus. Environ. Cell C 4, 625—632. doi: 10.1128/EC.4.625-632.2005

Beardsley, J., Halliday, C. L., Chen, S. C.-A., and Sorrell, T. C. (2018). Responding to the emergence of antifungal drug resistance: perspectives from the bench and the bedside. Future Microbiol. 13, 1175—1191. doi: 10.2217/fmb-2018-0059

Blosser, S. J., and Cramer, R. A. (2012). SREBP-dependent triazole susceptibility in Aspergillus fumigatus is mediated through direct transcriptional regulation of erg11A (cyp51A). Antimicrob. Agents Chemotherap. 56, 248—257. doi: 10.1128/AAC.05027-11

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. Nat. Method 12, 59—60. doi: 10.1038/nmeth.3176

Buil, J. B., Hire, R. K., Zwaan, B. J., Arendrup, M. C., Melchers, W. J. G., and Verweij, P. E. (2019). The fading boundaries between patient and environmental routes of triazole resistance selection in Aspergillus fumigatus. PLoS Pathog. 15:e1007858. doi: 10.1371/journal.ppat.1007858

Carolan, H., Piersson, S., Lagrou, K., and Van Dijck, P. (2020). Amphotericin B and other polyenes-discovery, clinical use, mode of action and drug resistance. J. Fungi. 6:321. doi: 10.3390/jof6040321

Castrasena, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540—552. doi: 10.1093/oxfordjournals.molbev.a026334

Chen, M., Zhong, G., Wang, S., Zhu, J., Tang, L., and Li, L. (2020a). tpo3 and dur3, aspergillus fumigatus plasma membrane regulators of polyamines, regulate polyamine homeostasis and susceptibility to itraconazole. Front. Microbiol. 11:563139. doi: 10.3389/fmicb.2020.563139

Chen, P., Liu, J., Zeng, M., and Sang, H. (2020b). Exploring the molecular mechanism of azole resistance in Aspergillus fumigatus. J. Mycol. Med. 30:100915. doi: 10.1016/j.jymed.2019.100915

da Silva Ferreira, M. E., Capelaro, J. L., dos Reis Marques, E., Malavazi, I., Perlin, D., Park, S., et al. (2004). In vitro evolution of itraconazole resistance in Aspergillus fumigatus involves multiple mechanisms of resistance. Antimicrob. Agents Chemotherap. 48, 4405—4413. doi: 10.1128/AAC.48.11.4405-4413.2004

de Vries, R. P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Uchima, C. A., et al. (2017). Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus Aspergillus. Genome Biol. 18:28. doi: 10.1186/s13059-017-1151-0

Denning, D. W. (2003). Echinocandin antifungal drugs. Lancet 362, 1142—1151. doi: 10.1016/S0140-6736(03)14472-8

Denning, D. W., and Bromley, M. J. (2015). Infectious disease. how to bolster the antifungal pipeline. Science 347, 1414—1416. doi: 10.1126/science.aaa6097

Desnos-Ollivier, M., Bretagne, S., Raoux, D., Hoinard, D., Dromer, F., Dannaoui, E., et al. (2008). Mutations in the Bsk1 gene in Candida albicans, C. tropicalis, and C. krusei correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. Antimicrob. Agents Chemotherap. 52, 3092—3098. doi: 10.1128/AAC.00088-08

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**SUPPLEMENTARY MATERIAL**

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Dos Santos, R. A. C., Silva, L. P., de Castro, P. A., Almeida de Lima, P. B., do Carmo, R. A., Marini, M. M., et al. (2018). The influence of genetic stability on aspergillus fumigatus virulence and azole resistance. 8, 285–278. doi: 10.1534/g3.117.300265

Dos Santos, R. A. C., Rivero-Menendez, O., Steenwyk, J. L., Mead, M. E., Goldman, G. H., Alastuey-Izquierdo, A., et al. (2020a). Draft genome sequences of four aspergillus section fumigati clinical strains. Microbiol. Resourc. Announc. 9:40. doi: 10.1128/MRA.00856-20

Dos Santos, R. A. C., Steenwyk, J. L., Rivero-Menendez, O., Mead, M. E., Silva, L. P., Bastos, R. W., et al. (2020b). Genomic and phenotypic heterogeneity of clinical isolates of the human pathogens Aspergillus Fumigatus, Aspergillus Lentulus, and Aspergillus Fumigatusfissiatus. Front. Gene. 11:459. doi: 10.3389/fgene.2020.00459

e Silva, A. P., Miranda, I. M., Branco, J., Oliveira, F., Faria-Ramos, I., Silva, L. M., et al. (2020). FK51 mutation associated with decreased echinocandin susceptibility of Aspergillus fumigatus following anidulafungin exposure. Sci. Rep. 10:11976. doi: 10.1038/s41598-020-68706-8

Emms, D. M., and Kelly, S. (2019). OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. 20:238. doi: 10.1186/s13059-019-1832-y

Enoch, D. A., Idris, S. F., Aliyu, S. H., Micallef, S., Ule, O., and Karas, J. A. (2014). Micafungin for the treatment of invasive aspergillosis. J. Infect. 68, 507–526. doi: 10.1016/j.jinf.2014.01.007

Esquivel, B. D., Rybak, J. M., Barker, K. S., Fortwendel, J. R., Rogers, P. D., and Fuller, K. K., Cramer, R. A., Zegans, M. E., Dunlap, J. C., and Loros, J. J. (2016). Multicentric analysis of the species distribution and antifungal susceptibility of cryptic isolates from aspergillus section fumigati. Antimicrob. Agents Chemotherap. 60:2271. doi: 10.1128/AAC.01374-20

Jeffares, D. C., Tomiczek, B., Sojo, V., and dos Reis, M. (2015). A beginners guide to estimating the non-synonymous to synonymous rate ratio of all protein-coding genes in a genome. Methods Mol. Biol. 1201, 65–90. doi: 10.1007/978-1-4939-1438-8_4

Jiménez-Ortigosa, C., Moore, C., Denning, D. W., and Perlin, D. S. (17). Emergence of echinocandin resistance due to a point mutation in the fks1 Gene of Aspergillus fumigatus in a patient with chronic pulmonary aspergillosis. Antimicrob. Agents Chemotherap. 61:17. doi: 10.1128/AAC.01277-17

Kalanvanamoorothy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., and Jermim, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14, 587–589. doi: 10.1038/nmeth.4285

Kath, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 21, 3157–3160. doi: 10.1093/molbev/mst018

Keller, N. P. (2017). Heterogeneity Confounds Establishment of “a” Model Microbial Strain [Review of Heterogeneity Confounds Establishment of “a” Model Microbial Strain]. MBio 8:135. doi: 10.1128/mBio.00135-17

Kowalski, C. H., Beattie, S. R., Fuller, K. K., McGurk, E. A., Tang, Y.-W., Hohl, T. M., et al. (2016). Heterogeneity among isolates reveals that fitness in low oxygen correlates with Aspergillus fumigatus virulence. MBio 7:16. doi: 10.1128/Mbio.01515-16

Kowalski, C. H., Kerkar, J. D., Liu, K.-W., Bond, M. C., Hartmann, R., Nadell, C. D., et al. (2019). Fungal biofilm morphology impacts hypoxia fitness and disease progression. Nat. Microbiol. 4, 2430–2441. doi: 10.1038/s41564-019-0558-7

Kück, P., and Meusemann, K. (2010). FASconCAT: Convenient orthology inference for comparative genomics. Genome Biol. 11:459. doi: 10.1186/gkb669

Li, Y., Zhang, C., Wang, H., Wei, X., Chen, P., et al. (2020). Mitochondrial dysfunctions trigger the calcium signaling-dependent calcium signaling-dependent fungal multidrug resistance. Proc. Natl. Acad. Sci. USA. 117, 1711–1721. doi: 10.1073/pnas.1911560116

Long, N., Xu, X., Zeng, Q., Sang, H., and Lu, L. (2017). Erg4A and Erg5B are required for condensation and azole resistance via regulation of ergosterol biosynthesis in Aspergillus fumigatus. Appl. Environ. Microbiol. 83, 16-16. doi: 10.26500/febs-0007236

Lyskova, P., Hubka, V., Svobodova, L., Barrs, V., Dhand, N. K., Yaguchi, T., et al. (2018). Antifungal susceptibility of the Aspergillus viridians complex:...
Tamiya, H., Ochiai, E., Kikuchi, K., Yahiho, M., Toyotome, T., Watanabe, A., et al. (2015). Secondary metabolite profiles and antifungal drug susceptibility of Aspergillus fumigatus and closely related species, Aspergillus lentulus, Aspergillus udagawae, and Aspergillus viridinutans. J. Infect. Chemother. Offic. J. Japan Soc. Chemotherap. 21, 385–391. doi: 10.1016/j.jiac.2015.01.005

Verweij, P. E., Chowdhary, A., Melchers, W. J. G., and Meis, J. F. (2016). Azole resistance in Aspergillus fumigatus: can we retain the clinical use of mold-active antifungal azoles? Clin. Infect. Dis. Offic. Publ. Infect. Dis. Soc. Am. 62, 362–368. doi: 10.1093/cid/civ885

Wassano, N. S., Goldman, G. H., and Damasio, A. (2020). Aspergillus fumigatus. Trends Microbiol. 28, 594–595. doi: 10.1016/j.tim.2020.02.013

Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M., and Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics, 25, 1189–1191. doi: 10.1093/bioinformatics/btp533

Wei, X., Chen, P., Gao, R., Li, Y., Zhang, A., Liu, F., et al. (2017). Screening and characterization of a Non-cyp51A Mutation in an Aspergillus fumigatus cox10 strain conferring azole resistance. Antimicrob. Agents Chemotherap. 61:116. doi: 10.1128/AAC.02101-16

Wei, X., Zhang, Y., and Lu, L. (2015). The molecular mechanism of azole resistance in Aspergillus fumigatus: from bedside to bench and back. J. Microbiol. 53, 91–99. doi: 10.1007/s12273-015-5014-7

Willger, S. D., Puttikamonkul, S., Kim, K.-H., Burritt, J. B., Grahl, N., Metzler, L. J., et al. (2008). A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in Aspergillus fumigatus. PLoS Pathog. 4:e1000200. doi: 10.1371/journal.ppat.1000200

Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 24, 1586–1591. doi: 10.1093/molbev/msm088

Yang, Z., Wong, W. S. W., and Nielsen, R. (2005). Bayes empirical bayes inference of amino acid sites under positive selection. Mol. Biol. Evol. 22, 1107–1118. doi: 10.1093/molbev/msi097

Conflict of Interest: AR is a scientific consultant for LifeMine Therapeutics, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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