UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

RENATO AUGUSTO CORRÊA DOS SANTOS

ANÁLISE GENÔMICA DE ESPÉCIES CRÍPTICAS DE *ASPERGILLUS*

GENOMIC ANALYSIS OF *ASPERGILLUS* CRYPTIC SPECIES

Campinas
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ANÁLISE GENÔMICA DE ESPÉCIES CRÍPTICAS DE *ASPERGILLUS*

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in Genetics and Molecular Biology the area of Bioinformatics.

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Genética e Biologia Molecular na área de Bioinformática.

Supervisor: Prof. Dr. Gustavo Henrique Goldman
Co-supervisor: Prof. Dr. Marcelo Falsarella Cazzarolle

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“Don’t panic”

Douglas Adams

(included here after a nice conversation with Dr. Alexandre Colato, who included the same sentence in his thesis - at the time of writing, I hadn't finished reading the book, a gift from Dr. rer. nat. Diego M. Riaño Pachón)

Resumo

Aspergillus fumigatus é um importante patógeno humano que causa aspergilose invasiva (AI). Adicionalmente, diversos outros membros da seção *Fumigati* que são morfologicamente próximos de *A. fumigatus* mas diferem genética e fenotipicamente, chamadas de espécies crípticas, também constituem importantes agentes causais de AI. O tratamento de pacientes com aspergilose é realizado principalmente com antifúngicos, mas há uma preocupação global sobre a emergência de resistência em diferentes isolados de *A. fumigatus* e de espécies crípticas. A crescente disponibilização de genomas fúngicos e informação fenotípica, como a suscetibilidade a antifúngicos e a virulência nos permite investigar diversos genes envolvidos em mecanismos biológicos de relevância clínica. No entanto, apenas alguns isolados de espécies crípticas na seção *Fumigati* com informação fenotípica associada estão disponíveis. Além disso, dados de genômica e transcriptômica que estão publicamente disponíveis são pouco explorados no contexto da resistência e resposta a antifúngicos, os quais poderiam ser explorados para levantar hipóteses genéticas e evolutivas. Trabalhos anteriores exploraram alta heterogeneidade fenotípica entre isolados de *A. fumigatus*, indicando que este poderia ser também o caso para a suscetibilidade a antifúngicos e que poderia ser estendido para espécies crípticas. Em relação aos dados de transcriptômica, embora estudos anteriores tenham explorado a expressão de genes codificadores de proteínas na resposta a drogas como itraconazol e voriconazol, nenhum deles integrou a resposta a diferentes azóis, tampouco exploraram genes não codificadores, como os RNAs longos não codificadores (lncRNAs). São três os objetivos desta tese: (i) estudar a heterogeneidade genômica e fenotípica de isolados clínicos de A. fumigatus e espécies crípticas relacionadas; (ii) identificar assinaturas de seleção positiva em genes envolvidos na resistência a antifúngicos usando isolados com genoma sequenciado da seção *Fumigati*, e (iii) explorar dados públicos de RNA-Seq para levantar novos candidatos na resposta geral a azóis, incluindo os lncRNAs. Interessantemente, foi encontrada alta heterogeneidade na suscetibilidade a antifúngicos e virulência entre isolados de *A. fumigatus* e duas espécies crípticas.

Analisando mutações em genes alvos de drogas, *cyp51A* (azóis) e *fks1* (equinocandinas), foram identificados isolados de A. fumigatus com mutações relevantes conferindo resistência a azóis, além de mutações exclusiva das espécies crípticas. Genômica comparativa e filogenética revelaram um parálogo em *A. fumigatiaffinis*, *cyp51C*, que é incomum em espécies da seção *Fumigati*. Análise de assinaturas de seleção natural em genes importantes nas espécies com genoma sequenciado de Fumigati revelaram sítios que não sobrepõem os conhecidos como conferindo resistência, incluindo *cyp51A*, o que sugere que a pressão seletiva ancestral provavelmente difere da presente nas espécies e isolados atuais. Por outro lado, o principal alvo de equinocandinas, para as quais análogos existem na natureza, apresentou sítios sob seleção (*fks1*). Análise de transcriptoma de *A. fumigatus* sob exposição a diferentes azóis revelou novos candidatos participantes na resposta a estes compostos, tanto codificadores como lncRNAs. Estes resultados ampliam nosso conhecimento sobre a biologia e a evolução de *A. fumigatus* e espécies relacionadas no contexto clínico.

Abstract

Aspergillus fumigatus is an important human pathogen that causes invasive aspergillosis (IA). Additionally, several closely related members of section *Fumigati* that are morphologically similar to *A. fumigatus* but differ genetically and phenotypically, so called ‘cryptic species’, also comprise important causal agents of IA. Treatment of patients with aspergillosis is mainly carried out with antifungals, but there is an increasing worldwide concern about the emergency of resistance in several isolates of *A. fumigatus* and in cryptic species. The increasing availability of fungal genomes and phenotypic information such as antifungal susceptibility and virulence allows us to investigate several genes involved in biological mechanisms of clinical relevance. However, only a few isolates of cryptic species in section *Fumigati* with associated phenotypic information are currently available. Additionally, the genomic and transcriptomic data that are publicly available are still underexploited in the context of resistance or the response to antifungals, and they could be exploited to raise genetic or evolutionary hypotheses. Previous studies reported that in *A. fumigatus*, a high phenotypic heterogeneity among different strains has been observed for several traits, indicating that this could also be the case for antifungal susceptibility, and that this might be extended to cryptic species. Regarding the transcriptomic data, even though previous studies analyzed the expression of protein-coding genes in responses to individual drugs such as itraconazole and voriconazole, none have integrated different data sets to understand the general response to azoles, nor have these studies exploited the noncoding genomic elements in the *A. fumigatus* genome, particularly the long-noncoding RNAs (lncRNAs). Three aims of this project were to (i) study genomic and phenotypic heterogeneity in clinical isolates of *A. fumigatus* and cryptic species; (ii) identify signatures of positive selection in genes involved in antifungal resistance using genome sequenced isolates of section *Fumigati* and; (iii) exploit public RNA-Seq data sets to raise novel gene candidates possibly involved in the general responses to azoles, including lncRNAs. Interestingly, we found high heterogeneity in antifungal susceptibility and virulence across strains of *A. fumigatus* and two cryptic species.

Analysing mutations in targets of antifungal drugs, *cyp51A* (azoles) and *fks1* (echinocandins), we identified strains of *A. fumigatus* with known relevant mutations related to resistance, and mutations exclusive to cryptic species. Comparative genomics and phylogenetics revealed a paralog in *A. fumigatiaffinis*, *cyp51C*, which is uncommon in section *Fumigati* and could be related to resistance. Analysis of signatures of natural selection across important genes in sequenced species of section *Fumigati* revealed sites that did not overlap known resistance mutations, including *cyp51A*, suggesting an ancient selective pressure that probably differs from those of current strains and species. On the other hand, the main target of echinocandins, for which analogous natural compounds exist in nature, did present sites under selection *(fks1*). Transcriptome analysis of *A. fumigatus* under different azoles revealed novel candidates in response to azoles, both coding genes and lncRNAs. These results improve our understanding of the biology and evolution of *A. fumigatus* and related species in the clinical context.

Resumen

Aspergillus fumigatus es un patógeno humano importante causante de la aspergilosis invasiva (AI). Además, existen varios miembros de la sección *Fumigati* estrechamente relacionados, que son morfológicamente similares a *A. fumigatus* pero difieren genéticamente y características fenotípicas, son las llamadas "especies crípticas", que también comprenden importantes agentes causales de IA. El tratamiento de los pacientes con aspergilosis se realiza principalmente con antifúngicos, pero existe una creciente preocupación mundial por el surgimiento de resistencias en varias cepas de *A. fumigatus* y en especies crípticas. La creciente disponibilidad de genomas fúngicos y datos fenotípicos, tales como la susceptibilidad antifúngica y la virulencia, nos permite investigar varios genes implicados en mecanismos biológicos de relevancia clínica. Sin embargo, actualmente solo se encuentran disponibles unas pocas cepas de especies crípticas en la sección *Fumigati* con información fenotípica asociada. Además, los datos genómicos y transcriptómicos que están disponibles públicamente aún están subexplorados en el contexto de la resistencia o la respuesta a los antifúngicos, y podrían ser examinados para plantear hipótesis genéticas o evolutivas. Estudios anteriores informaron que se ha observado en *A. fumigatus* una alta heterogeneidad fenotípica entre diferentes cepas para varios características, lo que indica que este también podría ser el caso de la susceptibilidad antifúngica, y que esto podría extenderse a las especies crípticas. En relación a los datos anteriores de transcriptómica, a pesar de que fue analizada la expresión de genes codificantes de proteínas en respuestas a fármacos individuales, tales como itraconazol y voriconazol, ninguno ha integrado diferentes conjuntos de datos para comprender la respuesta general a los azoles, así como tampoco han explorado los elementos genómicos no codificantes en el genoma de *A. fumigatus,* particularmente los ARN no codificantes largos (lncRNA). De esta forma, este proyecto propuso tres objetivos: (i) estudiar la heterogeneidad genómica y fenotípica en cepas clínicas de *A. fumigatus* y especies crípticas; (ii) identificar sitios de selección positiva en genes relacionados a la resistencia antifúngica, utilizando cepas con genoma secuenciado, perteneciente a la sección

Fumigati y, finalmente; (iii) explorar datos públicos de RNA-Seq para generar nuevos genes candidatos posiblemente implicados en las respuestas generales a los azoles, incluidos los lncRNA. Curiosamente, encontramos una alta heterogeneidad en la susceptibilidad antifúngica y la virulencia entre cepas de *A. fumigatus* y dos especies crípticas. Analizando mutaciones en dianas de fármacos antifúngicos, *cyp51A* (azoles) y *fks1* (equinocandinas), identificamos cepas de *A. fumigatus* con mutaciones relevantes conocidas relacionadas con la resistencia y mutaciones exclusivas de especies crípticas. La genómica comparativa y el análisis filogenético revelaron un parálogo en *A. fumigatiaffinis, cyp51C*, que es poco común en la sección *Fumigati* y podría estar relacionado con la resistencia. El análisis de sitios de selección natural en genes relevantes en especies secuenciadas de la sección *Fumigati* reveló sitios en los cuales no se superponen las mutaciones de resistencia conocidas, incluido *cyp51A*, lo que sugiere una presión selectiva antigua que probablemente difiere de las de las cepas y especies actuales. Por otro lado, el objetivo principal de las equinocandinas, para las cuales existen compuestos naturales análogos en la naturaleza, sí presentó sitios bajo selección (*fks1*). El análisis del transcriptoma de *A. fumigatus* bajo diferentes azoles reveló nuevos candidatos en respuesta a estas drogas, tanto genes codificantes como lncRNAs. Estos resultados mejoran nuestra comprensión de la biología y la evolución de *A. fumigatus* y especies relacionadas en el contexto clínico.

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strains form a separated group that is closely related to *A. novofumigatus*. All *A. lentulus* strains in this work group together and share an ancestor with *A. lentulus* IFM54703, the only sequenced strain in this species to date. The *A. fumigatus* strains sequenced in this work form different internal groups in the clade with other strains in the species (e.g., strains CNM-CM8714 and CNM-CM8812 group together and strains CNM-CM8686 and CNM-CM8689 form another group). **(B)** *A. fumigatiaffinis* and *A. lentulus* shares the highest number of common orthogroups and *A. fumigatiaffinis* displays the highest number of species-specific orthogroups. We considered species-specific orthologs those that were present in at least one strain of a given species, with no representative from another species. **(C)** Orthogroups shared by all and "all but one" strains are the most frequent in three closely related *Aspergillus* pathogens. *A. lentulus*, *A. fumigatus*, and *A. fumigatiaffinis* have 9,008, 8,321, and 9,423 orthologous genes present in all strains, respectively. The five largest combinations of orthogroups are shown. As expected, the most frequent combination of orthogroups are those in all strains but one.

Figure 6 - Changes in important genes related to antifungal susceptibility in the three *Aspergillus* pathogens. **(A)** Products of genes related to antifungal resistance, *Cyp51A* (azoles) and *Fks1* (echinocandins), display species- and strain-specific polymorphisms. Only the positions with changes in at least one strain are shown (substitutions or insertions/deletions). Blue triangles highlight important amino acid changes in positions 98, 121, and 289 in Cyp51A and in hot spot 2 (HS2) of *Fks1*. Red triangles indicate insertions/deletions. **(B)** Promoter region of the *cyp51A* gene displays strain-specific mutations among Spanish strains of three closely related *Aspergillus* pathogens. Well-known tandem repeat regions in antifungal-resistant strains of *A. fumigatus* are shown between positions 70–140 in the alignment (i.e., TR34 and TR46, observed in CNM-CM8714 and CNM-CM8057, respectively, delimited by two blue arrows in upper part). Polymorphisms in cryptic species were also identified, for instance, the short deletions exclusively found in the cryptic species (either in *A. fumigatiaffinis* or *A. lentulus*) around positions 230–250. Red arrow and red font indicate the start codon. **(C)** Phylogeny of Cyp51 gene family

(protein sequences) reveals three different members (Cyp51A, Cyp51B, and the putative Cyp51C) in *A. fumigatiaffinis*. Ultrafast Boostrap Approximation and SH-aLRT support values are shown.

Figure 7 - Orthogroups for virulence determinants reveals variable number of paralogs among the three closely related *Aspergillus* pathogens. We searched for 215 known genetic determinants of virulence in *A. fumigatus* Af293 in the species of interest and found they were grouped into 203 orthogroups. 146/203 were found in single copy across all strains and are not shown here. The cladogram above the species reflects similarities between strain presence/absence patterns. *A. fumigatus* Af293 shows a different pattern compared to other strains of *A. fumigatus*, grouping with one of the *A. lentulus* strains (CNM-CM8927). This may reflect the phylogenetic divergence of *A. fumigatus* strain Af293 from other species members. Conidial pigment polyketide synthase *alb1* (Afu2g17600) is one of the genetic determinants of virulence with highest number of copies in cryptic species (n = 7) when compared to *A. fumigatus* strains (n = 4). Gene identifiers in *A. fumigatus* Af293 are highlighted in bold. Color scale indicates the number of genes found within the orthogroup.

Figure 8 - Maximum-likelihood phylogenetic tree of the four strains sequenced in this announcement (in bold) and related species in section *Fumigati*, based on the analysis of the four markers β-tubulin gene (*benA*), calmodulin gene (*CaM*), actin gene (*act*), and RNA polymerase II second-largest subunit gene (*RPB2*), commonly used in *Aspergillus* taxonomy (Hubka et al. 2018); sequences were obtained from reference Hubka et al. (2018) except for the sequences of the four newly sequenced strains, which were obtained by searching for markers of each strain in orthogroups generated by OrthoFinder v2.3.3 (Emms and Kelly 2019) using *A. fumigatus* Af293 (Nierman et al. 2005) as the reference. Each marker was aligned with MAFFT v7.397 (Katoh and Standley 2013), and a supermatrix was generated with FASconCAT v1.11 (Kück and Meusemann 2010). Tree inference was carried out on IQ-TREE v2.0.3 (Minh et al. 2020) with partitions with the option “MFP+MERGE,” which employs ModelFinder to find the best partition scheme. The final tree was edited in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Support values are based

on 1,000 bootstrap replicates. A. clavatus (section *Clavati*) was used to root the tree. Note that the species *A. parafelis*, *A. pseudofelis*, and *A. felis* were merged (synonymized) into a single species, *A. felis* (Hubka et al. 2018); thus, we infer that the two sequenced strains belong to *A. felis*.

Figure 9 - 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.

Figure 10 - Protein annotation and annotated domains of genes for which the likelihood ratio tests (LRT) rejected the null hypothesis suggestive of positive selection among these genes. Among the eleven genes with significant LRT values, 10 presented sites under positive selection. Annotation of domains was carried out using the online Pfam database (http://pfam.xfam.org/). Proteins with long peptide stretches without any significant matches to Pfam domains were shortened (indicated by two bars with blank space in between). The gene products in *Aspergillus fumigatus* A1163 were used as reference.

Figure 11 - Length distribution of 5’ (left) and 5’ (right) UTRs in *A. fumigatus* Af293, as annotated in FungiDB.

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1 Introduction

1.1 Aspergillosis: a group of diseases caused by fungi of the genus *Aspergillus*

Most fungal infections occur on skin and nails, accounting for approximately one billion infections worldwide (Rodrigues and Nosanchuk 2020). Mucosal infections also account for a high incidence in humans, particularly vulvovaginal candidiasis (caused by *Candida* spp.). At least one episode of vulvovaginitis occurs in women during the childbearing years, and in general about 75 million women suffer from at least four episodes yearly (Sobel 2007).

Systemic fungal infections kill two million people annually, according to the 2020 report of the Global Action Fund or Fungal Infections (https://gaffi.org/annual-reports/). Despite the availability of antifungals they still present mortality rates over 50% (Brown et al. 2012). Invasive fungal diseases are particularly important due to their high mortality rates, although their incidences are much lower than superficial mycoses (Brown et al. 2012). Among the agents that cause invasive fungal infections, more than 90% of diseases are caused by *Cryptococcus*, *Pneumocystis*, *Candida*, and *Aspergillus*, in particular in immunocompromised patients (Brown et al. 2012). Other examples of causal agents of invasive fungal disease include *Blastomyces* ssp. (blastomycosis), *Coccidioides* ssp. (coccidioidomycosis), *Paracoccidioides* ssp. (paracoccidioidomycosis), and *Histoplasma capsulatum* (histoplamosis) (Almeida, Rodrigues, and Coelho 2019).

Among the most relevant invasive fungal infections are the spectrum of diseases caused by members of the genus *Aspergillus*, in particular *A. fumigatus*, but some close relatives are also clinically relevant, including *A. udagawae, A. felis*, *A. fischeri, A. fumigatiaffinis*, *A. thermomutatus*, *A. hiratsukae*, *A. laciniosus*, *A. lentulus*, *A. novofumigatus*, *A. fumisynnematus, A. pseudoviridinutans*, *A. spinosus*, and *A. viridinutans* (Arastehfar et al. 2021). *A. fumigatus* can reach lungs when humans inhale its conidia, which may be present in both indoor and outdoor environments (**Figure 1**). Concentration of conidia vary but can reach up to 10^8 per m^3 (Wéry 2014). It is common to identify *Aspergillus* spp. in lungs without any reaction or

allergic response, as indicated by a study which showed that *Aspergillus* or *Penicillium* DNA were found in lung biopsy of approximately 37% of samples in healthy adults (David W. Denning et al. 2011).

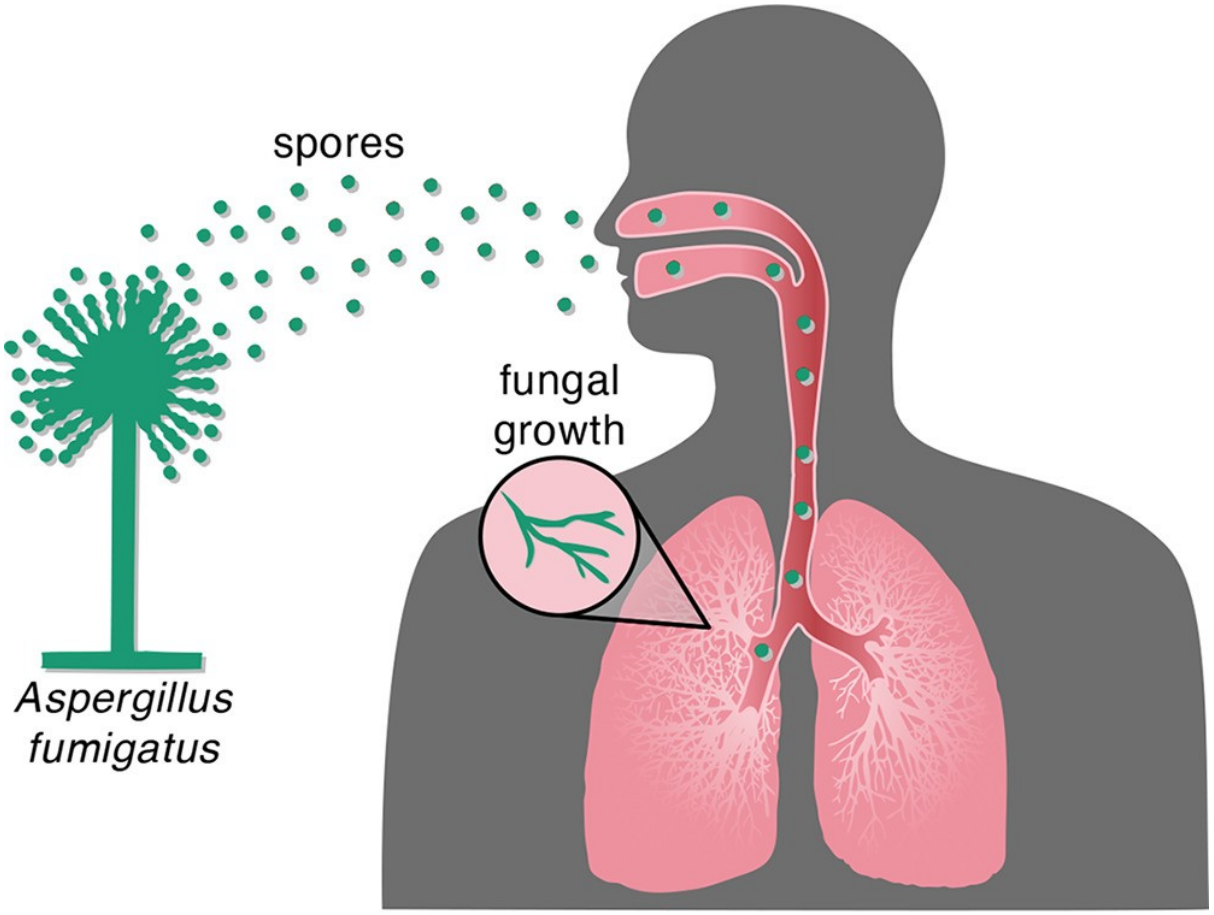

Figure 1. Spores of *A. fumigatus* can reach lungs and eventually infect the patient (colonization of other parts can also occur). Source: (Steenwyk, Mead, de Castro, et al. 2020).

The spectrum of diseases caused by *Aspergillus* in humans is wide. Besides affecting at least 16 million people yearly (David W. Denning et al. 2016; David W. Denning, Pleuvry, and Cole 2013), invasive aspergillosis has high mortality rates that range from 30 to 90%. These diseases are classified into three groups with distinct pathogenetic mechanisms, clinical manifestations, and overlapping features, depending on the status of the host immune system (Latgé and Chamilos 2019). In immunocompetent patients, *Aspergillus* species can cause chronic, noninvasive diseases like a fungus ball (also called “aspergilloma”) or chronic inflammation

accompanied by a fibrotic process. In atopic patients, in particular those with cystic fibrosis or genetic predisposition, sensitization to *Aspergillus* allergens leads to the several forms of aspergillosis called allergic bronchopulmonary aspergillosis (ABPA). The third group of aspergillosis comprises invasive pulmonary aspergillosis (IPA), which affects immunocompromised patients. Importantly, this is more common in patients with chronic diseases such as asthma and cystic fibrosis, with cancer (e.g., leukemia), and those that have received hematopoietic stem cells transplantation (HSCT).

Aspergillosis can happen in co-infection with viruses, including cytomegalovirus and influenza (Schauwvlieghe et al. 2018). Given the high impact of the Coronavirus Disease outbreak in 2019 (COVID-19) global pandemic, it is important to mention that aspergillosis is of concern in patients with COVID-19 because of its opportunism characteristic. Until May 2021, at least 100 cases of COVID-19-associated invasive pulmonary aspergillosis (CAPA) had been reported (Arastehfar et al. 2021). There are numerous pieces of evidence that infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) elevates the risk of having a fungal infection. For instance, the use of glucocorticoids has been described in an elevated number of COVID-19 patients with acute respiratory distress syndrome (close to 50%) (D. Wang et al. 2020). Immune dysregulation associated with damaged epithelial lung may facilitate the infection by *Aspergillus* spp (Arastehfar et al. 2021).

1.2 Diagnosis of aspergillosis

Early diagnosis of fungal disease is extremely important to its outcome (Rodrigues and Nosanchuk 2020). Laboratory methods exist for this purpose such as microscopic examination, microbial culture, antigen detection, and molecular tests, although some factors make the process difficult (Backx, White, and Barnes 2014). Since *Aspergillus* spp. are ubiquitous in nature, it is a challenge to differentiate cases where the pathogen is present simply because the patient inhaled the conidia from

those in which the fungus is detected as the causal agent of infection. In aspergillosis, methods for diagnosis have been recently reviewed. It is usually made by culturing and microscopic analyses, but these methods have low sensitivity. Alternatives available comprise the detection of a cell wall component, galactomannan, that is present in *Aspergillus* species, by either enzyme-linked immunosorbent assays (ELISAs) or more recently Point-of-care tests (POCTs). Additional methods include detection of *Aspergillus* DNA by real time PCRs (although standardization is still necessary), and the detection of *Aspergillus* antibodies (e.g., IgG) using methods like ELISA or POCTs (Arastehfar et al. 2021; Richardson and Page 2018).

Importantly, DNA sequencing would be necessary to distinguish between different fungal species, including *Aspergillus* cryptic species (*Aspergillus* complex). These species are of main concern in this thesis and details about them will be properly presented further in the text. The rDNA internal transcribed spacer (ITS) comprises the main fungal barcode, but in *Aspergillus* species (among others) additional taxonomic *loci* are necessary to achieve species resolution. However, although these methods are already in large use in research laboratories, commercialization is still difficult due to high costs and lack of expertise (Wickes and Wiederhold 2018). The correct identification of fungal species is important and may involve important decisions during clinical treatment in cases where resistance is observed. For instance, if an isolate is resistant to a first line drug (e.g., azole, for aspergillosis), then a decision would be necessary concerning the use of an alternative drug (e.g., echinocandins). However, the correct identification is also important to help improve our understanding of the real epidemiology of species causing the disease.

1.3 Antifungal treatment against aspergillosis

Besides early diagnosis, an effective therapy can be reached with early initiation of proper antifungal treatment. These drugs include a few chemical classes. The three main classes of antifungals target ergosterol or enzymes in its biosynthesis

pathway: azoles and polyenes (Alcazar-Fuoli and Mellado 2012). The first-line treatment is based on azoles, especially the triazoles. They act on the ergosterol production pathway by competitively interacting with an enzyme called sterol-demethylase. Interrupting the activity of this enzyme leads to the accumulation of toxic sterols, especially 14,α-methylated sterols (Rybak, Fortwendel, and Rogers 2019). Reduction of fungal membrane rigidity, interruption of functions of integral membrane proteins, and chitin synthases have also been reported (Song, Zhang, and Lu 2018). Triazoles include itraconazole, voriconazole, posaconazole, and isavuconazole. These drugs were introduced in agriculture in 1973, and in the 1990s they started to be used in clinical treatment for humans (Maertens 2004; Morton and Staub 2008).

Alternative options include polyenes and echinocandins, both constituting a “salvage therapy” (Novak et al. 2020). Among the polyenes, amphotericin B (AMB) comprises a broad-spectrum drug that causes depolarization of the fungal cell membrane and leakage, due to binding to ergosterol (Eta E. Ashu et al. 2018). One of the main concerns with the application of AMB in clinical treatment is its toxicity, in particular nephrotoxicity. Lipid AMB formulations have been developed with less associated toxicity and better toleration but are still not available in many countries but the added limitation of high costs (Faustino and Pinheiro 2020).

Echinocandins are lipopeptides that inhibit β-1,3-D-glucan synthase, an enzyme involved in the biosynthesis of β-1,3-D-glucan, a major component of fungal cell wall (Onishi et al. 2000). In *Aspergillus* spp. these drugs are fungistatic, lysing tips of hyphae, altering its morphology, and modifying cell wall organization and composition (Bowman et al. 2006, 2002). Importantly, echinocandins were initially isolated from natural sources in the 1970s, comprising secondary metabolites produced by fungi such as *Aspergillus nidulans* and *A. rugulosus*, and several other natural echinocandins have been isolated and characterized since then (Emri et al. 2013). Several aspects of natural echinocandins make them unsuitable for clinical applications, including hemolytic activity, narrow spectra, and low water solubility and stability. Therefore, semisynthetic drugs have been developed and approved by the

U. S. Food and Drug Administration (FDA): caspofungin (derived from pneumocandin B_0), anidulafungin (from echinocandin B), and micafungin (from FR901379) (Emri et al. 2013).

Since antifungal resistance has been a concern worldwide, the development of novel drugs is necessary. Several new antifungals have been developed in the last years and are now in different phases of clinical development (Rauseo et al. 2020). one of them, the orotomides comprise a novel class of antifungals in which the representative antifungal is olorofim, is of broad-spectrum (including *Aspergillus* spp.), and have been tested in cryptic species (Rivero-Menendez, Cuenca-Estrella, and Alastruey-Izquierdo 2019).

1.4 Taxonomy of the genus *Aspergillus*

Aspergillus is a fungal genus described in 1729 by Pier Antonio Micheli, an Italian priest. The name *Aspergillus* was given because of morphological features that resembled an aspergillum, i.e., a sprinkler of holy water (Arastehfar et al. 2021). Although macroscopic (e.g., conidial color) and microscopic characters (e.g., cell shape) were initially used to classify these fungi, today's taxonomy of the genus is polyphasic, which integrates phenotypic, genotypic, and phylogenetic information. Several examples of recent works employing a polyphasic taxonomy of the genus are presented in a recent review (Arastehfar et al. 2021).

The genus is placed in family *Aspergillaceae* and has currently more than 440 described species (J. Houbraken et al. 2020), and is one of the most important fungal genera, because it includes members with positive and negative impact on human activities (R. A. Samson et al. 2014). Some species may be of medical or industrial relevance, including *Aspergillus fumigatus* and closely related species (Renato A. C. Dos Santos et al. 2020). Other species are involved in food spoilage, including *A. flavus*, the main producer of aflatoxins (Tai et al. 2020). In industry, these fungi are also highlighted, that includes *A. niger* as an important producer of citric acid in industry (Cairns, Nai, and Meyer 2018). Lastly, the important study model *A. nidulans* helps us improve the understanding of cell biology and molecular processes

in eukaryotes (Etxebeste and Espeso 2020). Recent phylogenomics works suggested that *Aspergillaceae* originated in the lower Cretaceous about 117.4 mya, and the genus *Aspergillus* probably originated 81.7 mya (Steenwyk et al. 2019).

Even though these terms are not used by medical mycologists (Arastehfar et al. 2021), the genus *Aspergillus* has been traditionally classified employing ranks such as sections and series, a classification that has evolved over time (Raper, Fennell, and Others 1965; Gams et al. 1986). Currently, the genus has 27 sections and 87 series (J. Houbraken et al. 2020). Due to the presence of “cryptic species” in the genus, several informal group names emerged over time, but their usage lacks a consensus (e.g., “cryptic species”, “cryptic *A. fumigatus*”, “*A. fumigatus*-clade”, or *A. fumigatus* complex). Therefore, following a formal naming nomenclature is encouraged (J. Houbraken et al. 2020).

1.5 *Aspergillus fumigatus*, a major opportunistic pathogen in section *Fumigati*

Aspergillus fumigatus was first described in 1863. It is located in section *Fumigati*. Species in this group have uniseriate columnar conidial heads, are green (of different shades), and have flask shaped vesicles. *A. fumigatus* is globally distributed, is a saprophytic fungus that grows vegetatively, and occurs ubiquitously in soils, on decaying organic matter. *A. fumigatus* strains can survive in a variety of pH conditions and temperature, and have cell structures that favors its efficient aerial dispersal. These factors, allied with others such as small asexual spore size, presence of melanin in cell walls and of sialic acid on cell surface contribute for this species to become an important opportunistic pathogen that causes infection, mainly the diseases known collectively as aspergillosis (David W. Denning, Pleuvry, and Cole 2013; Latgé and Chamilos 2019; Abad et al. 2010; Kwon-Chung and Sugui 2013).

The spread of this fungus occurs largely by asexual sporulation. Asexual conidia are produced in phialides that emerge from conidiophores in conidial heads. Although it was believed that asexual reproduction occurred exclusively, with eventually undergoing a parasexual cycle resulting from hyphal fusion, recent studies

also provide evidence that a sexual cycle also exists - in particular based on evidences of recombination based in population studies (Losada et al. 2015; Teixeira, Amorim, and Araujo 2015; Eta Ebasi Ashu et al. 2017; Abdolrasouli et al. 2015). Of relevance is that sex appears to be specific to *A. fumigatus*, because attempted crosses between strains of this species with another of *A. lentulus* (a closely related species) failed to produce cleistothecia (Swilaiman et al. 2013). This observation is important, because it suggests a reduced gene flow between *A. fumigatus* and species that have shown increased antifungal resistance, which is the case of *A. lentulus* (Latgé and Chamilos 2019).

The main isolates of *A. fumigatus* that are studied in the laboratory derived from a few strains. Af293 is a strain derived from lung biopsy in 1993 (Bertuzzi et al. 2021). The Af293 was genetically modified (generating the Af293.1 strain) to study itraconazole resistance (Osherov 2001). Curiously, this was the first clinical isolate to have the genome sequenced. Another important strain commonly used in the laboratory is CEA10, which was isolated from a patient with aspergillosis in the ’90s and is more pathogenic than Af293. Several mutants have been derived from CEA10 and have been used in laboratories across the globe, including CEA17 (an auxotrophic derivative of CEA10), and A1163 (which harbors a functional *pyrG*), whose genome, like Af293, was also sequenced (Fedorova et al. 2008). Additional important *A. fumigatus* laboratory strains are ATCC46645 and D141, and their derivatives (Bertuzzi et al. 2021).

1.6 Cryptic species in section *Fumigati* include important human pathogens and non-pathogenic species

The traditional classification of *Aspergillus* has always been relying on several morphological characters, including cellular structures and morphology, as well as physiology (Pitt 1979; Stolk and Samson 1986). Several *Aspergillus* species are described as important pathogens, including *A. flavus*, *A. niger*, *A. terreus*, *A. versicolor*, *A. calidoustus*, and *A. nidulans*. In addition to these “true” species, some

designations usually describe a spectrum of species that are closely related but cannot be distinguished morphologically, the so-called “cryptic species”. For instance, section *Fumigati* includes more than 30 cryptic species closely related to *A. fumigatus* (R. A. Samson et al. 2014; J. Houbraken et al. 2020), including important pathogens and non-pathogenic species (Rokas et al. 2020).

Importantly, as previously described besides *A. fumigatus* section *Fumigati* comprises other clinically relevant species, including *A. felis*, *A. fischeri, A. fumigatiaffinis* (Hong et al. 2005), *A. fumisynnematus*, *A. hiratsukae*, *A. laciniosus*, *A. lentulus* (Balajee et al. 2005), *A. novofumigatus*, *A. pseudoviridinutans*, *A. spinosus*, *A. thermomutatus*, *A. udagawae* (Horie et al. 1995) and *A. viridinutans* (Sugui et al. 2014; Frisvad and Larsen 2015). Some are thermotolerant (including *A. fumigatus*), growing at 50º C, but other species have lower maximum growth temperature, such as *A. udagawae* (Horie et al. 1995). For *A. lentulus*, a previous case of fatal invasive aspergillosis after heart transplantation has been associated with infection by this species (Zbinden et al. 2012). The same species was further identified after renal transplantation (Gürcan et al. 2013). Importantly, in both case reports authors emphasize the difficulties associated with the identification of such species in clinical cases (or assigning isolates to *A. fumigatus*), that possibly leads to underrepresentation. Cases involving *A. udagawae* include chronic pulmonary aspergillosis associated with difficulties in antifungal treatment (Vinh et al. 2009), endobronchial infection (Posteraro et al. 2011), and keratitis (Gyotoku et al. 2012). Cases involving other species such as *A. viridinutans, A. felis, A. fischeri,* and *A. thermomutatus* have been included in a review (Lamoth 2016). Clinical cases have been associated with strains of *A. hiratsukae*, including one reported in Brazil (Guarro et al. 2002; Koutroutsos et al. 2010). *A. felis* has been associated with cases in humans and domestic animals (Barrs et al. 2013).

Even though in the past *Aspergillus* species were classified based exclusively on morphological characters, the advances in molecular methods and sequencing have allowed us to consider them in taxonomy studies. These groups have been classified based on sections and series (J. Houbraken et al. 2020).

Historically, the internal transcribed spacers (ITS) regions of the ribosomal DNA have been used in fungal taxonomy as the barcode (Schoch et al. 2012). To improve the taxonomy and resolution for discrimination of *Aspergillus* species, several studies have been employing multilocus phylogenies with additional markers and comparative genomics methods. Although no standard is currently considered in the fungal kingdom, the protein-coding gene markers used in species taxonomy of *Aspergillus* include β-tubulin (*benA*) and calmodulin (*CaM*) genes (Jos Houbraken, de Vries, and Samson 2014). Complementarily or alternatively to these markers, the genes encoding hydrophobin (*rodA*) (Yaguchi et al. 2007) and RNA polymerase II second largest subunit (*RPB2*) have also been used (J. Houbraken et al. 2020).

An important concern about these *Aspergillus* cryptic species is that they probably account for more than 10% of aspergillosis cases and some have been reported to have increased minimum inhibitory concentrations to antifungals (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014).

1.7 Components of the membrane and cell wall in *Aspergillus fumigatus*

Understanding the composition of fungal cell membrane and cell wall is essential not only to a better comprehension of *Aspergillus* physiology in its natural environment or in culture, but also to understand the mechanisms infection, interactions with the host, mechanisms of action of antifungals used in therapy, or those involved in resistance to the same compounds (David W. Denning and Bromley 2015).

The cell membrane is composed of sterols, which are important components of the eukaryotic cells. Ergosterol is the type of sterol that is a main component of membranes in fungi, is involved in biological functions such as membrane fluidity, regulation, activity of protein membranes, and cell cycle control (Alcazar-Fuoli and Mellado 2012).

The ergosterol pathway is well studied in *Saccharomyces cerevisiae* (Fryberg, Oehlschlager, and Unrau 1973). In *A. fumigatus*, the ergosterol

biosynthesis has more than 20 genes, and has been reviewed elsewhere (Alcazar-Fuoli and Mellado 2012). Initial genomic analyses pointed to the existence of two genes encoding 14-alpha-demethylases (cytochrome P450 monooxygenases), *cyp51A* and *cyp51B*, and three C-5 sterol desaturases, *erg3A*, *erg3B*, and *erg3C* (Alcazar-Fuoli and Mellado 2012). Additional enzymes in the ergosterol pathway have been genetically characterized in *A. fumigatus*, including Erg4 (sterol C24 reductase), Erg10 (acetyl-CoA acetyltransferase), Erg24A and its paralog Erg24B (sterol C-14 reductases), and Erg25 (C-4 methyl sterol oxidase) (Y. Li et al. 2021). Interestingly, the deletion of both *cyp51A* and *cyp51B* is lethal in *A. fumigatus*, but the deletion of either one or the other is not (Alcazar-Fuoli et al. 2008). Similarly, the deletion of *erg10*, or of *erg24A* and *erg24B* were also lethal (Y. Li et al. 2021).

Besides the sterols, sphingolipids are also part of the fungal cell membrane and several studies in yeast provide evidence of their importance, together with ergosterol, in the fungal plasma membrane structure. They participate in the signal transduction process and in stress responses (Song, Liu, and Li 2020). The basic structure of a sphingolipid is a backbone linked to fatty acid by an amide bond with the 2-amino group and to a polar head group at the C-1 position via an ester bond (Del Poeta et al. 2014). Its biosynthesis begins in endoplasmic reticulum and genes involved in biosynthesis have been studied extensively (Song, Liu, and Li 2020). In *A. fumigatus*, two types of sphingolipids are present, acidic and neutral, and it has been reviewed recently in the light of its putative importance to infection (Fontaine 2017).

Besides the cell membrane, the fungal cell wall is also of great importance. It comprises the main defense to the hostile environment found by fungal cells, and is the point connecting with the host and its immune system during infection (Abad et al. 2010). In *A. fumigatus*, the cell wall consists of proteins, lipids, and other compounds. The most abundant components are polysaccharides, including alpha- and β-glucans, chitin, galactomannan. The importance of the cell wall in *A. fumigatus* includes the maintenance of cell structure, the interaction with surfaces, production of biofilms, and is involved in the transportation of compounds into and out of the

cellular environment (Lee and Sheppard 2016). Components of cell wall and synthesis have been reviewed (Lee and Sheppard 2016). One of the major cell wall components is β-1,3-glucan, which is synthesized by the β-1,3-glucan synthase complex. β-1,3-glucan is present in prokaryotes,chromista, plants, and protozoa, but absent in vertebrates (Bacic, Fincher, and Stone 2009). In *A. fumigatus*, it is formed by *Fks1*, a glycosytransferase, and a regulatory subunit of *Rho* family of GTPases (Lee and Sheppard 2016). Deletion of gene *fks1* was not lethal in *A. fumigatus*, but strains were devoid of β-1,3-glucan. In addition, mutant strains were more susceptible to compounds such as calcofluor white and sodium dodecyl sulfate, there are cell-wall perturbing substances (Dichtl et al. 2015).

1.8 Disease models to study pathogenicity and virulence in *Aspergillus fumigatus*

Different infection models have been proposed in studies of pathogenicity and virulence in *Aspergillus fumigatus* strains. Recently, a study employed type II A549 lung epithelial cells, amoeba, larvae of the greater wax moth *Galleria mellonella*, and zebrafish embryos to evaluate virulence of strains of *A. fumigatus* isolated from dogs and the human clinical isolates, Af293, CEA10, and ATCC46645 (Keizer et al. 2021). They showed that each infection model has a different predictive value, which reflects the heterogeneity in either strains used in experiments as well as in the different hosts.

Choosing a single model is necessary to make comparisons among the different strains. Given the international initiatives aimed at reducing the use of rodent models in laboratory experiments and the increase in articles exploring model and non-model invertebrates, the latter have been increasingly exploited in literature to study fungal virulence, as emphasized in a recent review (Durieux et al. 2021). Among the invertebrates, *G. mellonella* has been used because they can be used in its larval stage, which is subject to microbial infection and develops an immune system which is similar with those in vertebrates (Trevijano-Contador and Zaragoza

2018). Other aspects that favor employing it as a model include the range of temperatures in which it grows, a genome which has already been sequenced (Lange et al. 2018). Additionally, studies with different fungi have shown a good correlation between virulence in *G. mellonella* and in murine models, including *Mucor circinelloides*, *Fusarium* spp., *Candida albicans*. In *A. fumigatus*, previous works indicated that the innate immune systems have functional similarities between *G. mellonella* and mammals, and exhibited similar survival curves that justify using the invertebrate as a model for virulence (Slater et al. 2011; Mead et al. 2019). However, recent research indicates that even though some correlation exist, experiments with moth require follow-up experiments with *M. musculus* (Durieux et al. 2021).

1.9 Phenotypic heterogeneity in *Aspergillus fumigatus* and closely related species

An important topic in the studies of fungal pathogenesis is the heterogeneity that exists and variation across clinical and environmental isolates, including strains that are considered reference in laboratory experiments (Keller 2017). For instance, results of studies on virulence in *A. fumigatus* may be confounded by a high variability in phenotype and genotype (Birch 1994). Several works have studied the heterogeneity in *A. fumigatus*. Given that this fungus is present in the environment and poses a threat to immunocompromised individuals, questions were raised about the probability of different strains to infect humans. An initial work revealed differences in virulence among different strains, that included clinical isolates of invasive and non-invasive aspergillosis, as well as environmental isolates (Mondon et al. 1996).

In 2014, comparing an environmental isolate of *A. fumigatus* with other isolates, (Amarsaikhan et al. 2014) observed correlations between radial growth, germination rates, and ability to establish colony growth. They also observed correlations between hyphal diameter and chitin content, proposing that changes in cell wall composition contribute to phenotype.

Different phenotypes have been recorded for experiments deleting genes

in the two laboratory *A. fumigatus* strains, Af293 and CEA10 (Beardsley et al. 2018). The presence of such heterogeneity allows researchers to test associations and correlations between different phenotypes. For instance, strain photobiological variability across different strains has been observed, and a heterogeneity in virulence that was independent of photoresponse (Fuller et al. 2016). In another study, it was shown that growth in low-oxygen environments underlies virulence (Kowalski et al. 2016); moreover, they showed a correlation between H/N ratio and increased virulence. Ries et al studied the nutritional heterogeneity that exists among several *A. fumigatus* isolates and concluded that changes in nitrogen catabolite repression are associated with fungal virulence (Ries et al. 2019).

Variability in growth rates, in the production of secondary metabolite production, and in response to chemical stresses were also encountered between Af293, CEA10, and two *A. fumigatus* strains isolated from the International Space Station (Knox et al. 2016). Additionally, Knox et al. (2016) also observed increased virulence of these isolates compared to the references. Phenotypic variability (based on morphology and colony) has also been observed in *A. fumigatus* infecting dogs, compared to environmental and human isolates (Valdes et al. 2018). Recently, a manuscript reporting genomic and phenotypic analyses of four COVID-19-associated *Aspergillus fumigatus* isolates was published by our research group (Steenwyk, Mead, de Castro, et al. 2020). This manuscript included the investigation of heterogeneity in both virulence and antifungal susceptibility across these strains.

Although there are numerous examples of studies that exploited the phenotypic and genotypic diversity in *A. fumigatus*, only a few studies have extended these analyses to closely related species, including the cryptic species in *Fumigati* (Sugui et al. 2014). No previous work analyzed the phenotypic and genomic diversity in isolates with sequenced genomes. In chapter 2 (see **Figure 2**), we study the genomic and phenotypic heterogeneity of three species of *Aspergillus*: *A. fumigatus*, and the cryptic species *A. lentulus* and *A. fumigatiaffinis*.

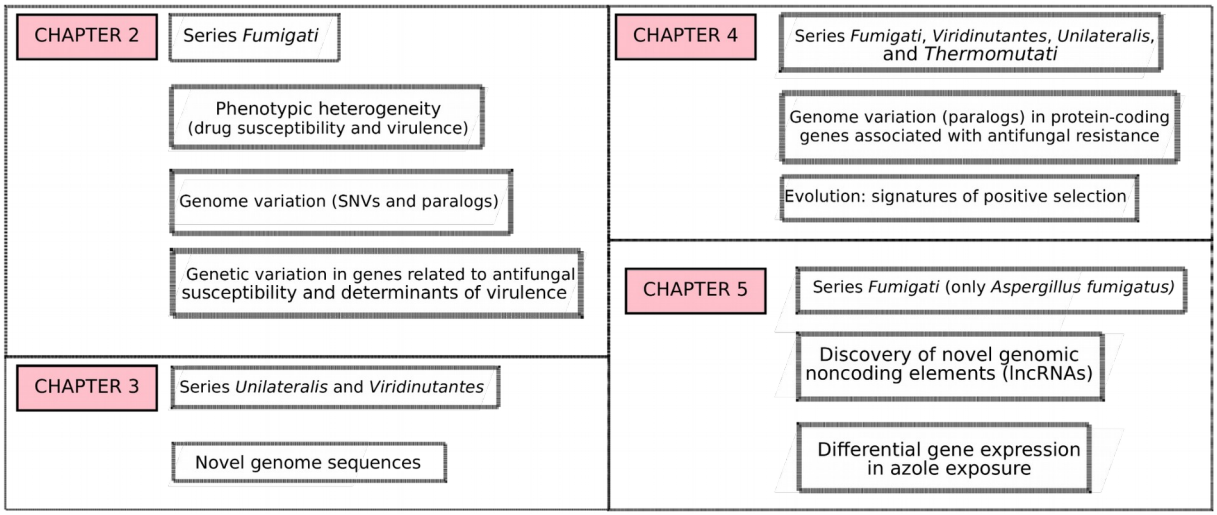

Figure 2. Subjects in each of the chapters.

1.10 Antifungal tolerance, resistance and susceptibility testing

The clinical antifungal resistance occurs when a patient fails or no longer responds to a drug at a given standard dose. Several factors affect the response to therapy, including drug, microbial and host conditions (e.g., the patient is immunocompromised). Before discussing the problem of resistance in *Aspergillus* and the evolution of drug resistance, it is important to provide clear definitions, including, resistance, tolerance, and persistence (or heteroresistance), and units involved in measurement of such metrics. The main measure to return the concentration of an antifungal that inhibits the growth of the fungus at some degree is called the minimum inhibitory concentration (MIC). An alternative measure for some drugs (as detailed further for echinocandins in *Aspergillus* spp) is the Minimum Effective Concentration (MEC), which is defined by alteration in multibranched rosette morphology and are commonly applied to molds (Berkow, Lockhart, and Ostrosky-Zeichner 2020; Meletiadis et al. 2019).

Berman and Krysan (2020) differentiate the two operational definitions in which we refer to antifungal resistance. In one situation, resistance in the context of clinical microbiology is defined if the minimum inhibitory concentration of a certain strain is higher than the established clinical breakpoint for a drug. In this case, the *in*

vivo efficacy of the drug is taken into account among other factors. A different scenario comprises the research lab in which a strain is compared to a control strain, to provide insights on whether the tested strain (not necessarily of the same species, but a species complex), in particular when a breakpoint is well established and a cutoff MIC / MEC value can be used to answer whether it is more or less resistant compared to the reference (Berman and Krysan 2020). Resistance can be intrinsic, defined by strains being naturally resistant to a given drug. On the other hand, acquired resistance comprises cases where mechanisms allow a susceptible strain to gain resistance under a selective pressure (e.g, in-patient antifungal treatment). Both cases are known in *Aspergillus* spp. (Arendrup 2014).

A different term, tolerance, is relatively new in medical mycology, and is defined by the ability of subpopulations (> 1%) of a given species to grow above the MIC, with growth being characterized as being “trailing”, whereas heteroresistance or persistence, which involves very rare cells (< 1%). Tolerance to azoles was mostly studied in *Candida* spp., whereas tolerance to echinocandins was more explored in *Aspergillus* spp (Arastehfar et al. 2021).

Concerns on antifungal resistance in fungi include *Candida* spp., (Whaley et al. 2016; Perlin 2015). Among *Aspergillus*, emergence of antifungal resistance in *A. fumigatus* was reported in 1989 in the United States (Wassano, Goldman, and Damasio 2020; D. W. Denning, Venkateswarlu, and Oakley 1997). In the Netherlands (Europe), the first triazole-resistant was in 1998 (Snelders et al. 2008). Today, there are several examples of resistant strains of *A. fumigatus*, including Asia, the Middle East, and Africa (Chowdhary et al. 2014; Badali et al. 2013; Vermeulen, Lagrou, and Verweij 2013). A recent review on *A. fumigatus* and aspergillosis raised an extensive list of references of antifungal resistance across the globe, including Brazil (Arastehfar et al. 2021).

Historically, two initiatives contributed to standardizing Antifungal Susceptibility Testing (AFST) methods. In 1968, the Clinical Laboratory Standards Institute was created. Likewise, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was created to provide standards in *in vitro* testing

as well as developing breakpoints (Berkow, Lockhart, and Ostrosky-Zeichner 2020). The first EUCAST publication was released in 2008 (Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST) 2008).

Several methods are currently available to recover the resistance level of strains that need to be tested (the AFSTs). These methods return the MICs and became increasingly important in mycology as increased resistance to the existing antifungals: first, only amphotericin B was available (in the 1950s); however, in the following decades antifungals such as 5-flucytosine, azoles, and echinocandins emerged, justifying testing for intrinsic and acquired resistance and take different therapeutic choices (Berkow, Lockhart, and Ostrosky-Zeichner 2020).

Broth microdilution methods are those in which a fungus is grown in liquid culture and for which the drug concentration is known. Both CLSI and EUCAST have developed standardized methods for yeast and molds. For azoles and amphotericin B, growth of conidia is totally inhibited, so the first well with 100% inhibition for molds denotes the MIC. On the other hand, treatment with echinocandins results in halted growth of the emerging hyphae for susceptible *Aspergillus* strains, and it makes the usage of MEC more appropriate (Berkow, Lockhart, and Ostrosky-Zeichner 2020; Meletiadis et al. 2019). Besides these broth reference methods, there are also commercial tests that will not be covered in this thesis, with some examples available in a recent review (Berkow, Lockhart, and Ostrosky-Zeichner 2020)).

1.11 Known mechanisms of antifungal resistance, in particular of azoles and echinocandins, and the prevalence in *A. fumigatus* and cryptic species

Among the main classes of antifungals, several mechanisms are known to confer resistance, including mutations, change in gene expression, among others. Azoles comprise drugs for which most of the mechanisms are studied in *A. fumigatus*. In echinocandins, however, most evidence for resistance mechanisms come from studies with *Candida spp.*, for which these antifungals are fungicidal. For

amphotericin B, no mechanism of resistance is known (Perlin, Rautemaa-Richardson, and Alastruey-Izquierdo 2017). From the microbial side, several factors contribute to emergence of resistance, including drug exposure in prophylaxis, or in repeated or long-term drug therapy. Microbial exposure to antifungals (fungicides) in agriculture has also been raised as a possible source of resistance.

The global distribution of *A. fumigatus* azole-resistant isolates has been studied. A recent work analyzed more than four thousand strains and their genetic relatedness using microsatellite loci. Resistant populations were shown to be less diverse using these markers. At the same time, the researchers found a global distribution of azole-resistant strains, in both clinical and environmental samples (Sewell et al. 2019).

The most common mechanism associated with resistance to azoles involves mutations in gene encoding the enzyme Cyp51A or its promoter. In *A. fumigatus*, changes in this gene include point-mutations in the coding region, such as positions G54, G138, P216, M220, and G448, a tandem repeat (TR) in the promoter, TR53, or combinations of TRs with associated SNPs in the coding region, including TR34/L98H and TR46/Y121F/T289A as the most commonly found in resistant isolates (Garcia-Rubio, Cuenca-Estrella, and Mellado 2017; Macedo et al. 2020; Sharma and Chowdhary 2017).

Overexpression of *cyp51* genes has also been associated with resistance. Interestingly, *cyp51A* expression responds to azole exposure, whereas *cyp51B* appears to be expressed in a constitutive manner (Hargrove et al. 2015). However, uncertainties still exist concerning their roles. A correlation between *cyp51A* expression and azole resistance does not always occur. At the same time, *cyp51B* expression was found overexpressed in non Wild Type strains, also questioning whether it is really not involved in azole resistance (Buied et al. 2013; Pérez-Cantero et al. 2020).

Two paralogs of the *cyp51* gene exist in *A. fumigatus*, *cyp51A* and *cyp51B*. However, some closely related species such as A. *flavus* also have a third copy of this gene, *cyp51C* (Pérez-Cantero et al. 2020). In phytopathology studies,

mutations in coding and promoter regions of the *cyp51B* gene have been reported in the wheat pathogen *Zymoseptoria tritici* in association with azole selection (Cools and Fraaije 2013). However, in *A. fumigatus* only a few studies have looked at the expression and mutations of *cyp51B*. Recently, Gonzalez-Jimenez et al (2020) analyzed a panazole resistant strain of *A. fumigatus* with a novel G457S mutation in *cyp51B* (Gonzalez-Jimenez et al. 2020).

As explained in previous sections, the cytochrome P450 14-α-demethylase is an important member of the ergosterol biosynthesis pathway and mechanisms of action by azoles involve interrupting the functioning of this enzyme. The cytochrome P450 heme-containing proteins are found in all biological kingdoms, and have undergone diversification in both molecular and functional levels, and presently have several classes and more then 250 represented families (Song, Zhang, and Lu 2018). Among the P450 proteins, the Cyp51 is considered one of the most ancient and has evolved in several kingdoms, bacteria, plants, animals, and fungi .

The main mechanism driving the evolution and diversity of *cyp51* is mutagenesis. Sequence similarity may be low between different species and between paralogs in the same species (e.g., *cyp51A* and *cyp51B*) (E. Mellado et al. 2001). *cyp51* is widely distributed in all phyla, with variations in number and subtypes. Yeast, basidiomycetes (e.g., *Cryptoccoccus* and *Ustilago*), and other filamentous fungi have only one copy of this gene (Gonzalez-Jimenez et al. 2020). Interestingly, in the Pezizomycotina subphylum of Ascomycota several important human and plant pathogens have two or three *cyp51* paralogs. Two paralogs exist in *A. fumigatus*, *Penicillium digitatum*, *A. nidulans*, and *Magnaporthe oryzae* (*cyp51A* and *cyp51B*); *Fusarium graminearum* and *A. flavus* have three paralogs (*cyp51A*, *cyp51B*, and *cyp51C*). In the genus *Aspergillus*, *A. oryzae*, *A. terreus*, and *A. carbonarius* comprise other species that have a third copy of *cyp51*, originating from duplication of either *cyp51A* or *cyp51B* (Pérez-Cantero et al. 2020).

Previous works verified that among the *cyp51* genes, *cyp51A* was lost multiple times in evolution, which raised the hypothesis that *cyp51B*, as it is present in all studied fungi, would be essential for ergosterol biosynthesis (Becher and Wirsel

2012). However, as previously explained, this hypothesis is not supported by functional studies which indicate neither one or the other paralog is essential.

Several studies showed that a high percentage of azole-resistant strains (20-50%) do not have any mutation in the *cyp51A* gene. These observations raised other candidates that must be involved in azole resistance (Dudakova et al. 2017). Transcription factors (TFs) are important in regulation of the ergosterol pathway, including the expression of *cyp51A*, and some have been studied. The ABC transporter regulator (or *atrR*) was shown to bind the *cyp51A* and *abcG1* (=*cdr1B/abcC*) promoter regions (Hagiwara et al. 2017; Paul et al. 2019). *abcG1* is an important gene which has previously been linked to an increased azole resistance (Fraczek et al. 2013). Another protein that is known to regulate expression of *cyp51A* and *cyp51B* in *A. fumigatus* is SrbA, a basic helix–loop–helix transcriptional activator. Mutants of this gene (*srbA*) are clearly more susceptible to azole drugs (Blosser and Cramer 2012). Besides c*yp51* genes, SrbA also regulates other genes in the ergosterol pathway, *erg3B* and *erg25A* in *A. fumigatus* and is involved in susceptibility to hypoxic conditions (Hagiwara, Watanabe, and Kamei 2016). A mutation in *hapE* gene, that encodes a CCAAT-binding TF complex subunit, was identified in azole-resistant strains not harboring *cyp51A* mutations. HapE forms a complex with HapB and HapC, that comprises a negative regulator of several genes involved in sterol biosynthesis (Furukawa et al. 2020). HapX is another transcriptional regulator that makes the action of the aforementioned complex possible (Schrettl et al. 2010). (Furukawa et al. 2020) screened a library of 484 transcription factors in *A. fumigatus* and identified twelve that were linked to antifungal resistance. Additionally, they described the role of regulators encoded by *nctA* and *nctB* genes, in which the loss of function results in azole resistance. Moreover, they found that loss of the NCT complex, which is formed by NctA and NctB, leads to multi-drug resistance phenotype, including resistance to azoles, amphotericin B, and terbinafine.

Deletion of *cybE* gene, which encodes a cytochrome b5 has also been linked to antifungal resistance (Misslinger et al. 2017). This gene plays a role in ergosterol biosynthesis because it is probably involved in the reaction carried out by

Cyp51 (cytochrome P450 enzyme). Evidences for this include the decreased ergosterol in *cybE*-deficient cells and accumulation of eburicol, a substrate of Cyp51s. *cybE* is regulated by iron availability via the HapX regulator.

A second group of genes that have been reported related to azole resistance comprises the transporters, particularly members of the Major Facilitator Superfamily (MFS) and the ATP-binding cassette (ABC) transporters (Cowen et al. 2014; Paul, Diekema, and Moye-Rowley 2017). The *A. fumigatus* genome has several annotated transporters in both classes (Meneau, Coste, and Sanglard 2016). Usually, they are associated with drug efflux, mediating the extrusion of toxic molecules to the extracellular space. In *A. fumigatus*, several transporter genes have been investigated for their role in antifungal resistance, including *cdr1B*, *mdr1*, *mdr2*, *abcD*, *abcE*, *atrI*, and several others (Pérez-Cantero et al. 2020). Genes *atrF*, *mrd3*, and *mdr4*, which encode transporters have been demonstrated to have increased expression leading to resistance to itraconazole (Slaven et al. 2002; Nascimento et al. 2003). Meneau et al tested the disruption of two ABC transporters, *atrF* and *atrI*, as well as a MFS, *mdrA*, in *A. fumigatus*. Mutants were all more susceptible to azoles, even though there were differences between voriconazole and itraconazole. They showed that the increased expression of *atrF* was associated with increase in azole resistance (Meneau, Coste, and Sanglard 2016).

Genes involved in stress-response are linked to resistance to antifungals. Azole exposure elicits the production of reactive oxygen species (ROS), which inhibits fungal growth. Damage resistance proteins (DapA, DapB, and DapC) are known to respond to azole and be involved in control of ergosterol biosynthesis (Song, Zhai, and Lu 2017). The involvement of other enzymes have also been verified in *A. fumigatus*. For instance, the deletion of a gene coding for RamA protein (*ramA*), a farnesyltransferase β-subunit that is involved in protein farnesylation (a post-translational prenylation modification), showed increased resistance to azoles (Norton et al. 2017). The findings indicate that resistance was *cyp51*-independent, since no differences in expression of *cyp51* paralogs were observed in mutants compared to the wild-type.

Studies indicate the mitochondrial genes are involved in azole-resistance. If mitochondrial dysfunction occurs, a cross-talk between nucleus and mitochondria initiates, which is known to lead to antifungal resistance (Y. Li et al. 2020). Studies involving mitochondrial fission mutants in *A. fumigatus* resulted in increased resistance to azoles, and possible link to the activity of Cyp51 and ergosterol biosynthesis (Neubauer et al. 2015). *cox10* and *cox15* genes encode important enzymes involved in heme biosynthesis, in the mitochondrial electron transport chain. Defects in these genes lead to mitochondrial dysfunction. Li et al showed that *cox10* and *cox15* mutants presented growth defects and reduced virulence, but showed increased resistance to multiple drugs, including azoles (Y. Li et al. 2020).

Besides ergosterol, it is known that other components are involved in composition of the fungal cell membrane. Recently, (Zhai et al. 2019) showed that the deletion of a gene encoding the regulator of sphingolipid production, *ormA*, in *A. fumigatus* affects the action of azoles (even though they are known for acting on the ergosterol biosynthesis). The null mutant resulted in azole susceptibility, also affecting the composition of sphingolipid ceramide components in the cell membrane, whereas overexpression resulted in azole-resistance.

Concerning the mechanisms of resistance to echinocandins, much of the information derives from studies in *Candida* (Perlin 2015; Kordalewska et al. 2018). As previously stated, these antifungals are fungicidal to yeasts and fungistatic to molds. In *A. fumigatus*, a few studies exist. For instance, previous works showed that mutations in the *fks1* gene resulted in elevated MEC in *A. fumigatus* (Rocha et al. 2007; Gardiner et al. 2005). More recent studies also associated mutations in this gens with resistance to echinocandins (Jiménez-Ortigosa et al. 2017; E Silva et al. 2020). In addition, it has also been proposed that the protein Hsp90 has an important role in resistance to both azoles and echinocandins. This protein is involved in folding, transport, maturation, degradation of several other proteins and is usually more expressed in response to environmental stresses. In *A. fumigatus*, the inhibition of Hsp90 increased the susceptibility to echinocandins (Steinbach et al. 2006).

As previously said, no mechanism of resistance to the polyene

amphotericin B has been proposed in *A. fumigatus*. However, studies suggest its activity is attributed to oxidizing action and the increase in the activity of enzymes such as catalases and hyperoxide dismutase could confer resistance (Sharma and Chowdhary 2017).

In section *Fumigati*, cryptic species have been reported in clinics and studies have associated them with increased resistance to different antifungals (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014; S. Imbert et al. 2020). For instance, members of the species *A. lentulus*, *A. fumigatiaffinis*, *A. viridinutans*, and *A. fischeri* showed high MIC to azoles and some were also high to amphotericin B (Perlin, Rautemaa-Richardson, and Alastruey-Izquierdo 2017). Importantly, they have been increasingly reported (Gautier, Normand, and Ranque 2016). Recently, a multicentre study registered the prevalence between 21% and 18.8% of cryptic species among isolated *Aspergillus* spp. (Sebastien Imbert et al. 2021).

Although there is now information about possible mechanisms of azole resistance in *A. fumigatus*, this is not the case for the cryptic species. For instance, at the beginning of the 2010s insights were gained about the role of *cyp51A* in antifungal resistance in *A. lentulus* (Alcazar-Fuoli et al. 2011; Emilia Mellado et al. 2011). Recently, a study in Canada provided information about four strains with resistance to azoles, including one *A. fumigatus* and three *Fumigati* cryptic species: *A. thermomutatus, A. lentulus, and A. turcosus*. Particularly, these authors do not find evidence of changes in cyp51A linked to resistance, but showed evidence of efflux-pump-mediated resistance in an *A. turcosus* isolate (Parent-Michaud et al. 2020).

1.12 Hypotheses on the evolution of antifungal resistance in *A. fumigatus*

Evolutionary biology and treatment of infectious diseases are fields concerned with the problem of antifungal resistance. In the former, resistant phenotypes provide information to model how evolution of resistance occurs; in the latter, the same resistant phenotypes pose a challenge in human health (Vincent et al. 2013). In *A. fumigatus* and closely related species, models of evolution can be

developed mainly based on what is known about the resistance to azoles. On the other hand, almost no information is available for developing models of resistance to echinocandins or polyene. Moreover, compared to azoles and echinocandins, polyenes have been refractory to evolution of resistance and resistant strains remain rare (Vincent et al. 2013; Revie et al. 2018).

Fungi are efficient organisms when it comes to the adaptation to challenges in the environment, including antifungal agents (Hokken, Zwaan, et al. 2019). The evolution of resistance to antifungals has been studied from different perspectives, including (i) the adaptation to environment, (ii) the fungal stress responses and adjustments in its cellular physiology, (iii) the modes of fungal reproduction, including sexual (genetic reshuffling and recombination), asexual (spontaneous mutations), and parasexual, (iv) ploidy changes, (v) genetic instability, and (vi) population dynamics (Hokken, Zwaan, et al. 2019).

In a general view of pesticides, (Hawkins et al. 2018) reviewed evolutionary origins of resistance. Among them, *de novo* mutations originate under a given selective pressure (e.g., presence of an antifungal) and can be selectively advantageous, spreading through the population or arising independently in different populations. A different scenario would be the case of a standing variation: polymorphisms that were previously present in the pathogen, but that become more frequent under a selective pressure (e.g., presence of an antifungal) if they confer selective advantage (that was previously considered neutral or deleterious). Other cases involve intrinsic resistance, in which before exposure to a selective pressure (e.g., antifungal) the organism was already resistant due to factors previously fixed in the population. Differently from a *de novo* mutation that results in emergence of resistance, intrinsic resistance is a “pre-existing adaptation”. Alternatively, a pre-existing adaptation can also be co-opted as a resistance mechanism (e.g., overexpression of a gene under exposure to antifungal). Interspecies transfer, although most common in bacteria, has also been observed in fungi and could be important in emergence of antifungal resistance genes, even though no evidence has been found until now (Martínez-Matías and Rodríguez-Medina 2018).

Response to the environment when conditions are not ideal may precede the development of novel mutations that can confer (genetic) resistance. These initial responses conferring drug resilience are called adaptive ‘phenotypic plasticity’. Differential gene expression that results in a compensatory mechanism comprises one such example (Stern et al. 2007; Hokken, Zwaan, et al. 2019). Another factor that increases the chance of gaining antifungal resistance is the increased mutation frequencies and rates, which are higher in stressful conditions such as the exposure to an antifungal. Most mutations are usually detrimental, but some are likely to favor the survival and reproduction of the fungus on antifungal (Hokken, Zwaan, et al. 2019).

Hypermutator strains may also be important to the emergence of resistance, and have been reported in other important pathogens, such as *Candida glabrata* and *Cryptococcus neoformans*. Although studies on hypermutators are more common in yeasts (Hokken, Zwaan, et al. 2019), in *A. fumigatus* the study by (Dos Reis et al. 2018) showed that null mutant strains of two important kinases, *atrA* and *atmA*, more likely gained resistance to voriconazole. A more recent work by Dos Reis et al. (2019) studied the protein Msh2, which binds to DNA mismatches and initiates the mismatch repair and its influence on azole resistance (Dos Reis et al. 2019).

Other evolutionary works focus on the existence of phenomena that favor the resistance phenotype under stress. For instance, some of them are based on the hypothesis that prolonged exposure of fungi to antifungal activates stress responses, which support adaptations to drugs in the short and in the long-term. A traditional view of evolution would state that mutations occur randomly and change in conditions, like the exposure to an antifungal would favor mutations that fit under this condition. One alternative hypothesis states that in stress conditions, fungi change their mutation rates, modulating the generation of mutations and recombination. Therefore, these increased genetic instabilities would favor the fungi in stress conditions. Besides studies using mathematical modelling, experimental evidence exists of increased genomic instability in fungi under stress conditions (Shor and Perlin 2015).

Emergence of resistance to azoles is known to be linked to two routes: through patient treatment and through the environment, during exposure to fungicides in agriculture. In cases where the patient obtains spores of susceptible strains from the environment, selection can be observed in-patient under azole therapy. A common way to track microevolutionary mechanisms of acquired resistance in patients involves the isolation of multiple isogenic isolates from respiratory samples (Bellete et al. 2010; J. Chen et al. 2005; Chryssanthou 1997; Camps, van der Linden, et al. 2012). Another evidence that selection of azole resistance occurs in patients is the correlation that was observed between the time of exposure to itraconazole during therapy and the MIC recorded for isolated strains (Tashiro et al. 2012). Acquisition of *A. fumigatus* azole resistance in patients is also associated with the group of diseases and the type of reproduction, as reviewed by (Verweij, Zhang, et al. 2016). For instance, in patients with chronic cavitary pulmonary aspergillosis or aspergilloma, the lung cavity can harbor conidiophores, indicating asexual reproduction which favors resistance mutations (Zhang et al. 2015). Employing *in vitro* experiments, it was confirmed that resistance levels to azoles increase in sporulating compared to non-sporulating strains (Zhang et al. 2015). Differently, in patients with invasive aspergillosis these conditions are usually not observed (growth in tissue is through hyphal elongation), including the inability to undergo a similar reproductive mode and gain of resistance is usually not observed (Shalhoub et al. 2015).

Concerning the antifungal resistance in agriculture, fungicide classes outnumber the antifungals used in medical settings but azoles remain the most commonly used in clinical, crop protection, and livestock. Facts that raise concerns include the parallel drivers of resistance and evolution in clinics and agriculture, the dual usage of these antifungals in clinics and agriculture, where common pathogens live, including *A. fumigatus*, and the naïve movement of people that help spreading important pathogens (Fisher et al. 2018). Importantly, use of fungicides in agriculture comprises a selective pressure to the generation of resistant strains that cause human diseases, damaging even more the treatment of patients, because the

number of antifungal classes approved for use in humans is already very limited (Sanglard 2019).

Regarding the global distribution of azole-resistant strains, a recent work showed that *A. fumigatus* resistant strains had a lower genetic diversity compared to wild-type populations, supporting selective sweeps that followed beneficial mutations (Sewell et al. 2019).

How can these evolutionary studies be carried out in pathogens such as *A. fumigatus*? Factors that are important to fungal adaptation include the genetic diversity, population size, and selective pressures. Some of the studies are focused on the impact of the environment on the evolution of resistance; environments are called “hotspots” if they support growth, reproduction, and genetic variation would favor mutations that emerge (Schoustra et al. 2019).

1.13 Genomics and comparative genomics of the genus *Aspergillus*, including *A. fumigatus* and closely related cryptic species

Omics technologies, which include the global analyses of the DNA (genomics), transcripts (transcriptomics), proteins, (proteomics), metabolites (metabolomics), and others, have revolutionized the studies of fungal biology and have been reviewed in (Ball, Langille, and Geddes-McAlister 2020). For instance, these studies contribute to the diagnosis of fungal diseases. Genomics contribute to understanding of epidemiology of emergent and rare species, genes and variants related to virulence and antifungal resistance in these pathogens.

Concerning the fungal genomes, members of the genus *Aspergillus* are among the first to be sequenced, including *A. fumigatus* and *A. oryzae* (Galagan et al. 2005; Machida et al. 2005; Nierman et al. 2005). In *A. fumigatus*, the main reference genome is of strain Af293 (Nierman et al. 2005). Since then, several genomes of *Aspergillus* have been sequenced and this increased availability resulted in comparative genomics projects. For instance, in 2017 our group published a large comparative genomic study of *Aspergillus* that revealed both the high diversity that exists in the genus and several specificities (de Vries et al. 2017).

There are several bioinformatics methods able to characterize genomic variation in fungal pathogens, including the analysis of whole assembled genomes, functional prediction, and analyses of gene family expansion. In addition, single-base changes, like SNPs (substitutions or insertion/deletion), to study recombination, and Copy Number Variation (CNV) can also be studied (Farrer and Fisher 2017).

As described previously, where *Aspergillus* cryptic species were introduced, several markers have been employed in the taxonomy of *Aspergillus*, including ribosomal genes, the ribosomal internal transcribed spacers (ITS), markers corresponding to loci encoding proteins, such as calmodulin and β-tubulin. Genome-scale phylogenetics of *Aspergillus* and closely related species has improved our understanding of the relationships between *Aspergillus* species and their evolutionary relationships with other species in the same or in closely related genera (e.g., *Penicillium*) (Steenwyk et al. 2019). Detailed analysis of *Aspergillaceae* genomes allowed not only to infer the phylogenomic time-tree with estimates for the emergence of the genera (using “clock-like” evolutionary rates), but also to study in detail the phylogenetic incongruences, phylogenetic signal across the phylogeny, as well as clades that in which uncertainties exist. According to the phylogenomic tree in Steenwyk et al. (2019), *Fumigati* section appeared recently in evolution (18.8 mya).

Genomic analyses have been employed to understand resistance in isolates from patients. A study from Japan used genome sequencing of 17 *A. fumigatus* isolates from patients with pulmonary aspergilloma and chronic necrotizing pulmonary aspergillosis (Takahashi-Nakaguchi et al. 2015). They called SNPs for all the 17 strains (compared to *A. fumigatus* Af293) but did not find correlation between sequence typing and pathological conditions. Another study involving genomics and the in-host evolution of resistance was carried out (Ballard et al. 2018). They isolated several isogenic isolates from the same patient, who had chronic granulomatous disease, comprising strains susceptible and resistant to azoles, and sequenced their genomes. Ballard identified 248 SNPs, including non-synonymous mutations that may be important to adaptation to the host environment in *cyp51A* and in other genes. Like other works, they did not find a correlation between gain of resistance

and increased virulence.

To understand the emergence of resistance in *A. fumigatus*, Losada et al. used two isogenic strains, identical in their genome sequences except the mating locus. The pair was used for *in vitro* selection of resistance to multiple azoles (itraconazole, posaconazole, and voriconazole) (Losada et al. 2015). They identified several mutated genes, including *cyp51A* and beyond. Among non-*cyp51A* genes, they were able to confirm the role of a mutation identified in *hmg1* by generating mutants and successfully recapitulating the resistance phenotype, a gene in which all strains resistant to voriconazole had mutations.

One of the first works that used genomic data to study susceptibility and resistance to azoles in *A. fumigatus* was Camps et al (2012). These isolates did not have any mutation associated with the *cyp51A* gene and, therefore, was an opportunity to study non-*cyp51A* mutations (Camps, Dutilh, et al. 2012). The genomic analysis after several crosses including azole-resistant isolates resulted in a mutation in *hapE*, encoding for the CCAAT-binding factor complex subunit HapE, as having a strong link to resistance. Studying strains of *A. fumigatus* from India, UK, and the Netherlands with sequenced genomes, they analyzed SNPs and CNVs. They identified deleted genomic regions that included genes associated with antifungals. They also identified mutations in the *cyp51A* gene and were able to find some well-described mutations of resistant strains such as the L98H substitution and TR promoter region, as well as mutations associated with both susceptible and resistant strains. More recently, a study (Sharma et al. 2019) analyzed four *A. fumigatus* strains from India that were resistant to azoles. They found mutations in several non-cyp51A genes, including the *abcE* (most variation), encoding a transporter, synonymous and non-synonymous mutations in *hmg1* (previously mentioned). Mutations in the *erg3*, *erg24*, *srbA* genes were also recorded.

To gain insights into the resistance mechanisms gained by strains of *A. fumigatus* that were not associated with *cyp51A*, (Hagiwara et al. 2018) sequenced their whole genomes. They found mutations in *erg6* and in *hmg1*, encoding a sterol 24-C-methyltransferase and a hydroxymethyglutaryl-CoA (HMG-CoA), both included

in the ergosterol biosynthesis pathway and therefore likely to play a role in azole resistance.

Genomics has also helped to improve our understanding of sexual and clonal reproduction in *Aspergillus fumigatus* and its relationship with antifungal resistance. Interestingly, genomic analyses with tandem repeats (microsatellite markers) resulted in evidence of both clonal reproduction and recombination (Eta Ebasi Ashu et al. 2017). The same study showed that a local clonal population was associated with increased prevalence of triazole resistance.

Recently, the availability of genomes of *A. fumigatus* allowed the development of microbial GWAS - genome-wide association studies, with focus on antifungal resistance. Zhao et al studied Japanese populations of *A. fumigatus* and raised possible SNPs that could play a role in azole resistance, integrating gene expression from the literature (RNA-Seq) and including the validation of mutations in gene Afu2g02220 using CRISPR/Cas9 (S. Zhao et al. 2020).

As described in previous paragraphs, most studies that associated genomics with antifungal resistance have been carried out with strains of *A. fumigatus*. However, the genomes of cryptic species have also been sequenced recently and they contribute to understanding mechanisms of resistance in the whole section *Fumigati* (Toyotome et al. 2018). The genome of *A. udagawae* was sequenced in 2015 (Kusuya et al. 2015), a species for which strains causing diseases in humans and cats had also been reported with decreased susceptibility to drugs, including amphotericin B and azoles (Kusuya et al. 2015). (Parent-Michaud et al. 2019b) sequenced the genomes of the cryptic species *A. turcosus* in Canada from bronchoalveolar lavage samples, including one azole-susceptible and one azole-resistant strain. Two strains of *A. fischeri* (IBT 3003 and IBT 3007) have been sequenced (Shu Zhao, Latgé, and Gibbons 2019), a species considered an agent of food spoilage and, like *A. fumigatus*, an opportunistic human pathogen.

1.14 Studying adaptive evolution with availability of genomes

In the past, selection studies focused on specific loci of interest, but the increasing availability of sequenced genomes in different species has allowed researchers to study genome evolution using probabilistic models, the detection of regions with accelerated evolution, or to use codon models to study evolution at the gene, branch, or branch-site specificities (Kosiol and Anisimova 2019; Jeffares et al. 2015).

Selection can occur in synonymous mutations, such as in cases where some codons are suboptimal. However, it is known that this type of selection in most genes, and for most organisms, are usually very weak (Jeffares et al. 2015). In particular with regard to adaptive evolution in protein-coding genes, these analyses are important as they provide information about interactions that could have shaped the evolution of species in a clade (Sackton 2020). However, it is known that the patterns of evolution in DNA sequences are highly heterogeneous, which makes it necessary to distinguish the different regions of a coding sequence to create models of their evolution. One strategy to study adaptive evolution in protein-coding regions comprises the use of codon-based models that distinguish nonsynonymous (in which there is AA replacement) from the synonymous (silent). The ratio of amino acid substitutions (nonsynonymous) relative to neutral substitutions, dN/dS (or omega, ω) is used as a measure of selection. These models assume that the omega values will be above 1 in cases where a certain mutation is beneficial (thus, having a higher chance of being fixation in the species) (Sackton 2020; Jeffares et al. 2015; Kosiol and Anisimova 2019). Importantly, these analyses are usually done employing one-to-one orthologs, since the presence of paralogs (homologous sequences generated by gene duplication) complicate the interpretation of dN/dS (Jeffares et al. 2015).

The codon-based models are divided into three classes: (i) site models, which assume only a subset of sites undergo selections and capture codons under positive selection (each position has its own omega); (ii) branch models, that assume lineage-specific rates of evolution (each branch can have its own omega, but the values for genes are fixed); (iii) branch-site models, in which omega vary across lineages and codons (Sackton 2020).

Today, it is known that most positions in protein-coding genes do not undergo positive selection, whereas only a small portion of sites have and probably shaped the evolution of some lineages in a tree. Evidence of positive selection with codon-based models are obtained from running a Likelihood Ratio Test (LRT) which compares a model that does not allow positive selection versus another that does (Jeffares et al. 2015). If the LRT statistics are significant, then sites under positive selection can be predicted. This is done by checking the posterior probabilities (PP, usually > 0.95) to predict if a site belongs to the model's class with omega > 1 (Jeffares et al. 2015).

As far as we are aware, studies on the evolution of individual sites of antifungal resistance genes had not been previously studied in several sequenced *Aspergillus* spp of section *Fumigati*, including the cryptic species. In chapter 4 (see **Figure 2**), we exploit the genomes of all sequenced species of *Aspergillus* in section *Fumigati*, including several cryptic species, and analyze signatures of positive selection in sites of genes known to be involved in antifungal response and resistance.

1.15 Transcriptomics data for analysis of response of *Aspergillus fumigatus* to azoles, and potential of public datasets to discover novel players such as long noncoding RNAs

An overview of the potential of genomics and comparative genomics in fungal biology and their applications to study *A. fumigatus* and related species was presented previously. Differently, transcriptomic analyses capture the fingerprint of gene expression in a given time point. Studies with transcriptomics on fungal pathogens have contributed to understanding of the regulatory networks involved in interaction with hosts, over different developmental stages, the impact of environmental (including stressing) conditions, and usage of nutrients (Ball, Langille, and Geddes-McAlister 2020).

For instance, to understand the role of transcription factors NctA and NctB

in antifungal resistance in *A. fumigatus*, (Furukawa et al. 2020) analyzed RNA-Seq experiments with the null mutants for both genes (*nctA* and *nctB*) and were able to come up with the suggestion that these TFs act cooperatively. Interestingly, these TFs regulate several genes in the ergosterol and siderophore biosynthesis and are also responsible for the regulation of other regulators of ergosterol biosynthesis, including *srbA*, *atrR*, and *hapC*.

Although these studies used RNA-Seq data to understand the response to azoles, no study so far has explored the noncoding elements of the genome in *A. fumigatus*. Of relevance, studies involving transcriptomics with RNA-Seq have been increasingly available in the SRA public database (NCBI), but their use has been mostly limited to the initial publication (Doughty and Kerkhoven 2020). Even though it is still not exploited in filamentous fungi of importance in human health, the epigenetic mechanisms are starting to be considered in studies of fungal antifungal resistance (Chang et al. 2019). Noncoding RNAs have been linked to antifungal susceptibility in fungi, including the lncRNAs. Investigations in *Schizosaccharomyces pombe* showed that ncRNA.1343, a lncRNA, is involved in the nucleosome density in the promoter region of *tgp1*, a gene encoding Tgp1 (glycerophosphodiester transporter 1). Deleting this lncRNA resulted in a hypersensitive strain to several broad-spectrum drugs such as caffeine, thiabendazole, and hydroxyurea (Ard, Tong, and Allshire 2014). Recently, transposon disruption was employed in *Candida auris* and researchers were able to identify a novel lncRNA, *DINOR,* that plays an important role in stress tolerance, including to the antifungals caspofungin (echinocandin) and amphotericin B (Gao et al. 2021).

Given the similarities that exist between lncRNAs and mRNAs, the former elements can be easily detected using RNA-Seq projects. Several publications reported the use of bioinformatic pipelines to discover lncRNAs in ascomycetes (W. Kim et al. 2018; Bu et al. 2020) and basidiomycetes (Borgognone et al. 2019). Exploiting public datasets to discover novel lncRNAs in the *A. fumigatus* genome and their differential expression in conditions of interest, including exposure to azoles, comprises a promising way for novel discoveries in medical mycology with focus on

Aspergillus spp and drug resistance. In chapter 5, we exploit several RNA-Seq datasets for the purpose of studying the response of *A. fumigatus* to azoles (see **Figure 2**).

2 Article 1: Genomic and phenotypic heterogeneity of clinical isolates of the human pathogens *Aspergillus fumigatus*, *Aspergillus lentulus*, and *Aspergillus fumigatiaffinis*

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2.1 Summary

Fungal pathogens are a global threat to human health. For example, fungi from the genus *Aspergillus* cause a spectrum of diseases collectively known as aspergillosis. Most of the >200,000 life-threatening aspergillosis infections per year worldwide are caused by *Aspergillus fumigatus*. Recently, molecular typing techniques have revealed that aspergillosis can also be caused by organisms that are phenotypically similar to *A. fumigatus* but genetically distinct, such as *Aspergillus lentulus* and *Aspergillus fumigatiaffinis*. Importantly, some of these so-called cryptic species are thought to exhibit different virulence and drug susceptibility profiles than *A. fumigatus*, however, our understanding of their biology and pathogenic potential has been stymied by the lack of genome sequences and phenotypic profiling of multiple clinical strains. To fill this gap, we phenotypically characterized the virulence and drug susceptibility of 15 clinical strains of *A. fumigatus*, *A. lentulus*, and *A. fumigatiaffinis* from Spain and sequenced their genomes. We found heterogeneity in drug susceptibility across species and strains. We further found heterogeneity in virulence within each species but no significant differences in the virulence profiles between the three species. Genes known to influence drug susceptibility (*cyp51A* and *fks1*) vary in paralog number and sequence among these species and strains and correlate with differences in drug susceptibility. Similarly, genes known to be important for virulence in *A. fumigatus* showed variability in number of paralogs across strains and across species. Characterization of the genomic similarities and differences of clinical strains of *A. lentulus*, *A. fumigatiaffinis*, *and A. fumigatus* that vary in disease-relevant traits will advance our understanding of the variance in pathogenicity between *Aspergillus* species and strains that are collectively responsible for the vast majority of aspergillosis infections in humans.

2.2 Introduction

Aspergillosis is a major health problem, with rapidly evolving epidemiology and new groups of at-risk patients (Patterson et al. 2016). Aspergillosis infections are usually caused by inhalation of airborne asexual spores (conidia) of *Aspergillus fumigatus* and a few other *Aspergillus* species (Rokas et al. 2020). Aspergillosis covers a spectrum of diseases (Latgé and Chamilos 2019). For example, non-invasive diseases caused by *Aspergillus*, such as aspergilloma, are currently classified as chronic pulmonary aspergillosis and are commonly associated to pulmonary tuberculosis (David W. Denning et al. 2016). In atopic patients, the most severe form of aspergillosis is allergic bronchopulmonary aspergillosis (ABPA), which develops following sensibilization to *A. fumigatus* allergens in atopic patients with cystic fibrosis or individuals with genetic predisposition to ABPA (Agarwal et al. 2013). However, the most common invasive type of infection is invasive pulmonary aspergillosis (IPA), whose risk is significantly increased in immunocompromised individuals, in patients with acute leukemia and recipients of hematopoietic stem cells transplantation, or in solid-organ transplant recipients (Brown et al. 2012). Importantly, IPA has recently been described in new groups of traditionally low-risk patients, such as patients in intensive care units recovering from bacterial sepsis (Latgé and Chamilos 2019).

Although *A. fumigatus* is the major etiologic agent of aspergillosis, a few other *Aspergillus* species, such as *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, and *Aspergillus nidulans*, can also cause infections (Zakaria et al. 2020). While most of these pathogens can be phenotypically easily distinguished, infections can also be caused by *Aspergillus* species that are morphologically very similar to *A. fumigatus* (Rokas et al. 2020). These close pathogenic relatives of *A. fumigatus* are considered sibling species or cryptic species because they are undistinguishable from each other and from *A. fumigatus* by classical identification methods (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014); these species vary mostly in their colony growth, robustness of the production of conidia, conidial surface markings, presence and absence of septation in phialides, and maximum growth

temperatures (Balajee et al. 2005; Katz et al. 2005; J. W. Taylor et al. 2000). As a result of their near identical morphological characteristics, most of these cryptic species have only recently been described. For example, *Aspergillus lentulus* was first described in 2005 in a case of human aspergillosis (Balajee et al. 2005). Similarly, *A. fumigatiaffinis*, another pathogenic species that is closely related to *A. fumigatus*, was first described in 2005 (Hong et al. 2005). Even though cryptic species were only discovered relatively recently, understanding their genetic and phenotypic similarities and differences from the major pathogen *A. fumigatus* is important for two reasons. First, their prevalence in the clinic has been estimated to be between 11 and 19% (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014; Negri et al. 2014; Balajee et al. 2009). Second, several of these species, including *A. lentulus* and *A. fumigatiaffinis*, have been shown to differ in their drug susceptibility to amphotericin B and azoles compared to *A. fumigatus* (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014).

Antifungal resistance is of worldwide concern in human pathogenic *Aspergillus* species as well as in many other human, animal, and plant fungal pathogens (Parker et al. 2014; Sharma and Chowdhary 2017). Several antifungal-resistance mechanisms have been proposed in fungi (Pérez-Cantero et al. 2020; Sharma and Chowdhary 2017). In azole-resistant *Aspergillus* strains, known mechanisms are particularly well-described in genes of the cytochrome P450 sterol 14 α-demethylase family (*cyp51*), and include sequence variants in diverse positions of the Cyp51A protein sequence (e.g., G54, G138, M220, G448, Y121, P216, F219, A284, Y431, G432, and G434; reviewed in (Pérez-Cantero et al. 2020; Wei, Zhang, and Lu 2015), as well as combinations of the aforementioned protein sequence changes with tandem repeat (TR) variants in the promoter region, such as TR34/L98H or TR46/Y121F/T289A (reviewed in (Wei, Zhang, and Lu 2015)). Non-*cyp51* based mechanisms of antifungal resistance, such as in multidrug efflux pumps and pathways such as ergosterol biosynthesis and stress response, have also been proposed (Pérez-Cantero et al. 2020). Mechanisms of echinocandin resistance have mostly been attributed to FKS subunits of glucan synthase (Sharma and Chowdhary

2017). While most of these studies are in *Candida* species (Garcia-Effron et al. 2008; Desnos-Ollivier et al. 2008), a recent study in *A. fumigatus* also observed mutations associated with echinocandin resistance (Jiménez-Ortigosa et al. 2017).

An emerging realization in the study of *Aspergillus* pathogens is the presence of phenotypic heterogeneity among strains of the same species (Keller 2017). For example, recent studies have shown how variation in hypoxic growth phenotypes is associated with virulence among *A. fumigatus* strains (Kowalski et al. 2016, 2019). Similarly, *A. fumigatus* strains have previously been shown to exhibit great quantitative and qualitative heterogeneity in light response (Fuller et al. 2016); in this case, heterogeneity in light response was not associated with heterogeneity in virulence. Finally, (Ries et al. 2019) found a high heterogeneity among *A. fumigatus* strains with regard to nitrogen acquisition and metabolism during infection and correlation between nitrogen catabolite repression-related protease secretion and virulence. These studies highlight the biological and clinical relevance of understanding strain heterogeneity in *Aspergillus* pathogens, especially with respect to virulence and antifungal drug susceptibility. However, comparisons of strain heterogeneity in virulence and drug resistance profiles among clinical strains in *A. fumigatus* and closely related cryptic species, such as *A. lentulus* and *A. fumigatiaffinis*, are lacking.

To address this gap in the field, we phenotypically characterized and sequenced the genomes of 15 clinical strains of *A. fumigatus*, *A. lentulus*, and *A. fumigatiaffinis* from Spain. At the phenotypic level, we found strain heterogeneity in both virulence and drug susceptibility profiles within each species as well as differences in drug susceptibility profiles between the three species. Interestingly, we found that the virulence profiles of the three species were similar. At the genomic level, we found that gene families known to influence drug susceptibility, such as *cyp51*, exhibit variation in their numbers of paralogs and sequence among these species and strains. Similarly, we found variability in the number of paralogs within and between species in many genes known to be important for virulence in *A. fumigatus*. Characterization of the genomic similarities and differences of clinical

strains of *A. lentulus*, *A. fumigatiaffinis*, *and A. fumigatus* that vary in disease-relevant traits will advance our understanding of the variation in pathogenicity between *Aspergillus* species and strains that are collectively responsible for the vast majority of aspergillosis infections in humans.

2.3 Materials and methods

2.3.1 Strains and Species Identification

To understand the degree of genomic heterogeneity among strains, we sequenced six clinical strains of *A. fumigatus*, five of *A. lentulus*, and four of *A. fumigatiaffinis* available in the Mycology Reference Laboratory of the National Center for Microbiology (CNM) in Instituto de Salud Carlos III in Spain (Supplementary Table S1). For initial species identification, we sequenced the Internal Transcribed Spacer region (ITS) and β-tubulin (*benA*) gene amplicons (primer pairs in Supplementary Table S2). We downloaded reference sequences for the type strains of *A. fumigatiaffinis* IBT12703 and *A. lentulus* IFM54703, and of *Aspergillus clavatus* NRRL1 (section *Clavati*), which we used as the outgroup. We aligned DNA sequences with MAFFT v.7.397 (Katoh and Standley 2013), followed by model selection and phylogenetic inference in IQ-TREE v.1.6.7 (Nguyen et al. 2015).

2.3.2 Characterization of Virulence and Antifungal Susceptibility Profiles

To understand the pathogenic potential of the 15 clinical strains, we carried out virulence assays using the moth *Galleria mellonella* model of fungal disease (Slater et al. 2011; Fuchs et al. 2010). Briefly, we obtained moth larvae by breeding adult moths that were kept for 24 h prior to infection under starvation, in the dark, and at a temperature of 37°C. We selected only larvae that were in the sixth and final stage of larval development. We harvested fresh asexual spores (conidia) from each strain from yeast extract-agar-glucose (YAG) plates in PBS solution and filtered through a Miracloth (Calbiochem). For each strain, we counted the spores using a hemocytometer and created a 2×10^8 conidia/ml stock suspension. We

determined the viability of the administered inoculum by plating a serial dilution of the conidia on YAG medium at 37°C. We inoculated 5 µl (1×10^6 conidia/larvae) to each larva (n = 10). We used as the control a group composed of larvae inoculated with 5 µl of PBS. We performed inoculations via the last left proleg using a Hamilton syringe (7000.5KH). After infection, we maintained the larvae in petri dishes at 37°C in the dark and scored them daily (i.e., recorded the number of dead larvae each day) during a 10-day period. We considered larvae that did not move in response to touch as dead.

We tested the virulence of each clinical strain by infecting 10 larvae, i.e., for each strain tested we have one experimental replicate with an sample size n of 10. We performed two sets of analyses. First, we statistically assessed if the survival curves of different strains in a given species are identical (null hypothesis of strain homogeneity) or different (alternative hypothesis of strain heterogeneity). Second, we used strains within each species as “biological replicates” and statistically assessed if the survival curves between species were similar or different. We performed these statistical assessments using the log-rank test implemented in the survival R package (Therneau, 2014), followed by multiple test correction of p-values (Benjamini and Hochberg). Scripts used to perform these analyses are available on the GitLab repository[1] under ‘experimentalData/’.

To measure the antifungal susceptibility of the clinical strains, we applied the EUCAST (European Committee for Antimicrobial Susceptibility Testing) reference microdilution method version 9.3.1 (Arendrup et al., 2017), in which fungi are grown on plates with increasing concentrations of antifungals and the first concentration in which fungal growth is inhibited (MIC) is recorded. For all strains, we tested their susceptibility to four antifungal drug classes: (a) Polyenes: amphotericin B (Sigma-Aldrich Quimica, Madrid, Spain); (b) Azoles: itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer SA, Madrid, Spain), and posaconazole (Schering-Plough Research Institute, Kenilworth, NJ, United States); (c) Echinocandins: caspofungin (Merck & Co. Inc., Rahway, NJ, United States), micafungin (Astellas Pharma Inc., Tokyo, Japan), and anidulafungin (Pfizer SA,

Madrid, Spain); and (d) Allylamines: Terbinafine (Novartis, Basel, Switzerland). The final concentrations tested ranged from 0.03 to 16 mg/L for amphotericin B, terbinafine, and caspofungin; from 0.015 to 8 mg/L for itraconazole, voriconazole and posaconazole; from 0.007 to 4 mg/L for anidulafungin; and from 0.004 to 2 mg/L for micafungin. *A. flavus* ATCC 204304 and *A. fumigatus* ATCC 204305 were used as quality control strains in all tests performed. MICs for amphotericin B, itraconazole, voriconazole, posaconazole, and terbinafine, and minimal effective concentrations (MECs) for anidulafungin, caspofungin, and micafungin were visually read after 24 and 48 h of incubation at 35°C in a humid atmosphere. To assess the relationship between antifungal susceptibility and strain/species identification, we carried out principal component analysis (PCA) with scaled MIC/MEC values with the R package FactoMineR (Lê, Josse, and Husson 2008), and data visualization with the factoextra v.1.0.6 package. Scripts used to perform these analyses are available on the GitLab repository (https://gitlab.com/SantosRAC/Santosetal2021_evolutionGenesAntifungalsFumigati) under 'experimentalData/'.

2.3.3 Genome Sequencing

To understand the genomic similarities and differences within and between these pathogenic *Aspergillus* species and how they are associated with differences in drug susceptibility and virulence profiles, we sequenced the genomes of all 15 strains. Each strain was grown in glucose-yeast extract-peptone (GYEP) liquid medium (0.3% yeast extract and 1% peptone; Difco, Soria Melguizo) with 2% glucose (Sigma-Aldrich, Spain) for 24 h to 48 h at 30°C. After mechanical disruption of the mycelium by vortex mixing with glass beads, genomic DNA of isolates was extracted using the phenol–chloroform method (Holden, 1994). The preparation of DNA libraries was performed using the Nextera® TM DNA Library PrepKit (Illumina Inc., San Diego, CA, United States) according to manufacturer's guidelines. DNA quantification was carried out using the QuantiFluor® dsDNA System and the QuantiFluor® ST Fluorometer (Promega, Madison, WI, United States) and its quality

was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, United States). Sequencing was performed in the Illumina platform NextSeq500, following the manufacturer's protocols (Illumina Inc., San Diego, CA, United States). We performed an initial quality analysis of the sequence reads using FastQC, v.0.11.7[2]. We inspected sequence reads for contaminants using BLAST (Altschul et al. 1990) and MEGAN5 (Huson and Weber 2013). We trimmed low quality bases (LEADING = 3; TRAILING = 3; SLIDINGWINDOW: windowSize = 4 and requiredQuality = 15), removing both short sequences (<90 bp) and Nextera adaptors, with Trimmomatic v.0.38 (Bolger, Lohse, and Usadel 2014).

2.3.4 Genome Assembly and Annotation

We assembled the genomes of all strains with SPAdes v3.12.0 (Bankevich et al. 2012). We corrected bases, fixed mis-assemblies, and filled gaps with Pilon, v.1.22 (Walker et al. 2014). We assessed genome assembly quality using QUAST, v.4.6.3 (Gurevich et al. 2013). We assessed genome assembly completeness using Benchmarking Universal Single-Copy Orthologs (BUSCO) (Simão et al. 2015) and the 4,046 Eurotiomycetes BUSCO gene set (genes from OrthoDB that are thought to be universally single copy). We carried out gene prediction with AUGUSTUS v.3.3.1 (Stanke et al. 2004) using the gene models of *A. fumigatus* Af293 strain (Nierman et al. 2005) as reference. We carried out functional annotation with InterProScan 5.34-73.0 (Jones et al. 2014).

2.3.5 Orthogroup Identification

To identify orthologs (and closely related paralogs) across strains, we performed all-vs.-all searches with blastp 2.7.1+ (Altschul et al. 1990) using the strains' predicted proteomes. We used OrthoFinder v.2.3.3 (Emms and Kelly 2019) to generate orthogroups using pre-computed BLAST results (-og option) and a Markov Clustering (MCL) inflation value of 1.5. We considered an orthogroup "species-specific" if it possessed one or more protein sequences from only one species. Information on performing these analyses is available on the GitLab wiki page

‘orthology-calling’.

2.3.6 Identification of Single Nucleotide Polymorphisms and Insertions/Deletions

To characterize genetic variation within and between the three pathogenic *Aspergillus* species, we assessed single nucleotide polymorphisms (SNPs) and insertions/deletions (indels). We used BWA-MEM v.0.7.17 (Heng Li and Durbin 2009) with default parameters to map reads to the reference genome sequences for *A. fumigatus*, *A. lentulus*, and *A. fumigatiaffinis* (CNM-CM8686, CNM-CM7927, and CNM-CM6805, respectively). We did not use type strains as reference genomes for the species under study, because they are not from Spain. Duplicate reads were identified using PICARD MarkDuplicates, v.2.9.2. We indexed genomes using SAMTOOLS v.1.8 (Heng Li et al. 2009) for subsequent variant detection analyses.

We used GenomeAnalysisTK (GATK) v.3.6 for SNP calling with the recommended hard filtering parameters (McKenna et al. 2010; DePristo et al. 2011). We used SnpEff v.4.3t (Cingolani et al. 2012) to annotate and predict the functional effect of SNPs and indels. Variants assumed to have high (disruptive) impact in the protein, probably causing protein truncation, loss of function or triggering nonsense mediated decay were classified as “high,” variants assumed that might change protein effectiveness but were non-disruptive were classified as “moderate,” and variants most likely to be harmless or unlikely to change protein behavior were classified as “low.” Finally, non-coding variants or variants affecting non-coding genes, where predictions are difficult or there is no evidence of impact, were classified as “modifier”. Details can be found on the SnpEff manual.

We aligned protein and coding sequences for genes of interest with MAFFT v.7.397 (Katoh and Standley 2013), using the –auto mode. We used Jalview v.2.10.3 (A. M. Waterhouse et al. 2009) to visualize SNPs, and a Python script to recover non-synonymous mutations compared to the reference, *A. fumigatus* A1163. Enrichment analysis of GO terms in genes with high impact SNPs and indels for each species was carried out with GOATOOLS v.0.9.9 (Klopfenstein et al. 2018). Scripts

used to perform these analyses are available on the GitLab repository (see text footnote 1) under ‘genomePolymorphisms/’ and ‘goatools/’.

2.3.7 Genetic Determinants Important for Virulence

To examine whether SNPs, indels, and number of paralogs in a given orthogroup were associated with virulence, we recovered 215 genes in *A. fumigatus* Af293 considered genetic determinants of virulence based on their presence in PHI-base (Winnenburg et al. 2006) and in previously published studies (Abad et al. 2010; Kjærbølling et al. 2018). We obtained functional annotation of these virulence-related genes from FungiDB (Basenko et al. 2018).

2.3.8 Maximum-Likelihood Phylogenomics

To reconstruct the evolutionary history of our 15 strains and closely related *Aspergillus* species, we first downloaded or assembled genomes of other strains of the three pathogenic species or their closely relatives that are publicly available. Specifically, we downloaded the genomes of *Aspergillus novofumigatus* IBT16806 (Kjærbølling et al. 2018), *Aspergillus lentulus* IFM 54703^{T} (Kusuya et al. 2016), *Aspergillus fischeri* NRRL181 (Fedorova et al. 2008), *Aspergillus udagawae* IFM46973 (Kusuya et al. 2015), and *Aspergillus viridinutans* FRR_0576 (GenBank accession: GCA_004368095.1). To ensure our analyses also captured the genetic diversity of *A. fumigatus*, we also included additional *A. fumigatus* genomes that spanned the known diversity of *A. fumigatus* strains (Lind et al. 2017). Specifically, we downloaded the genomes of *A. fumigatus* A1163 (Fedorova et al. 2008) and *A. fumigatus* Af293 (Nierman et al. 2005). Additionally, we obtained the raw reads of *A. fumigatus* strains 12-750544 and F16311 (SRA accessions: SRR617737 and ERR769500, respectively). To assemble these genomes, we first quality-trimmed the sequence reads using Trimmomatic, v0.36 (Bolger, Lohse, and Usadel 2014) using parameters described elsewhere (leading:10, trailing:10, slidingwindow:4:20, and minlen:50). The resulting quality-trimmed reads were then used for genome assembly using SPAdes, v3.8.1 (Bankevich et al. 2012), using the ‘careful’ parameter

and the ‘cov-cutoff’ parameter set to ‘auto.’ Altogether, we analyzed a total of 24 genomes.

To identify single-copy orthologous genes among the 24 genomes, we implemented the BUSCO, v.2.0.1 pipeline (R. M. Waterhouse et al. 2013; Simão et al. 2015). Specifically, we used the BUSCO pipeline to identify single-copy orthologous genes from genomes using the Eurotiomycetes database of 4,046 orthologs from OrthoDB, v9 (R. M. Waterhouse et al. 2013). Among the 4,096 orthologs, we identified 3,954 orthologs with at least 18 taxa represented and aligned the protein sequence each ortholog individually using Mafft, v7.294b (Katoh and Standley 2013), with the same parameters as described elsewhere (Steenwyk et al. 2019). We then forced nucleotide sequences onto the protein alignment with a custom Python, v3.5.2 script (indicated on the Gitlab repository README.md file) using BioPython, v1.7 (Cock et al. 2009). The resulting nucleotide alignments were trimmed using trimAl, v1.4 (Capella-Gutiérrez, Silla-Martínez, and Gabaldón 2009), with the ‘gappyout’ parameter. The trimmed alignments were then concatenated into a single matrix with 7,147,728 sites. We then used the concatenated data matrix as input into IQ-TREE, v1.6.11 (Nguyen et al. 2015), with the ‘nbest’ parameter set to 10. The best-fitting model of substitutions was automatically determined using the Bayesian information criterion. The best-fitting model was a general time general time-reversible model with empirical base frequencies, a discrete Gamma model with 4 rate categories, and a proportion of invariable sites (GTR+I+F+G4) (Tavaré, 1986) (Vinet and Zhedanov 2011; Z. Yang 1994, 1996). Lastly, we evaluated bipartition support using 5,000 ultrafast bootstrap approximations (Hoang et al. 2018).

In order to build the phylogeny with Cyp51 paralogs, we recovered protein sequences from two orthogroups that included Cyp51A and Cyp51B from *A. fumigatus* Af293 (Afu4g06890 and Afu7g03740, respectively). We generated a maximum-likelihood phylogeny in IQ-Tree v. 1.6.12 (Nguyen et al. 2015), using 1000 Ultrafast Bootstrap Approximation (UFBoot) replicates. The LG+G4 model was chosen as the best according to Bayesian Information Criterion. The protein sequences and tree files are available on the GitLab repository (see text footnote 1)

under ‘AntifungalGenes/’.

2.4 Results

2.4.1 Clinical Strains Show Varying Antifungal Drug Susceptibility

To study susceptibility to antifungals across all strains of the three *Aspergillus* pathogens, we employed the EUCAST reference microdilution method with the four different known classes of antifungal drugs (**Table 1**). By performing PCA on the antifungal drug susceptibility values of all 15 strains, we found that the strains exhibited high heterogeneity in their drug resistance profiles (**Figure 3A**). In many cases, we found that strains from different species were more similar to each other (e.g., strain CNM-CM8686 from *A. fumigatus* with strain CNM-CM6069 from *A. lentulus*) than to other strains from the same species (e.g., strain CNM-CM8686 with strain CNM-CM8057 from *A. fumigatus*), highlighting the magnitude of heterogeneity in drug susceptibility of these species and strains. Principal component 1 (PC1) explained 37.2% of the variation and separated almost all *A. fumigatus* strains from those of the other two species. Principal component 2 (PC2) explained 21% of the variation, but did not separate species. The individual contributions of each antifungal drug to each PC are shown in Supplementary Figure S1. Finally, we found that the susceptibility of amphotericin B (in the polyenes class) was negatively correlated with micafungin (echinocandins) and terbinafine (allylamines), whereas anidulafungin (echinocandins) and voriconazole (azoles) were positively correlated (Supplementary Figure S2). Interestingly, the drugs exhibiting these negative or positive correlations are from different classes (e.g., polyenes *versus* allylamines or echinocandins *versus* azoles).

Table 1. Susceptibility profile of cryptic *Aspergillus* species isolated in the Mycology Reference Laboratory of Spain.

Species	Strain identifier	MIC (mg/L)				MEC (mg/L)			
		AMB	***ICZ***	***VCZ***	***PCZ***	***TRB***	***CPF***	***MCF***	***AND***
Aspergillus lentulus	CNM-CM6069	8	0.5	2	0.12	0.5	1	0.015	0.015
	CNM-CM6936	16	0.5	4	0.25	2	2	0.03	0.03
	CNM-CM7927	8	0.5	2	0.12	0.5	0.06	0.015	0.007
	CNM-CM8060	0.12	0.25	1	0.12	0.5	2	0.06	0.03
	CNM-CM8694	2	0.12	0.25	0.06	32	0.03	0.03	MD
	CNM-CM8927	16	2	1	0.25	2	0.25	0.015	0.015
Aspergillus fumigatiaffinis	CNM-CM5878	1	0.25	0.5	0.06	0.25	1	0.03	0.015
	CNM-CM6457	16	16	2	0.25	1	0.25	0.03	0.007
	CNM-CM6805	16	0.25	2	0.12	0.25	0.5	0.03	0.03
	CNM-CM8980	16	0.5	2	0.5	0.5	0.12	0.007	0.015
Aspergillus fumigatus	CNM-CM8057	0.25	>8	>8	1	16	0.5	0.06	0.12
	CNM-CM8714	0.25	>8	4	1	4	0.25	0.007	0.03
	CNM-CM8812	0.25	0.25	0.5	0.12	1	0.25	0.03	0.03
	CNM-CM8686	0.5	0.25	0.25	0.12	2	0.25	0.015	0.015
	CNM-CM8689	1	1	8	0.25	16	0.5	0.125	0.03
	Af293	0.5	1	1	0.125	2	0.125	0.007	0.007
One-way ANOVA (between species)	*P*-value	0.025*	0.435	0.209	0.171	0.492	0.364	0.462	0.242
Tukey multiple comparisons of means	*Aspergillus fumigatus – Aspergillus fumigatiaffinis*	0.0245507*	–	–	–	–	–	–	
	Aspergillus lentulus - Aspergillus fumigatiaffinis	0.5595621	–	–	–	–	–	–	
	Aspergillus lentulus - Aspergillus fumigatus	0.0982057	–	–	–	–	–	–	

*AMB, amphotericin B; ICZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; CPF, caspofungin; MCF, micafungin; AND, anidulafungin; TRB, terbinafine. *P-values < 0.05 were considered significant.*

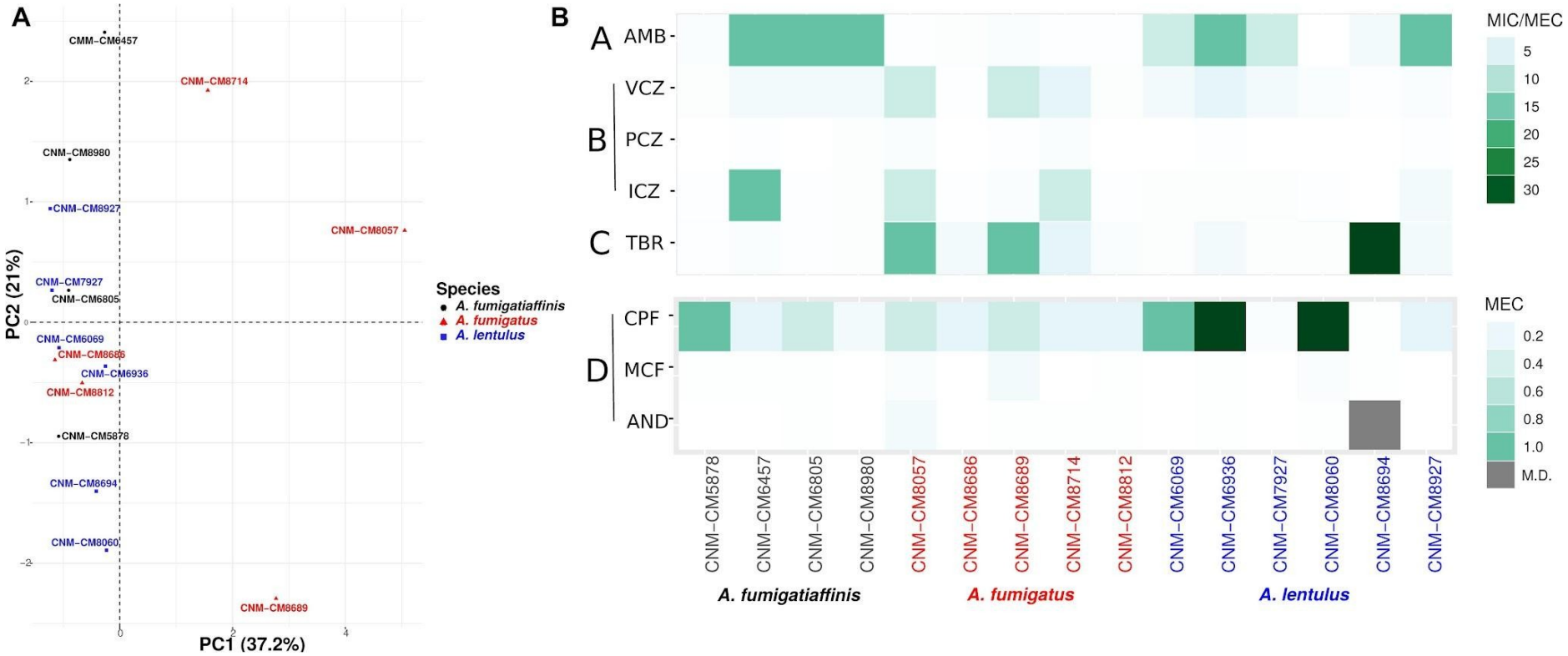

Figure 3. High heterogeneity in drug susceptibility profiles among Spanish strains of three closely related *Aspergillus* pathogens. **(A)** Principal component analysis (PCA) carried out for antifungal susceptibility testing. PC1 (Dim1) explains most of the variation (37.2% of the variation) and is able to separate *A. fumigatus* from other two species, whereas an overlap is observed in cryptic species (*A. lentulus* and *A. fumigatiaffinis*). **(B)** Antifungal susceptibility testing was carried out using the EUCAST reference microdilution method. The minimum inhibitory concentration (MIC) was obtained for AMB, VCZ, PCZ, and ICZ and the minimum effective concentration (MEC) was obtained for TRB, CPF, MCF, and AND. A lower scale is shown for echinocandins (bottom panel). Antifungal classes are A: polyenes; B: azoles; C: allylamines; D: echinocandins. AMB, amphotericin B; ICZ, itraconazole; VCZ, voriconazole; PCZ, posaconazole; CPF, caspofungin; MCF, micafungin; AND, anidulafungin; TRB, terbinafine.

We also looked at the differences in susceptibility between strains for each antifungal drug (**Figure 3B**). Our data show that clinical strains of *A. fumigatus* exhibit lower MICs to amphotericin B compared to *A. lentulus* and *A. fumigatiaffinis*, albeit different levels are observed among different strains (one-way ANOVA; $\alpha < 0.05$; Tukey multiple comparisons of means for amphotericin B) (**Table 1**). With the exception of susceptibility of *A. fumigatus* and *A. lentulus* to amphotericin B, for which a significant difference is observed between these two species, we observed high heterogeneity among strains of different species for the other drugs **(Table 1**). Among azoles, itraconazole and voriconazole displayed higher levels of variability across strains. With respect to terbinafine, the four *A. fumigatiaffinis* strains exhibited low MICs, whereas four *A. fumigatus* strains displayed higher MICs (MIC values >1 mg/L) and the other two *A. fumigatus* strains even higher; finally, one *A. lentulus* strain (CNM-CM8694) displayed the highest MICs across all strains (albeit other strains showed in general lower MICs). Among echinocandin drugs, caspofungin showed high MECs for the three species. In particular, one strain of *A. fumigatiaffinis* and three of *A. lentulus* were notable in exhibiting very high MECs (MECs ≥ 1 mg/L). MECs for micafungin and anidulafungin were low (≤0.125 mg/L) for all strains.

2.4.2 Clinical Strains Within Each Species Show Varying Levels of Virulence

Given functional similarities of the greater wax moth *Galleria mellonella* innate immune system with that of mammals, and prior work showing that moth larvae and mice exhibit similar survival rates when infected with *A. fumigatus* (Slater et al. 2011; Mead et al. 2019), we infected *G. mellonella* larvae with all 15 strains to assess their virulence profiles (**Figure 4**).

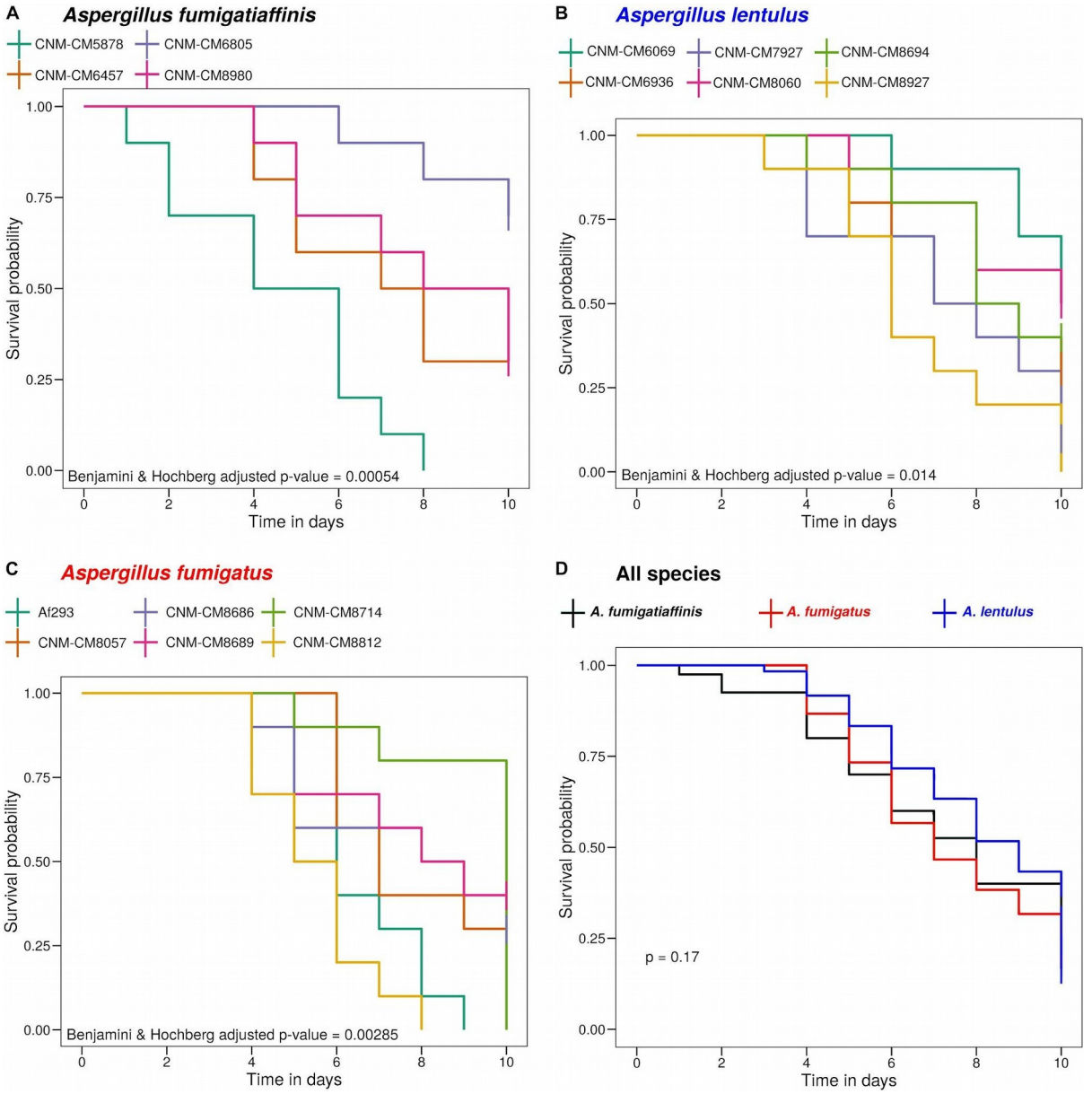

Figure 4. High heterogeneity of virulence levels among Spanish strains of three closely related *Aspergillus* pathogens. We found significant heterogeneity in the survival curves between strains within *A. fumigatiaffinis* **(A)**, *A. lentulus* **(B)**, and *A. fumigatus* **(C)** (Benjamini and Hochberg adjusted *p*-values using the log-rank test are shown). We also tested differences between species (considering each strain as a biological replicate), and found that the virulence profiles of Spanish strains of all three species are not significantly different **(D)** (*p*-value using the log-rank test is shown).

Survival curves revealed high heterogeneity in virulence across clinical strains within each of the three species (**Figure 4**). We observed highly virulent strains for which all ten larvae were dead at day 10, such as *A. fumigatus* Af293 (one of our reference strains), *A. fumigatiaffinis* CNM-CM5878 and *A. lentulus* CNM-CM8927. In contrast, other strains were less virulent and >25% larvae survived to the last day of data collection, such as *A. lentulus* CNM-CM6069 and CNM-CM8060.

Moreover, we found significant heterogeneity in the survival curves between strains within each species (Benjamini and Hochberg adjusted *p*-values: 0.00285 in *A. fumigatus*, 0.00054 in *A. fumigatiaffinis*, and 0.014 in *A. lentulus*; log-rank test) (**Figures 4A–C**). We also tested differences between species (considering each strain as a biological replicate), and observed no significant difference between the kill curves of the various species (*p*-value = 0.17; log-rank test) – that is, we found that both *A. lentulus* and *A. fumigatiaffinis* were as virulent as *A. fumigatus* (**Figure 4D**).

2.4.3 Genomic Variation Within and Between Spanish Strains of *A. fumigatus*, *A. lentulus*, and *A. fumigatiaffinis*

To begin exploring the potential genetic underpinnings of species and strain variation in drug susceptibility and virulence, we conducted comparative genomic analyses. The genomes of all 15 strains were of high quality and contained 97–98% of expected complete and single-copy BUSCOs (Supplementary Table S3). *A. lentulus* and *A. fumigatiaffinis* genomes had larger gene repertoires (9,717–9,842 and 10,329–10,677, respectively) than *A. fumigatus* (8,837–8,938), consistent with previous genome studies of *A. lentulus* and *A. fumigatus* (Nierman et al. 2005; Fedorova et al. 2008; Kusuya et al. 2016). A genome-scale phylogenetic analysis using the nucleotide sequences of BUSCOs with previously sequenced strains (**Figure 5A**) supports the close relationship between *A. lentulus* and *A. fumigatiaffinis*.

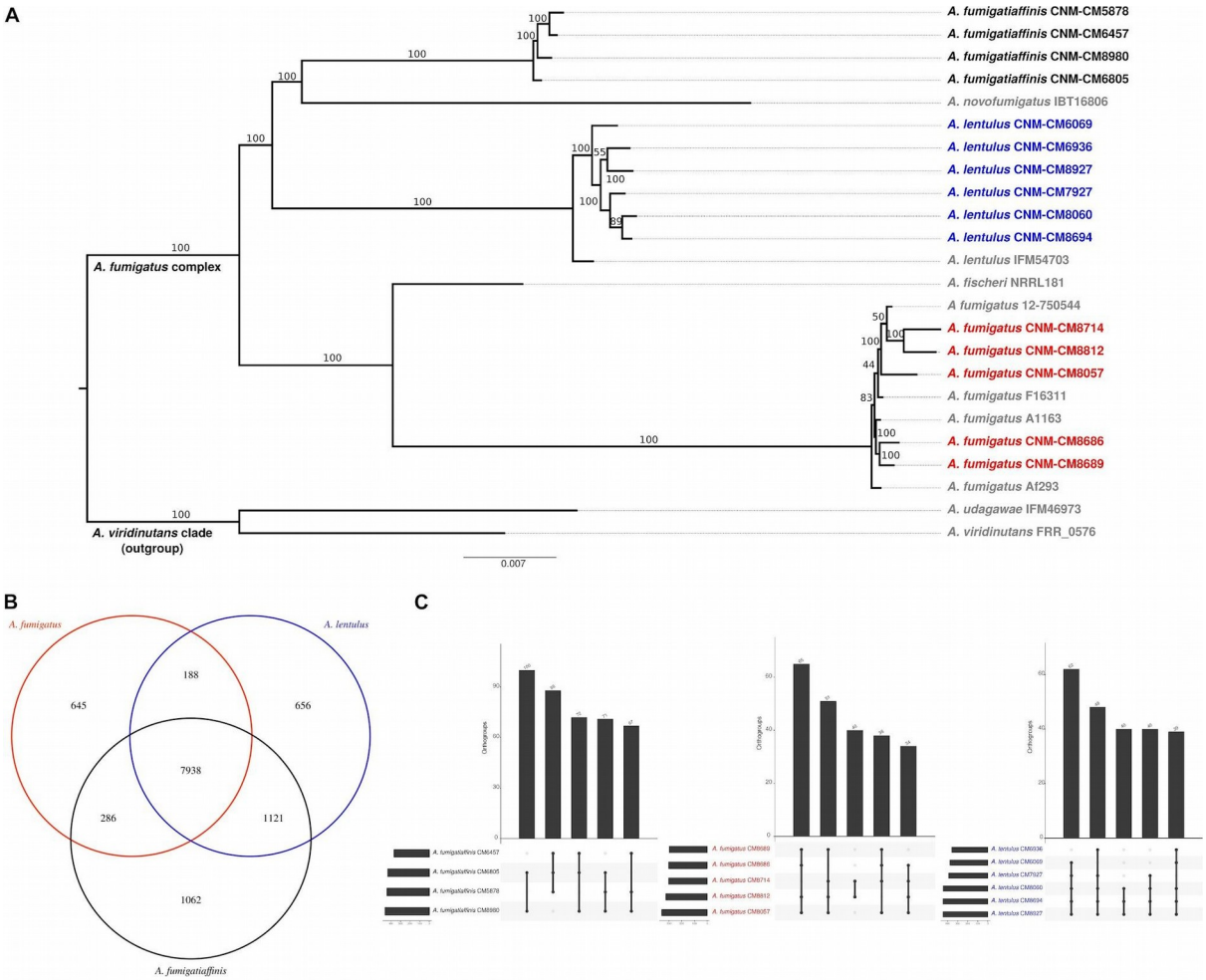

Figure 5. Genomics of the three closely related *Aspergillus* pathogens. **(A)** Genome-scale phylogeny of the section *Fumigati* species used in this study and additional species with sequenced genomes. The *A. viridinutans* clade is presented as a sister clade. Spanish strains sequenced in this work are colored in red (*A. fumigatus*), blue (*A. lentulus*) and black (*A. fumigatiaffinis*). The newly sequenced *A. fumigatiaffinis* strains form a separated group that is closely related to *A. novofumigatus*. All *A. lentulus* strains in this work group together and share an ancestor with *A. lentulus* IFM54703, the only sequenced strain in this species to date. The *A. fumigatus* strains sequenced in this work form different internal groups in the clade with other strains in the species (e.g., strains CNM-CM8714 and CNM-CM8812 group together and strains CNM-CM8686 and CNM-CM8689 form another group). **(B)** *A. fumigatiaffinis* and *A. lentulus* shares the highest number of common orthogroups and *A. fumigatiaffinis* displays the highest number of species-specific orthogroups. We considered species-specific orthologs those that were present in at least one strain of a given species, with no representative from another species. **(C)** Orthogroups shared by all and "all but one" strains are the most frequent in three closely related *Aspergillus* pathogens. *A. lentulus*, *A. fumigatus*, and *A. fumigatiaffinis* have 9,008, 8,321, and 9,423 orthologous genes present in all strains, respectively. The five largest combinations of orthogroups are shown. As expected, the most frequent combination of orthogroups are those in all strains but one.

2.4.4 Genome Diversity Among and Within Species Across Clinical Strains

Examination of orthogroups across the 15 strains and three species revealed that most genes (7,938) are shared by all three species (**Figure 5B**). *A. fumigatiaffinis* has a larger set of species-specific genes (1,062) than *A. lentulus* (656) or *A. fumigatus* (645), consistent with its larger genome size and gene number. The numbers of shared genes between *A. lentulus* and *A. fumigatiaffinis* are also higher than intersections between each of them with *A. fumigatus*, consistent with their closer evolutionary relationship (**Figure 5A**). Within each species, most orthogroups are found in all strains (9,008, 8,321, and 9,423 in *A. lentulus*, *A. fumigatus*, and *A. fumigatiaffinis*, respectively); approximately 5.4–6.13% of genes in each species appear to vary in their presence between strains (Supplementary Figure S3). Among these, we noted that orthogroups that are present all but one strain are usually the most frequent (**Figure 5C**).

We identified a total of 114,378, 160,194, and 313,029 SNPs in *A. fumigatus*, *A. fumigatiaffinis*, and *A. lentulus*, respectively. We identified 406, 493, and 747 SNPs in *A. fumigatus*, *A. fumigatiaffinis*, and *A. lentulus*, respectively, as high-impact polymorphisms; these polymorphisms are those whose mutation is presumed to be highly deleterious to protein function. Similarly, out of a total of 11,698 (*A. fumigatus*), 20,135 (*A. fumigatiaffinis*) and 34,506 (*A. lentulus*) indels segregating within each species, we identified 615, 1,739, and 1,830 high-impact indels in *A. fumigatus*, *A. fumigatiaffinis*, and *A. lentulus*, respectively.

Gene ontology (GO) enrichment analysis was carried out for genes with high impact SNPs and indels ($\alpha = 0.05$). *A. fumigatus* only showed GO terms identified as underrepresented in “cellular process” and several cellular compartments (“protein-containing complex,” “intracellular organelle part,” “organelle part,” “cytoplasmic part,” “cell part”). *A. lentulus* had “nucleoside metabolic” and “glycosyl compound metabolic processes” enriched, and *A. fumigatiaffinis* showed enriched terms for “modified amino acid binding,” “phosphopantetheine binding,” “amide binding,” “transition metal ion binding,” “zinc ion binding,” “chitin binding,” and “ADP binding.” *A. lentulus* and *A. fumigatiaffinis* genes with high impact SNPs and indels also showed underrepresented GO terms (Supplementary Table S4). We also analyzed SNPs and indels separately (Supplementary Table S4).

2.4.5 Polymorphisms in Major Antifungal Target Genes Correlate With

Antifungal Susceptibility

Given the observed variation within and between species in antifungal drug susceptibility, we examined DNA sequence polymorphisms in genes known to be involved in antifungal susceptibility to azoles and echinocandins. In particular, we examined patterns of sequence variation in the 14α-sterol demethylase gene *cyp51A* (Afu4g06890) and in the β-1,3-glucan synthase catalytic subunit gene *fks1* (Afu6g12400). Using *A. fumigatus* A1163 as reference, we identified important species- and strain-specific polymorphisms in both *cyp51A* and *fks1* (**Figure 6A** and **Table 2** shows a detailed breakdown of all SNP and indel polymorphisms per strain).

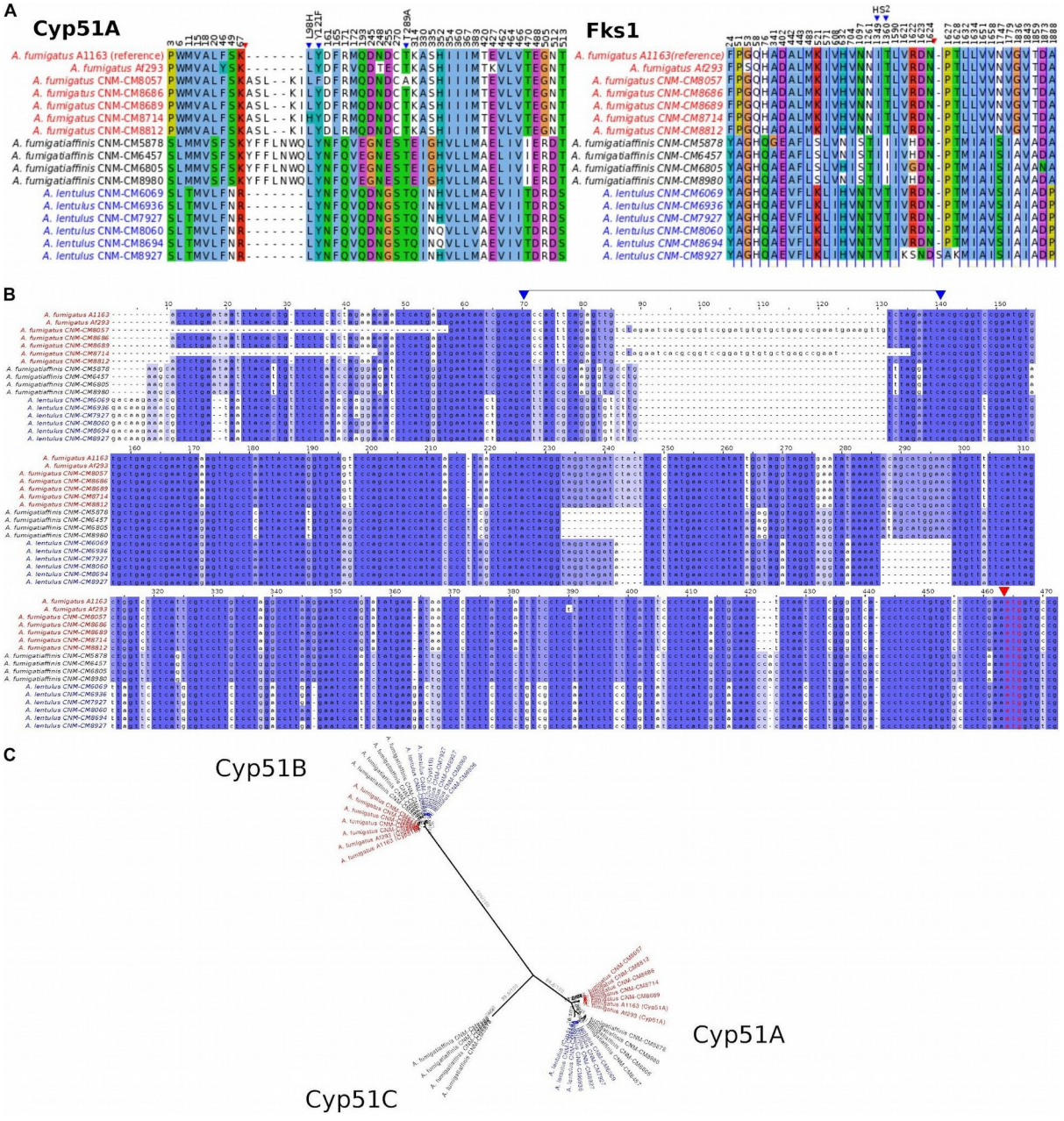

Figure 6. Changes in important genes related to antifungal susceptibility in the three *Aspergillus* pathogens. **(A)** Products of genes related to antifungal resistance, *Cyp51A* (azoles) and *Fks1* (echinocandins), display species- and strain-specific polymorphisms. Only the positions with changes in at least one strain are shown (substitutions or insertions/deletions). Blue triangles highlight important amino acid changes in positions 98, 121, and 289 in Cyp51A and in hot spot 2 (HS2) of *Fks1*. Red triangles indicate insertions/deletions. **(B)** Promoter region of the *cyp51A* gene displays strain-specific mutations among Spanish strains of three closely related *Aspergillus* pathogens. Well-known tandem repeat regions in antifungal-resistant strains of *A. fumigatus* are shown between positions 70–140 in the alignment (i.e., TR34 and TR46, observed in CNM-CM8714 and CNM-CM8057, respectively, delimited by two blue arrows in upper part). Polymorphisms in cryptic species were also identified, for instance, the short deletions exclusively found in the cryptic species (either in *A. fumigatiaffinis* or *A. lentulus*) around positions 230–250. Red arrow and red font indicate the start codon. **(C)** Phylogeny of Cyp51 gene family (protein sequences) reveals three different members (Cyp51A, Cyp51B, and the putative Cyp51C) in *A. fumigatiaffinis*. Ultrafast Boostrap Approximation and SH-aLRT support values are shown.

Table 2. Single-nucleotide polymorphisms and insertions/deletions in *cyp51* family and *fks1* genes in each species individually.

Species	Polymorphism type	Gene	High impact variant	Low impact variant	Moderate impact variant	Modifier impact variant
A. fumigatus	INDELs	*cyp51A*	0	0	0	6
A. fumigatus	SNPs	*cyp51A*	0	0	5	23
A. lentulus	INDELs	*cyp51A*	0	0	0	10
A. lentulus	SNPs	*cyp51A*	0	8	3	92
A. fumigatus	INDELs	*cyp51B*	0	0	0	1
A. fumigatus	SNPs	*cyp51B*	0	1	1	7
A. lentulus	INDELs	*cyp51B*	0	0	0	1
A. lentulus	SNPs	*cyp51B*	0	0	0	2
A. fumigatiaffinis	INDELs	*cyp51C*	0	0	0	28
A. fumigatiaffinis	SNPs	*cyp51C*	0	10	1	157
A. fumigatiaffinis	INDELs	*fks1*	0	0	0	45
A. fumigatiaffinis	SNPs	*fks1*	0	23	5	143
A. fumigatus	INDELs	*fks1*	0	0	0	3
A. fumigatus	SNPs	*fks1*	0	4	0	10
A. lentulus	INDELs	*fks1*	4	0	0	50
A. lentulus	SNPs	*fks1*	0	43	5	218

HIGH = The variant is assumed to have high (disruptive) impact in the protein, probably causing protein truncation, loss of function or triggering nonsense mediated decay. MODERATE = A non-disruptive variant that might change protein effectiveness. LOW = A variant that is most likely to be harmless or unlikely to change protein behavior. MODIFIER = Usually non-coding variants or variants affecting non-coding genes, where predictions are difficult or there is no evidence of impact. Details can be found on the SnpEff manual (http://snpeff.sourceforge.net/SnpEff_manual.html).

An alignment of Cyp51A protein sequences in the three species shows possible insertions in different sites (**Figure 6A** – red arrow). We observed substitutions in at least one of the clinical strains in the three species in 42 positions that might be correlated with the strains' varying drug susceptibility levels. For instance, Cyp51A in *A. fumigatus* CNM-CM8714 revealed a well-documented substitution related to azole resistance at position 98 (L98H) (**Figure 6A** – blue arrows), which might be correlated to its lower susceptibility to itraconazole or voriconazole compared to other *A. fumigatus* strains (**Figure 3B**).

We also looked at the promoter region of the *cyp51A* gene (**Figure 6B**) and identified the TR insertions TR34 and TR46 (region highlighted between blue arrows), previously reported in antifungal resistant strains (Dudakova et al. 2017). These changes were specific to certain clinical strains of *A. fumigatus* and were previously reported in combination with specific point mutations leading to amino acid substitutions. For example, *A. fumigatus* CNM-CM8714 carries the TR34 promoter insertions combined with L98H (**Figure 6A** – blue arrow), whereas *A. fumigatus* CNM-CM8057 has a TR46 insertion combined with Y121F/T289A (**Figure 6A** – blue arrow). There are other variants (short indels) that were exclusive to either *A. lentulus* or *A. fumigatiaffinis*, or both.

Examination of the Fks1 protein sequence alignment from strains of the three species also revealed substitutions in 39 sites (**Figure 6A**). We also observed an insertion at position 1,626 of *A. lentulus* CNM-CM8927 (red arrow). Fks1 also showed substitutions at positions comprising an important hot-spot 2 (HS2) (blue arrows): all *A. lentulus* strains have a substitution at position 1,349 (I1349V) and all *A. fumigatiaffinis* have a substitution at position 1,360 (T1360I).

Examination of orthogroups revealed that the orthogroup that includes the *cyp51A* gene (Afu4g06890) contained additional paralogs of the *cyp51* family in *A. fumigatiaffinis*. Thus, we carried out a phylogenetic analysis with the amino acid sequences with the orthogroups containing *cyp51A* and *cyp51B* genes in *A. fumigatus* Af293 (**Figure 4C**) that comprises the three species in this work. We observed three well-defined clades. The *A. fumigatiaffinis* paralog related to *cyp51A* is likely to represent *cyp51C*, which has been previously reported in other *Aspergillus* species, such as *A. flavus* and *A. oryzae* (Pérez-Cantero et al. 2020; Hagiwara et al. 2016). Sequence identity between the putative Cyp51C protein in *A. fumigatiaffinis* CNM-CM6805 and Cyp51C (XM_002383890.1) and Cyp51A (XM_002375082.1) of

A. flavus (W. Liu et al. 2012) is 471/512 (92%) and 391/508 (77%), respectively.

2.4.6 Genetic Determinants Involved in Virulence: Single-Nucleotide Polymorphisms, Insertions/Deletions Across Strains and Within Species Conservation

To explore the genetic underpinnings of the observed strain heterogeneity in virulence we next examined the SNPs and indels in 215 genes that have previously been characterized as genetic determinants of virulence in *A. fumigatus* (Supplementary Table S5).

Most virulence genetic determinants (146 genes) were found in single-copy in all strains (Supplementary Table S6), whereas 57 genes varied in their number of paralogs across clinical strains (**Figure 7**). We also identified four virulence determinants that had no orthologs in either *A. lentulus* or *A. fumigatiaffinis*, such as Afu6g07120 (*nudC*), which is an essential protein involved in nuclear movement (Morris et al. 1998), and considered an essential gene in *A. fumigatus* (Hu et al. 2007). Interestingly, we noted 17 virulence determinants that are present in *A. fumigatus* and *A. fumigatiaffinis* but absent in *A. lentulus* (**Figure 7** – top panel), such as Afu8g00200 (*ftmD*), one of the genes in the fumitremorgin biosynthetic gene cluster (Abad et al. 2010).

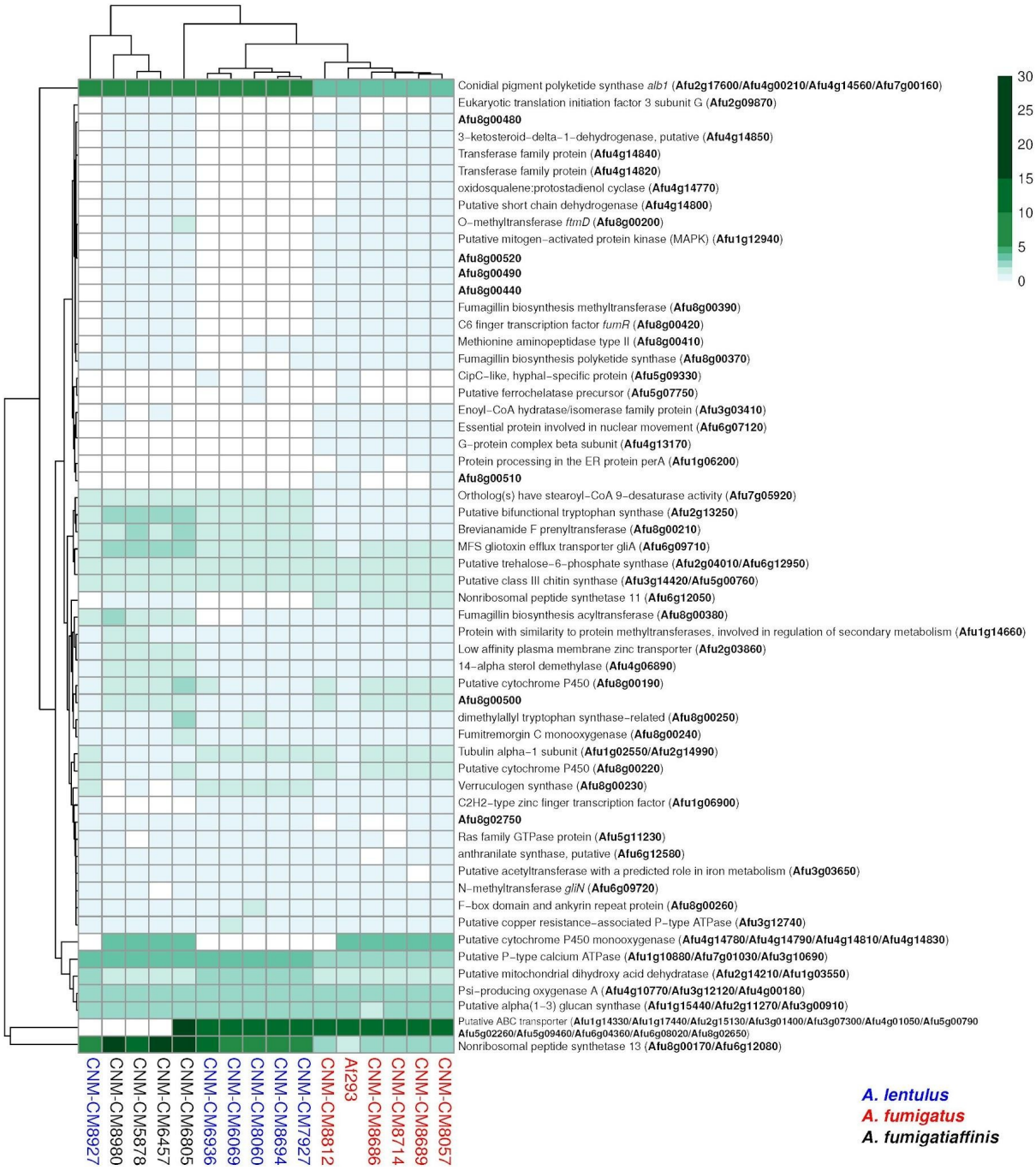

Figure 7. Orthogroups for virulence determinants reveals variable number of paralogs among the three closely related *Aspergillus* pathogens. We searched for 215 known genetic determinants of virulence in *A. fumigatus* Af293 in the species of interest and found they were grouped into 203 orthogroups. 146/203 were found in single copy across all strains and are not shown here. The cladogram above the species reflects similarities between strain presence/absence patterns. *A. fumigatus* Af293 shows a different pattern compared to other strains of *A. fumigatus*, grouping with one of the *A. lentulus* strains (CNM-CM8927). This may reflect the phylogenetic divergence of *A. fumigatus* strain Af293 from other species members. Conidial pigment polyketide synthase *alb1* (Afu2g17600) is one of the genetic determinants of virulence with highest number of copies in cryptic species (n = 7) when compared to *A. fumigatus* strains (n = 4). Gene identifiers in *A. fumigatus* Af293

are highlighted in bold. Color scale indicates the number of genes found within the orthogroup.

Several virulence determinants exhibited larger numbers of paralogs in one or more species. For example, the conidial pigment polyketide synthase *alb1* (Afu2g17600), which is involved in conidial morphology and virulence (Tsai et al. 1998), is one of the determinants with highest number of paralogs in *A. lentulus* and *A. fumigatiaffinis* (*n* = 7) when compared to *A. fumigatus* strains (*n* = 4). For determinants that contained a gene in at least one strain, we tested correlations between number of paralogs and virulence (lethal time 50: day at which 50% of the larvae were dead, or “ND-end”: the number of dead larvae at the end of the experiment) and we observed no significant correlation suggesting paralog number does not associate with virulence.

2.5 Discussion

A. fumigatus and the closely related species *A. lentulus* and *A. fumigatiaffinis* are important causal agents of aspergillosis (Lamoth 2016; Zbinden et al. 2012). Importantly, the emergence of antifungal resistance is of increasing worldwide concern (Fisher et al. 2018) and antifungal resistant strains of *A. lentulus* and *A. fumigatiaffinis* (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014) have been identified. Heterogeneity in virulence across different strains of *A. fumigatus* has also been known for some time (Mondon et al. 1996). Analyses of strain phenotypic and genetic heterogeneity allow us to identify correlations between phenotype and genotype in strains of *Aspergillus* pathogens.

We found that high heterogeneity exists in drug susceptibility and virulence across different strains of *A. fumigatus*, *A. lentulus*, and *A. fumigatiaffinis* (**Figures 3, 4**). For one specific antifungal drug, amphotericin B, our results confirmed previous findings that *A. fumigatus* is more susceptible to amphotericin B than strains of cryptic species (Balajee et al. 2004). Studies on the intrinsic resistance to amphotericin B reported for *A. terreus* highlight the importance of stress response pathways, in particular heat shock proteins (such as Hsp90 and Hsp70), as well as enzymes detoxifying reactive oxygen species (Posch et al. 2018). Future work involving genomics on the cryptic species will be able to exploit changes in genetic determinants involved in amphotericin B susceptibility, although this drug is not commonly used in clinical settings. Interestingly, our PCA identified pairs of positively

and negatively correlated antifungal drugs from different classes (Supplementary Figure S2), suggestive of potential synergistic effects (resistance to one drug leads to resistance to the other) and trade-offs (resistance to one drug leads to susceptibility to the other), which could be important for clinical applications.

Comparison of the three species showed that the virulence profiles of *A. fumigatiaffinis* and *A. lentulus* strains were not significantly different from the virulence profiles of *A. fumigatus* strains (**Figure 4**). This finding is in contrast to a previous comparison of survival curves of the type strains of *A. lentulus* and *A. fumigatus*, which found that *A. fumigatus* is significantly more virulent than *A. lentulus* (Sugui et al. 2014). The likely explanation for this is our finding that there is significant strain heterogeneity within each species (**Figure 4**), suggesting that comparisons of individual strains between species are not going to be representative of the variation in virulence within species. While additional testing using diverse models of fungal disease will be required to test the validity of these observations, our findings reinforce the emerging view (Kowalski et al. 2016, 2019; Ries et al. 2019; Bastos et al. 2020) that examining within-species variation in *Aspergillus* pathogens is an important, yet poorly studied and understood, dimension of fungal virulence.

The advent of whole genome sequencing boosted our understanding of the biology of the genus *Aspergillus* (de Vries et al. 2017). Several studies have previously analyzed genomic data of *A. fumigatus* strains (Abdolrasouli et al. 2015; Takahashi-Nakaguchi et al. 2015), uncovering *cyp51A* mutations in *A. fumigatus* populations (Abdolrasouli et al. 2015). Some studies have also used population genomic data for strains of *A. fumigatus* to gain insights on antifungal drug susceptibility (Garcia-Rubio, Alcazar-Fuoli, et al. 2018) or virulence potential (Puértolas-Balint et al. 2019). Correlations between phenotypic traits, such as antifungal susceptibility or virulence and genetic traits have been also studied in other well-studied pathogens, such as the opportunistic yeast *Candida albicans* (Hirakawa et al. 2015). However, to our knowledge, this is the first study that examines the phenotypic and genetic heterogeneity among strains of species closely related to *A. fumigatus*.

The three main classes of antifungal drugs comprise polyenes, azoles, and echinocandins, involved in ergosterol composition of fungal membrane, ergosterol biosynthesis, and the cell wall biopolymer (1,3)-β-D-glucan, respectively (Robbins, Caplan, and Cowen 2017). Due to toxicity to host cells, polyenes are only

used in exceptional cases, and first-line prophylaxis and treatment of aspergillosis is usually carried out with azoles (Garcia-Vidal et al. 2019; Garcia-Rubio, Cuenca-Estrella, and Mellado 2017). Mechanisms of azole resistance involving mutations in the *cyp51* genes have been identified in diverse fungi, including in multiple animal and plant pathogens (Parker et al. 2014). In *A. fumigatus*, research has focused on azole susceptibility testing and correlation with point mutations in the *cyp51A* gene and TR insertions in its promoter region (P. Chen et al. 2020; Zakaria et al. 2020). Major changes in protein *Cyp51A* that correlated with azole resistance include point mutations, such as in positions G54, G138, M220, and G448, or combination of point mutations with TRs in the promoter region, such as the TR34/L98H and the TR46/Y121F/T289A (Beardsley et al. 2018; Wei, Zhang, and Lu 2015). In previous studies, alterations such as the insertion of TR34 and TR46 (Dudakova et al. 2017) were only found in the *cyp51A* promoter of *A. fumigatus* strains, but these have also been found in other pathogens, such as the wheat pathogen *Zymoseptoria tritici* (Cools et al. 2012). Our work explored the promoter region of the *cyp51A* gene in *A. lentulus* and *A. fumigatiaffinis*, two closely related pathogenic species, and identified these promoter region changes only in two strains of *A. fumigatus* and not in either of the two cryptic species. Interestingly, two of the *A. fumigatus* strains in this work presented the combined TR34/L98H and the TR46/Y121F/T289A. We also identified other changes in proteins encoded by *cyp51A* and *fks1* that can be used in the future to generate mutants and test the effect of mutations in well-studied wild-type strains of *A. fumigatus* (P. Chen et al. 2020).

The evolution of the gene families that contain genes involved in drug resistance might also give us clues on how drug resistance evolves in fungal populations; previous studies (Hawkins et al. 2014; Zheng et al. 2019) report two paralogs of c*yp51* in diverse species, including *A. fumigatus*, *A. nidulans*, *Penicillium digitatum*, and *Magnaporthe oryzae*, while *Fusarium graminearum*, *A. flavus*, and *A. oryzae* have three *cyp51* genes (Dudakova et al. 2017). Recently, a study proposed the existence of *cyp51C* gene arising from a duplication in *cyp51B* in *A. terreus* and *A. carbonarius* (Pérez-Cantero et al. 2020). Interestingly, our study found a paralog of the *cyp51A* gene in *A. fumigatiaffinis* that likely corresponds to *cyp51C*. Whole-genome sequence analysis in *A. flavus* reported substitutions in the three paralogous genes (*cyp51A, cyp51B*, and *cyp51C*) in the context of antifungal resistance (Sharma et al. 2018). Novel substitutions identified in *cyp51C* and modeling of protein changes

suggested possible effects on drug binding. Next steps in studies of azole susceptibility in *A. fumigatiaffinis* strains could include the analysis of this putative *cyp51C* gene and its role in the organism's observed drug susceptibility profile.

Studies on echinocandins have focused on the (1,3)-β-D-glucan synthase enzyme, encoded by the *fks1* gene (Robbins, Caplan, and Cowen 2017). Particularly, two hot-spots have been studied (Gonçalves et al. 2016). Although most studies report mutations in *Candida* (Desnos-Ollivier et al. 2008; Garcia-Effron et al. 2008), previous work reported point mutations in *fks1* hot spot 1 associated with echinocandin resistance in *A. fumigatus* (Jiménez-Ortigosa et al. 2017). Our work did not find changes among *A. fumigatus* clinical strains, and didn't find any mutation in the hot spot 1 of sequences of the cryptic species, which is in agreement with previous study that analyzed *fks1* sequences in *A. lentulus* (Staab, Kahn, and Marr 2010). However, we did observe changes in hot spot 2 that were specific to the cryptic species; further examination of these changes with respect to echinocandin susceptibility is an interesting future avenue of research.

Although this study focused on polymorphisms in genes *cyp51A* and *fks1*, there is also increasing research on non-*cyp51* (Zakaria et al. 2020) and non-*fks1* (Szalewski et al. 2018) genetic changes. Future exploitation of genomic data on strains of *A. fumigatus* and closely related species could also exploit these additional genes. These future studies could also exploit new antifungal drugs, such as olorofim, which has also been tested on cryptic species of *Aspergillus* (Rivero-Menendez, Cuenca-Estrella, and Alastruey-Izquierdo 2019). Finally, future phenotypic and genomic analyses can help us to better understand more complex topics involving antifungal drugs, such as resistance, persistence, and tolerance (as well as the role of tolerance in resistance) (Berman and Krysan 2020). Given possible emergence of antifungal resistance in agriculture (Hawkins et al. 2018), future work could also exploit correlations in antifungals and the origin of these isolates.

3 Article 2: Draft Genome Sequences of Four *Aspergillus* Section *Fumigati* Clinical Strains

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3.1 Abstract

Aspergillus fungi in section *Fumigati* include important human pathogens. Here, we sequenced the genomes of two strains of *Aspergillus hiratsukae* and two strains of *Aspergillus felis*. The average genome sizes are 29.5 Mb for *A. hiratsukae* and 31.8 Mb for *A. felis*.

3.2 Announcement

Aspergillus is a highly diverse genus of industrially and medically important fungi (Gibbons and Rokas 2013; de Vries et al. 2017). The genus is taxonomically divided into 27 sections (J. Houbraken et al. 2020). Section *Fumigati* contains the major human pathogen *Aspergillus fumigatus* (Rokas et al. 2020) and several so-called cryptic species, such as *Aspergillus hiratsukae* and *Aspergillus felis* (Guarro et al. 2002; Koutroutsos et al. 2010; Barrs et al. 2013), which are morphologically similar but genetically distinct from A. fumigatus. Cryptic species account for over 10% of cases of *Aspergillus* infection (Alastruey-Izquierdo, Alcazar-Fuoli, and Cuenca-Estrella 2014). Here, we sequenced the genomes of two clinical strains of *A. hiratsukae*, CNM-CM5793 and CNM-CM6106, from nail and ear infections, respectively, both from Spain. We also sequenced two clinical strains of *A. felis*, strain CNM-CM7691 from an ear infection in Spain and strain CNM-CM5623 from Portugal. All four isolates were recovered from clinical samples following standard procedures and sent to the Medical Mycology Reference Laboratory (at the National Center for Microbiology, Instituto de Salud Carlos III, Madrid, Spain) for identification and susceptibility testing. Except for infection type, no clinical data were recorded. Therefore, the fungal isolates were judged to be exempt from informed consent of the patients and institutional review board approval.

Species assignment was based on a maximum-likelihood phylogenetic analysis (**Figure 8**). For genome sequencing, we grew all strains in glucose-yeast extract-peptone (GYEP) liquid medium (0.3% yeast extract and 1% peptone; Difco, Soria

Melguizo) with 2% glucose (Sigma-Aldrich, Spain) for 24 to 48 h at 30°C. The mycelium was mechanically disrupted by vortex mixing with glass beads and used to extract genomic DNA using the phenol-chloroform method (Holden, 1994). DNA was quantified using the QuantiFluor double-stranded DNA (dsDNA) system and the QuantiFluor ST fluorometer (Promega, Madison, WI, USA). DNA quality was checked with the Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). DNA libraries were prepared using the Nextera DNA library prep kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's guidelines. Paired-end sequencing (2 × 150 bp) was performed using the NextSeq 500 platform following the manufacturer's protocols (Illumina, Inc.).

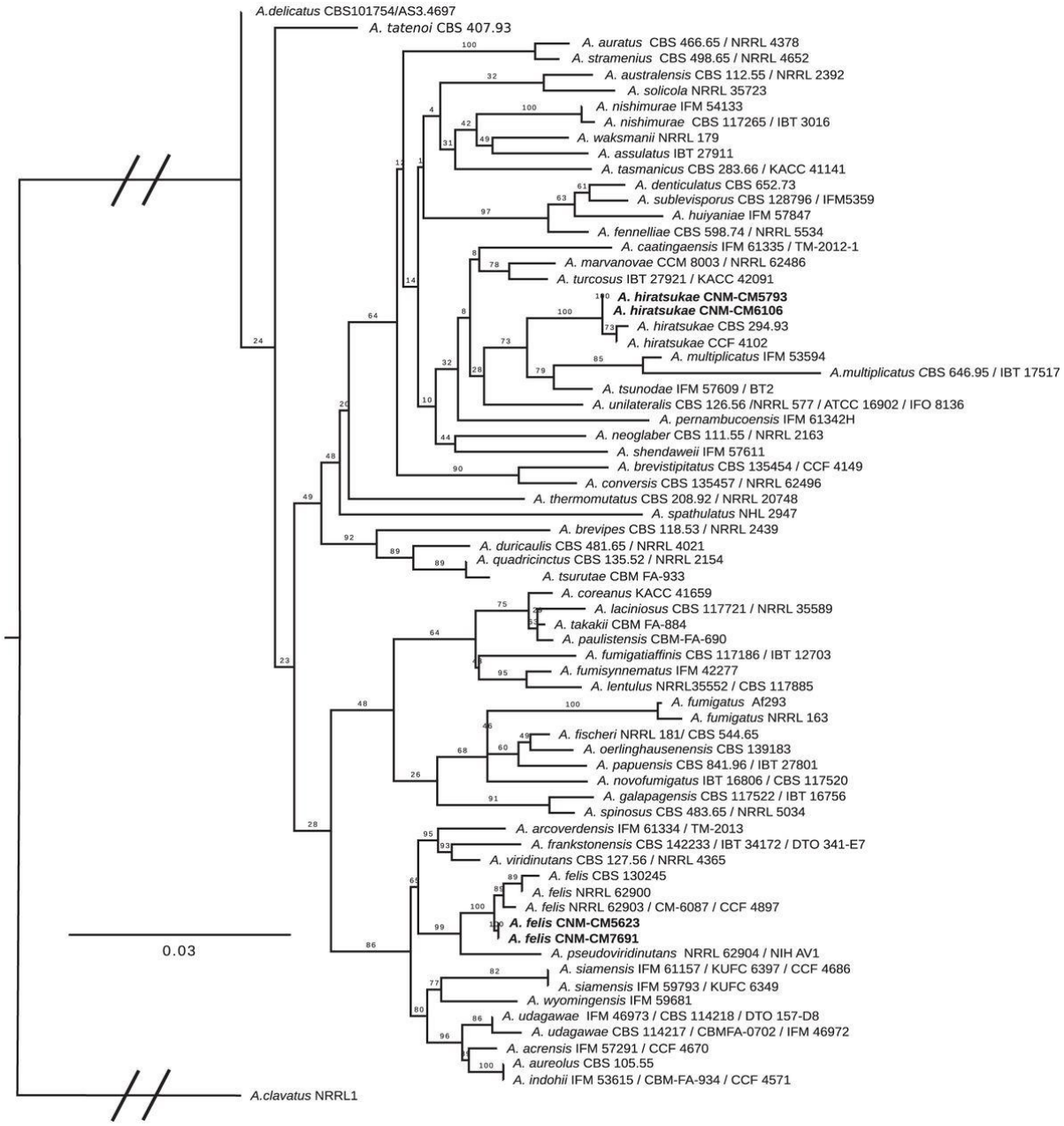

Figure 8. Maximum-likelihood phylogenetic tree of the four strains sequenced in this announcement (in bold) and related species in section *Fumigati*, based on the analysis of the four markers β-tubulin gene (*benA*), calmodulin gene (*CaM*), actin gene (*act*), and RNA polymerase II second-largest subunit gene (*RPB2*), commonly used in *Aspergillus* taxonomy (Hubka et al. 2018); sequences were obtained from reference Hubka et al. (2018) except for the sequences of the four newly sequenced strains, which were obtained by searching for markers of each strain in orthogroups generated by OrthoFinder v2.3.3 (Emms and Kelly 2019) using *A. fumigatus* Af293 (Nierman et al. 2005) as the reference. Each marker was aligned with MAFFT v7.397 (Katoh and Standley 2013), and a supermatrix was generated with FASconCAT v1.11 (Kück and Meusemann 2010). Tree inference was carried out on IQ-TREE v2.0.3 (Minh et al. 2020) with partitions with the option “MFP+MERGE,” which employs ModelFinder to find the best partition scheme. The final tree was edited in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/

figtree/). Support values are based on 1,000 bootstrap replicates. A. clavatus (section *Clavati*) was used to root the tree. Note that the species *A. parafelis*, *A. pseudofelis*, and *A. felis* were merged (synonymized) into a single species, *A. felis* (Hubka et al. 2018); thus, we infer that the two sequenced strains belong to *A. felis*.

For all software, default parameters were used except where otherwise noted. The numbers of sequencing read pairs generated for strains CNM-CM5793, CNM-CM6106, CNM-CM7691, and CNM-CM5623 were 7,733,508, 5,237,901, 9,555,248, and 6,768,577, respectively. Quality control of the sequence reads was performed with FastQC v0.11.7 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were cleaned with Trimmomatic v0.38 (Bolger, Lohse, and Usadel 2014) with the following parameters: NexteraPE-PE.fa:2:30:10:2:keepBothReads, SLIDINGWINDOW:4:15, LEADING:3, TRAILING:3, and MINLEN:90. The genome sequences were assembled with SPAdes v3.14.0 (Bankevich et al. 2012) employing multiple k-mers (31, 41, 51, 61, 71, 81, and 91) and the --careful parameter. The genomic reads were mapped to the assembly with Bowtie v2.3.4.1 (Langmead and Salzberg 2012), followed by a single iteration of Pilon v1.22 (Walker et al. 2014) used in base correction to fix misassemblies and for gap filling. Overall statistics of the final assemblies were assessed with QUAST v4.6.3 (Gurevich et al. 2013). Genome assembly completeness was assessed by examining for the presence of 3,546 universal single-copy orthologs in Eurotiomycetes (eurotiomycetes_odb10) with BUSCO v4.0.4 (Simão et al. 2015). We used AUGUSTUS v3.3.1 (Stanke et al. 2004) for prediction of protein-coding genes using the A. fumigatus Af293 gene models as a reference (Nierman et al. 2005).

Assembly sizes, numbers of contigs, GC content, N_{50} contig values, gene numbers, percentages of complete and single-copy BUSCOs, and percentages of fragmented BUSCOs for all four strains are reported in **Table 3**. We summarized the genome statistics based on contigs of greater than 1,000 bp but included all contigs of greater than 200 bp in the assemblies submitted to GenBank. Genomic information of *Aspergillus* strains that are closely related to major human pathogens is important for understanding the origin and evolution of opportunistic human pathogenicity in the genus *Aspergillus*.

Table 3 Overall genome assembly, completeness, and annotation statistics

Strain	Assembly size[a] (bp)	No. of contigs >1,000 bp	Avg genome coverage (×)	GC content (%)	N_{50} (bp)	No. of genes	No. (%) of complete and single-copy BUSCOs	No. (%) of fragmented BUSCOs
A. hiratsukae CNM-CM5793	29,562,918	745	63	50.38	100,935	9,685	3,487 (98.33)	31 (0.87)
A. hiratsukae CNM-CM6106	29,374,270	922	39	50.37	71,695	9,663	3,466 (97.74)	38 (1.07)
A. felis CNM-CM5623	31,643,783	663	47	49.93	112,776	10,161	3,494 (98.53)	26 (0.73)
A. felis CNM-CM7691	31,957,614	559	70	49.93	138,232	10,243	3,503 (98.78)	18 (0.51)

[a] Based on contigs with more than 1,000 bp.

3.3 Data availability

The draft genome sequences of *A. felis* strains CNM-CM5623 and CNM-CM7691 and *A. hiratsukae* strains CNM-CM5793 and CNM-CM6106 are deposited in GenBank under the accession numbers JACBAE000000000, JACBAG000000000, JACBAD000000000, and JACBAF000000000, respectively. The raw reads of *A. felis* strains CNM-CM5623 and CNM-CM7691 and *A. hiratsukae* strains CNM-CM5793 and CNM-CM6106 are deposited in the NCBI Sequence Read Archive (SRA) under accession numbers SRR11804853, SRR11804830, SRR11802685, and SRR11802449, respectively. Genome assemblies and raw data are associated with BioProject number PRJNA633131.

3.4 Acknowledgments

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4 Article 3: Examining signatures of natural selection in antifungal resistance genes across *Aspergillus* fungi

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4.1 Abstract

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.

4.2 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 (Rokas et al. 2020;

Steenwyk et al. 2019). 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, Alcazar-Fuoli, and Cuenca-Estrella 2014).

The first-line drugs against aspergillosis are the azoles, with echinocandins or amphotericin B as alternative or complementary treatments (David W. 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(Enoch et al. 2014; Nishiyama et al. 2005).

Antifungal resistance is a worldwide concern, both in the clinic and in the field (Hagiwara et al. 2016; Verweij, Chowdhary, et al. 2016; Wassano, Goldman, and Damasio 2020; Fisher et al. 2018; Garcia-Rubio, Cuenca-Estrella, and Mellado 2017; Perlin, Rautemaa-Richardson, and Alastruey-Izquierdo 2017). 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, Zhang, and Lu 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; P. Chen et al. 2020; 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 with azole 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, Zhang, and Lu 2015; Beardsley et al. 2018). Changes in *cyp51A* expression have also been reported in several cases of drug-resistant *Aspergillus* species (Osherov 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 (P. Chen et al. 2020; Pérez-Cantero et al. 2020). For example, genes involved in transport, including the ATP-binding cassette (ABC) and the Major Facilitator Superfamily (MFS) (Nascimento et al. 2003; Slaven et al. 2002; da Silva Ferreira et al. 2004; Fraczek et al. 2013; M. Chen et al. 2020; Meneau, Coste, and Sanglard 2016; Paul, Diekema, and Moye-Rowley 2013), ergosterol biosynthesis (e.g., *hmg1*, the HMG-CoA reductase enzyme that participates in the regulation of sterol synthesis in eukaryotes) (Rybak et al. 2019; Arai et al. 2021), stress response (e.g. calcium signaling pathway) (P. Chen et al. 2020), mitochondrial processes (Wei et al. 2017; Y. Li et al. 2020), and regulatory genes (Hortschansky et al. 2020; Hagiwara et al. 2017; Furukawa et al. 2020; Willger et al. 2008; Blosser and Cramer 2012; Song et al. 2016; Song, Zhai, and Lu 2017) 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* (Carolus 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, Alcazar-Fuoli, and Cuenca-Estrella 2014). Isolates of *A. fumigatus* are phenotypically heterogeneous (Kowalski et al. 2019, 2016; Fuller et al. 2016; Keller

2017; Ries et al. 2019; Steenwyk, Mead, Knowles, et al. 2020; Steenwyk, Mead, de Castro, et al. 2020), 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. fumigatiaffinis* (Renato A. C. Dos Santos et al. 2020), needing examination of multiple isolates per species. Previous genomic examinations of azole resistance solely focused on *A. fumigatus* (Abdolrasouli et al. 2015; Garcia-Rubio, Monzon, et al. 2018). Recently, Parent-Michaud et al (2020) explored mechanisms of antifungal resistance in the pathogenic species *A. thermomutatus* and *A. turcosus*, 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.

4.3 Methods and methods

4.3.1 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 S1). We followed a recent classification of *Aspergillus* (J. 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, Xie, and Huson 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.

4.3.2 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(Renato Augusto Corrêa Dos Santos et al. 2020). 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, Guinea, and Cuenca-Estrella 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(Renato Augusto Corrêa Dos Santos et al. 2020), we tested their susceptibility to four antifungal drug classes as previously described(Renato A. C. Dos Santos et al. 2020).

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 S2). 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.

4.3.3 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 (*benA*), 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), followed by generation of a supermatrix consisting of the individual gene partitions with FASconCAT (Kück and Meusemann 2010). The supermatrix was used as input in IQ-TREE v.2.0.3 option '-m MFP+MERGE' (Minh et al. 2020), with model selection based on the greedy strategy in ModelFinder (Lanfear et al. 2012; Kalyaanamoorthy et al. 2017), and using 1,000 bootstrap replicates to assess bipartition support (IQ-TREE -b 1000 option). The interactive web tool iTOL(Letunic and Bork 2019) was used for tree visualization.

4.3.4 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 Table S3 and Supplementary Table S4). 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, Longden, and Bleasby 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, Torrents, and Bork 2006). Alignments were visually inspected with Jalview v.2.10.3 (A. M. Waterhouse et al. 2009).

4.3.5 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 $\omega > 1$ are suggestive of positive selection, whereas $\omega = 1$ and $\omega < 1$ are suggestive of neutral evolution and negative selection, respectively. To determine dN and dS values, we used codeml from PAML v.4.9i (Ziheng 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 (β model) that considers one ω across all isolates, plus ten site classes with ω <= 1, and the M8 model (β 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: $0 \leq \omega < 1$ and $\omega = 1$) 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 (ln*L*) 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 $\chi 2$ value(Jeffares et al. 2015). For genes that rejected the null hypothesis, the Bayes Empirical Bayes (BEB) analysis (Ziheng Yang, Wong, and Nielsen 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 9**) 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.

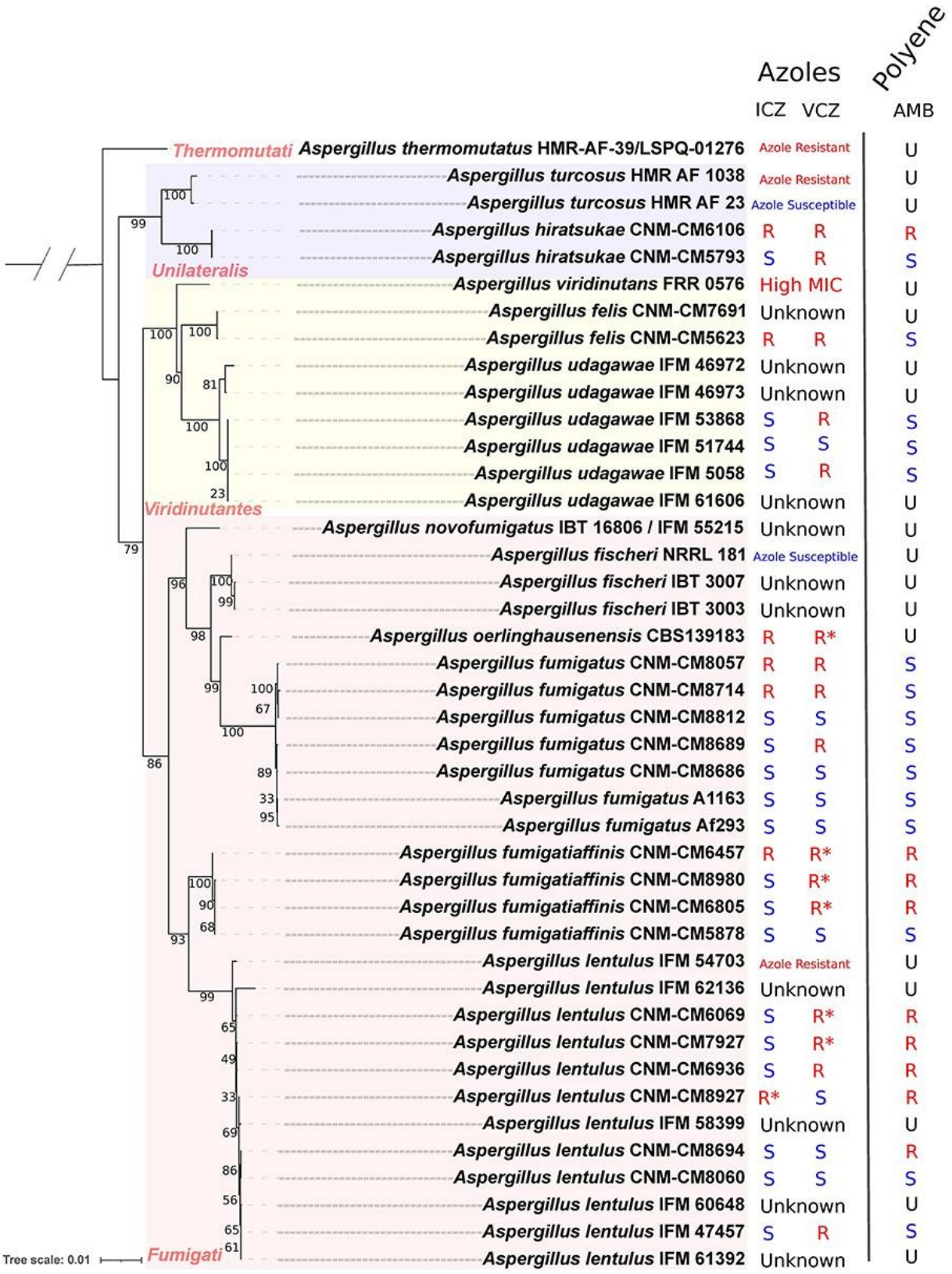

Figure 9. Phylogenetic reconstruction of strains in section *Fumigati* with sequenced genomes and the different susceptibility levels to different antifungals (the outgroup is not shown). We observed that the four sequenced Series (*Fumigati, Unilateralis, Viridinutantes, Thermomutati*) have members 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.

4.4. Results

4.4.1 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 Table S1 and Supplementary Table S2).

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 9**). Our phylogeny is consistent with previous studies (J. Houbraken et al. 2020; Renato Augusto Corrêa Dos Santos et al. 2020; Renato A. C. Dos Santos et al. 2020) 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 S2; **Figure 9**). 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 (Jos Houbraken et al. 2016; Parent-Michaud et al. 2019b, [a] 2019; Lyskova et al. 2018; Tamiya et al. 2015; Renato A. C. Dos Santos et al. 2020; Kusuya et al. 2016; Fedorova et al. 2008;

Talbot et al. 2019) 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 9**). 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* (Renato A. C. Dos Santos et al. 2020). 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 VCZ, but was susceptible to AMB. We also phenotyped two *A. hiratsukae* (series *Unilateralis*) isolates that were recently sequenced by our group(Renato Augusto Corrêa Dos Santos et al. 2020). 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 9** and the full data are shown in Supplementary Table S1 and Supplementary Table S2.

4.4.2 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 Table S3 and Supplementary Table S4). 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 S4), 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 S4). 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 S4); 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 4**), suggesting these genes have undergone positive selection ($\omega > 1$). 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. In addition, we found that the gene encoding a drug target for echinocandins, *fks1*, has sites under positive selection.

Table 4. Genes with signatures of positive selection (the Likelihood Ratio Test rejected the null hypothesis).

Gene	Category	Antifungal class	Justification of selection
abcA	Transporter	Azole	Overproduction of *AbcA* in *A. fumigatus* yielded increase resistance; *S. cerevisiae* expressing *abcA* were more resi fluconazole than the PDR5-deleted background strain.
abcF	Transporter	Azole	*Saccharomyces cerevisiae* expressing *abcF* had the stro efflux activities, had the broadest range of substrate spec and were more resistant to fluconazole.
atmA	Kinase	Azole	*atmA* and *atrA* mutant populations grown under sub-inhi drug concentration resulted in voriconazole resistance ar discrete alterations in *cyp51A* and/or the *Cdr1B* efflux tra these kinases are likely involved in genetic stability
atrA	Kinase	Azole	*atmA* and *atrA* mutant populations grown under sub-inhi drug concentration resulted in voriconazole resistance ar discrete alterations in *cyp51A* and/or the *Cdr1B* efflux tra these kinases are likely involved in genetic stability
erg4B	Reductase	Azole	*erg4A* and *erg4B* null mutant displays remarkable increas susceptibility to antifungal azoles (but not their isolated m
fks1	Drug target	Echinocandin	hot spot FKS1 mutation E671Q might be responsible for reduced susceptibility
mdr3	Transporter	Azole	Overexpression of Mdr3, Mdr4 or both drug efflux pump *A. fumigatus* and/or selection of drug target site mutation linked to high levels of itraconazole resistance
mot1	Transcriptional regulator/ modulator	Azole	Involved in regulation of the negative cofactor 2 complex, a key regulator of drug resistance in *A. fumigatus*
ramA	Enzyme	Azole	Loss of *ramA* led to a Cyp51A/B-independent increase in resistance to triazole antifungal drugs
tpo3	Transporter	Azole	*tpo3* and *dur3* played important roles in susceptibility to potential mechanism (*tpo3* and *dur3* → polyamine home → ROS content → ITC susceptibility)
dapB	Damage resistance protein	Azole	Overexpression of *dapB* and *dapC* causes dysfunction of *erg11*, resulting in abnormal accumulation of sterol intern and further accentuating the sensitivity of Δ*dapA* strains

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 10**). 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.

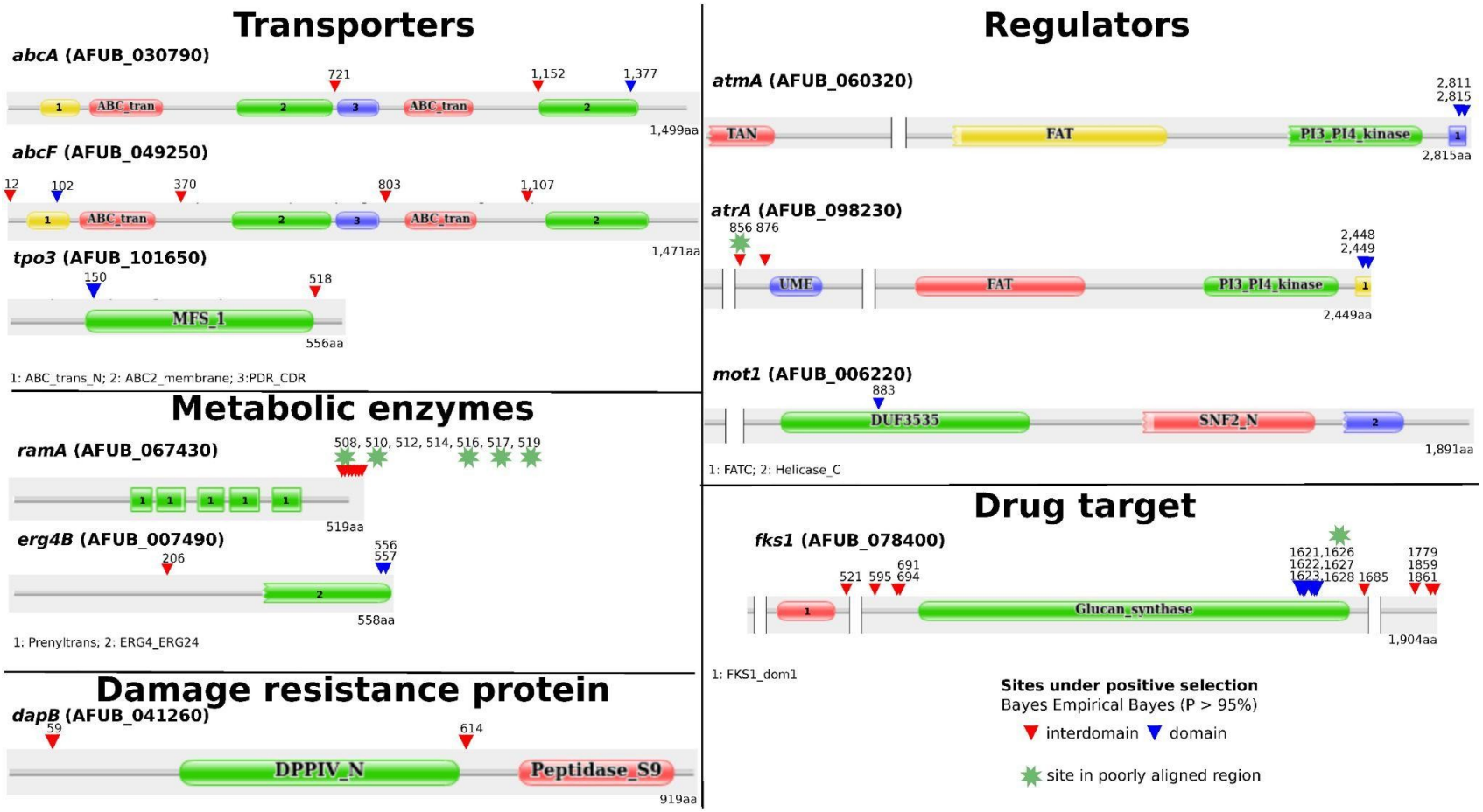

Figure 10 - Protein annotation and annotated domains of genes for which the likelihood ratio tests (LRT) rejected the null hypothesis suggestive of positive selection among these genes. Among the eleven genes with significant LRT values, 10 presented sites under positive selection. Annotation of domains was carried out using the online Pfam database (http://pfam.xfam.org/). Proteins with long peptide stretches without any significant matches to Pfam domains were shortened (indicated by two bars with blank space in between). The gene products in *Aspergillus fumigatus* A1163 were used as reference.

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 10**; Supplementary Table S5). 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 9**). 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 S5). 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.

4.5 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 (Ziheng 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, Rautemaa-Richardson, and Alastruey-Izquierdo 2017; S. 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* (J. 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 (Renato A. C. Dos

Santos et al. 2020). 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*) (Renato Augusto Corrêa Dos Santos et al. 2020; Renato A. C. Dos Santos et al. 2020), 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*) (Renato A. C. Dos Santos et al. 2020), and these differences are highlighted in **Figure 9**. 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 *Viridinutantes*) (Renato Augusto Corrêa Dos Santos et al. 2020). 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 (Schoustra et al. 2019; Buil 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 S3). 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 (Pérez-Cantero et al. 2020; Wei, Zhang, and Lu 2015), as well as a mutation in *cyp51B* (G457S) (Gonzalez-Jimenez et al. 2020). Studying mitochondrial genes, (Y.

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 eukaryotic transcription factor (Hortschansky et al. 2020). Mutations (F262del, S305P, I412S, F390L) 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 (Haitao 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 9**). 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* (M. Chen et al. 2020). 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, Foiani, and Kumar 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 analogues are not known), echinocandin has structural analogues of natural origin (David W. 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(Renato A. C. Dos Santos et al. 2020), 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.

4.6 Data availability statement

All genomes included in this study are available from the NCBI GenBank database. Assembly accession numbers are presented in Supplementary Table S1, including the reference manuscript 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.

4.7 Authors contributions

RS, JS, MM, AA-I, GG, 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.

4.8 Funding

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5 The identification of Aspergillus fumigatus putative new players in azole antifungal response

5.1 Introduction

Aspergillus fumigatus is an important opportunistic pathogen, responsible for aspergillosis (Latgé and Chamilos 2019). The main treatment for this disease comprises antifungal therapy, including azoles which are the first-line class of antifungals (Rybak, Fortwendel, and Rogers 2019). However, concerns have increased worldwide due to increased resistance of *A. fumigatus* and closely related species to these available drugs (Nywening et al. 2020).

Various epigenetic mechanisms are able to change the expression of target genes without altering DNA. They are grouped in two categories: RNA-based and chromatin-based (Chang et al. 2019). Long noncoding RNAs (lncRNAs) comprise RNAs that are longer than 200bp. In general, they can have poly(A)-tail, 5'methylguanosine cap, and post transcriptional modifications, but these are not the rules (Till, Mach, and Mach-Aigner 2018). It is also commonly observed that these RNAs have very low expression and are poorly conserved. LncRNAs are involved in different cellular processes, including the physical interference with transcription *in cis*, nucleosome repositioning, modifying histones, recruiting chromatin modelling factors. They can also act in *trans*, by interacting with proteins, RNAs, and DNA. They can also act as scaffold to attachment of factors. In general, they are negative regulators, but positive regulation has also been reported. Most of these characteristics are better described in humans or mammals in general.

Recent revisions have been made about the role of noncoding RNAs in response to drugs (Dhingra 2020; Chang et al. 2019). Investigations in *Schizosaccharomyces pombe* showed that a lncRNA, ncRNA.1343, is involved in the nucleosome density in the promoter region of a neighboring gene, *tgp1*, which encodes the glycerophosphodiester transporter 1 (Tgp1). Deletion of this lncRNA in *S. pombe* resulted in a strain that is hypersensitive to several broad-spectrum drugs, including thiabendazole, caffeine, and hydroxyurea (Ard, Tong, and Allshire 2014). Recently, a study involving transposon disruption identified an intergenic site harboring a long noncoding RNA, *DINOR,* that plays an important role in stress

tolerance in *Candida auris* and was upregulated in response to caspofungin (echinocandin) and amphotericin B (Gao et al. 2021). Tolerance to azoles is relatively well studied in *Candida* spp., but no information is available for *A. fumigatus* (Arastehfar et al. 2021). Given that previous research showed that lncRNAs play important roles in antifungal tolerance and susceptibility in yeast, it is possible that these elements are also involved in antifungal responses in molds, such as *A. fumigatus*.

Transcriptomic analyses have the potential to uncover genes involved in response to antifungals. Previous works used RNA-Seq data to study the responses involved in azole-induced stress. Interestingly, (M. Chen et al. 2020) discovered two transporters which expression was induced in azoles (*dur3* and *tpo3*) that are involved in polyamine transport, a compound that is involved in the resistance to itraconazole in *A. fumigatus*. In another study, (Marion Aruanno et al. 2021) transcriptomic analysis was applied to understand the differences in responses to azoles after generation of a pan-azole strain, in which the authors could identify increased expression of several genes involved in ergosterol biosynthesis as well as transporters and regulators. (Hagiwara et al. 2017) used transcriptomics of *A. fumigatus* mutants (*srbA* and *atrR*) to understand their differential responses to miconazole, as well as identifying genes that were regulated by either SrbA or AtrR, or both transcription factors.

Although these studies analyze the coding portion of the genome, none have exploited the noncoding RNAs in *A. fumigatus*, including the lncRNAs. Of relevance is the fact that there is an increase in the availability of public projects of RNA-Seq, but they are underexplored (Doughty and Kerkhoven 2020). Using such datasets to uncover the noncoding RNAs in *A. fumigatus* and reanalyze them considering these noncoding transcripts would provide relevant information about the role of these elements in antifungal response. Previous works have used RNA-Seq data and bioinformatics tools to identify noncoding RNAs in fungi. Among them, Kim et al (W. Kim et al. 2018) identified lncRNAs in *Fusarium graminearum* to study their involvement in fruiting body development. Recently, a manuscript described the discovery of lncRNAs in *Aspergillus sydowii* and identified three that are involved in the biosynthesis of anthocyanins (Bu et al. 2020). Although *A. fumigatus* is an important pathogen that has been studied for decades and for which several RNA-Seq datasets are currently available, for our knowledge there is still no work

describing lncRNAs in this fungus. Due to the differences in evolutionary constraints in lncRNAs compared to mRNAs and as consequence a lack of sequence conservation (Ulitsky 2016), methods for discovering novel lncRNAs in *A. fumigatus* and for ranking their importance to study putative roles in antifungal response are needed.

To fill the gaps that exist concerning the discovery of noncoding RNAs in *A. fumigatus* and the understanding of their roles in antifungal response, in this work we exploited public RNA-Seq datasets to answer two questions. First, what are the putative lncRNAs expressed by *A. fumigatus*? Using RNAsamba (Camargo et al. 2020), we trained a model based on fungal ncRNAs, that was able to identify novel transcripts in fungi (ascomycete and basidiomycete). This model was able to identify novel transcripts, including several with high potential to be noncoding. Second, what are the common responses to different azoles and what lncRNAs could be playing a role in this response? Using three different datasets under exposure (two for itraconazole and one for voriconazole), we tested the differential gene expression and analyzed the genes that were up- and downregulated when A. fumigatus is exposed to these drugs. Interestingly, we identified novel genes coding protein products that are potentially involved in response. Additionally, several intergenic lncRNAs were upregulated across treatments and one was downregulated. These novel putative players can help shedding light on our understanding of tolerance and resistance to azoles in *A. fumigatus*.

5.2 Materials and Methods

5.2.1 Reference genome and public transcriptome sequencing data

We used the FungiDB *A. fumigatus* Af293 genome release 47 (Basenko et al. 2018; Nierman et al. 2005) in all downstream analyses. We recovered all bioprojects in NCBI that were associated with RNA-seq experiments and the species *Aspergillus fumigatus*, including different contrasting conditions to favor co-expression analyses (**Table 5**).

5.2.2 Training RNAsamba for discovery of fungal lncRNAs

To classify transcripts in coding and noncoding, we employed RNAsamba `v.0.2.0` (Camargo et al. 2020). To train RNAsamba for classification of fungal transcripts, we used a dataset available in Ensembl Fungi v.47 (Howe et al. 2019). For training coding sequences, we relied on 1,000 fungal sequences, but included a step of recovering sequences without start and stop codons, that allows RNAsamba to consider sequences with UTRs. For training noncoding, we included only noncoding sequences in the ncRNA Ensembl Fungi dataset. Due to overrepresentation of tRNAs, miRNAs, snRNAs, rRNAs, and several others from classes that do not comprise the lncRNAs, we first recovered the sequences classified as biotype 'ncRNA'. Next, we used the `easy-search` (with options `-e 1e-3 -c 0.5`) software in mmseqs2 11.e1a1c (Steinegger and Söding 2017) to identify RNAs matching Uniprot sequences and ensure we had only a dataset that do not match protein sequences. From the final set, we randomly selected 1,000 sequences with the `fasta-subsample v.5.1.0` script included in MEME suite (Bailey et al. 2009). To test whether the newly trained RNAsamba model would work well for the classification of fungal sequences, we tested it using previously identified lncRNAs in fungi: the ascomycete *Fusarium graminearum* (W. Kim et al. 2018) and the basidiomycete *Serpula lacrymans* S7.9 (Borgognone et al. 2019), and compared to the original pre-trained model that included only human sequences.

5.2.3 Discovery of novel lncRNAs in *Aspergillus fumigatus*

We downloaded the sequencing reads for samples associated to five bioprojects, which included single- and paired-end, unstranded and strand-specific reads (**Table 5**). Sequencing reads were downloaded to the Kahuna server (UNICAMP) using grabseqs (L. J. Taylor, Abbas, and Bushman 2020). Sequencing quality was analyzed with FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). In particular, we were interested in identifying adapters to be removed in downstream analysis. Trimmomatic v0.39 (Bolger, Lohse, and Usadel 2014) was used to remove sequencing adapters, but not to remove low-quality reads (discussions on pros and cons were recently presented in a publication by our research group at UNICAMP; see (de Carvalho et al. 2019). The *A. fumigatus* Af293 genome was indexed using

hisat2-build, followed by mapping reads with HISAT2 v2.1.0 (D. Kim, Langmead, and Salzberg 2015). Information about strand specificity is not always easily available in published manuscripts and this information is not provided in NCBI either. It is also common not to have responses from authors when such information is requested (personal notes from the graduate student). Therefore, an initial round mapping of mapping reads with HISAT2, followed by the python script `infer_experiment.py` in RSeQC v3.0.1 (L. Wang, Wang, and Li 2012) was carried out to predict the best experimental design. RSeQC results matched responses received by email from authors. RSeQC requires a BED file (instead of a GFF, as provided by FungiDB), so the interconversion was carried out using BEDOPS - `gff2bed v2.4.39` (Neph et al. 2012). After RSeQC, HISAT2 was used again with the adjusted parameters (`--rna-strandness` option, with `R` for stranded single-end reads or `RF` for stranded paired-end reads). Mapping files (BAM) were used in StringTie v2.1.3 to assemble all transcripts (with `--rf` parameter used for strand-specific reads). To select transcripts with suitable length (> 200bp), we used the `-m 200` option. StringTie was used with the `--merge` option to generate a final annotation file with transcripts coming from different conditions and samples.

To identify novel RNAs in *A. fumigatus* among the transcripts assembled with StringTie, we used a combination of different computational methods. First, we used Gffcompare (Pertea and Pertea 2020) to recover the classes of assembled transcripts based on the genome annotation for the reference (*A. fumigatus* Af293). From the several classes in gffcompare output (see project webpage), we selected transcripts fully contained in introns annotated in reference as well as intergenic. In brief, `gffread v0.12.3` from the cufflinks suite (Trapnell et al. 2012) was used to recover the transcripts sequences and the EMBOSS v6.6.0 (Rice, Longden, and Bleasby 2000) `seqret` script was used to select only transcripts in classes of our interest. RNAsamba `classify` (Camargo et al. 2020) was used to identify possible noncoding sequences. `easy-search` (with options `-e 1e-3 -c 0.5`) in mmseqs2 11.e1a1c was used to identify transcripts matching Uniprot sequences. Finally, we also used a Perl script, followed by the `intersect` tool in BEDTOOLS `v2.27.1` (Quinlan and Hall 2010) to compare annotation files (`-A` indicated GFF with discovered transcripts, `-B` indicated GFF with the FungiDB reference including *A.*

fumigatus coding genes and UTR annotation, `-v` to indicate user wanted to recover only discovered transcripts that did not overlap gene +/- UTR length mean) recover transcript sequences that were not contained in possible 3' or 5' UTRs in *A. fumigatus*. Venn diagram was generated online (http://bioinformatics.psb.ugent.be/webtools/Venn/) and IGV `2.8.13` (Thorvaldsdóttir, Robinson, and Mesirov 2013) was used to visualize the results of noncoding annotation with both strategies of using reference or not in transcript assembly with StringTie. After verifying that there is consistent overlap between results of both StringTie strategies (with and without a reference annotation), we used StringTie with the `--merge` option to merge the transcriptomes into one unique annotation file.

5.2.4 Differential gene expression in response to azoles

RNA-seq data for three previously published experiments employing azoles (Furukawa et al. 2020; Marion Aruanno et al. 2021; M. Chen et al. 2020) (**Table 5**) and available on NCBI SRA were used. FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to remove adapters where present. Ribosomal sequences were cleaned with SortMeRNA v.2.1b (Kopylova, Noé, and Touzet 2012). Software check_strandedness (Signal and Kahlke 2021; L. Wang, Wang, and Li 2012) was employed in order to verify or confirm strandness of RNA-seq libraries. Cleaned reads were pseudoaligned to the FungiDB *A. fumigatus* Af293 genome release 47 (Basenko et al. 2018; Nierman et al. 2005) using kallisto 0.46.1 option quant (Bray et al. 2016) with the index generated with kallisto option index. Differential gene expression was tested with the Wald Test, as implemented in Sleuth (Pimentel et al. 2017). In order to test whether kallisto was sensitive to libraries with huge differences in read counts between compared conditions, we tested a subset of reads in bioproject PRJNA551498, for which there was a ~6-fold difference between treatment and control conditions.

5.2.5 Coexpression analysis

The coexpression analyses were carried out with the clust software (Abu-

Jamous and Kelly 2018). In summary, normalized expression matrices (TPM and TMM) were generated using the Perl script `abundance_estimates_to_matrix`.pl available as part of Trinity v2.12.0 (Haas et al. 2013). In order to select conditions to include in coexpression analyses, we analyzed the heatmaps using expression in condition (TPM) for each BIOPROJECT using the pheatmap R package. Experiments representing triplicates with adequate clustering were used in coexpression analyses. The TMM matrix was used in clust, which was employed with default parameters (normalization = 101 3 4; tightness weight = 1.0). The matrix generated with the Trinity script was used to recover genes that were either turned on or off on antifungals using the script recoverDEGthresholdFromMatrix.py, by selecting ‘TMM < 1 in untreated samples’ AND ‘TMM > 3 in azoles’ (turned on during drug exposure) and ‘TMM > 5 in untreated samples’ AND ‘TMM < 2 in azoles’ (turned off during drug exposure).

5.2.6 Data availability

Jupyter notebook with metrics used to evaluate the performance of the models of RNAsamba, R notebooks describing differential expression analyses: https://gitlab.com/SantosRAC/santosetal2021_lncrnasafumigatusantifungals

5.3 Results

5.3.1 Exploitation of public RNA-Seq projects allowed the identification of novel lncRNAs in *Aspergillus fumigatus*

Several protein-coding genes are important in antifungal response and susceptibility have been previously studied in *A. fumigatus* (dos Santos et al. 2021; Marion Aruanno et al. 2021). Although long noncoding RNAs (lncRNAs) also have potential roles in the regulation of antifungal response, much less is known about these genomic elements. In order to prospect putative lncRNAs involved in antifungal response in *A. fumigatus*, in this work we exploited publicly available RNA-Seq datasets (**Table 5**).

Table 5 - *A. fumigatus* RNA-Seq datasets used for discovery of novel putative lncRNAs, coexpression networks, and differential gene expression on azoles.

Strain	Conditions	Antifungal	Layout	Strandness	Reference	BIOPROJECT	Identifiers in this work
Af293	-	-	PAIRED	Stranded	(Stewart et al. 2020)	PRJNA601094	hyphaebrlANeg (HBN), hyphaebrlAPos (HBP), hyphaeWTNeg (HWN), hyphaeWTPos (HWP)
CEA10	-	-	SINGLE	Unstranded	(Chung et al. 2014)	PRJNA240563	deltaSrbA, deltaSrbB, WT_CEA10
Af293	-	-	SINGLE	Unstranded	(Kurucz et al. 2018)	PRJNA374516	Af_control (AFC), Fe_starved (FES), H2O2 (H22), FeSt_H2O2 (FEH)
KU80	-	-	SINGLE	Stranded	(M. Aruanno et al. 2019)	PRJNA486252	HSP90CAS, HSP90, KU80_exp2, KU80CAS
A1160 Δku80 pyrG+ / MFIG001	In total, 1 × 106 spores/ml of the wild-type (MFIG001), the nctA null or the nctB null mutant was grown in 50 ml of Vogel's minimal medium containing 1.0% glucose for 18 h at 37 °C on a rotary shaker (180 rpm). Mycelia were collected by filtration, and washed twice with distilled water. About 1.0 g of wet mycelia were transferred into 50 ml of RPMI-1640 medium containing 2.0% glucose and 165 mM MOPS buffer (pH 7.0), and the cells were incubated for 4 h in the absence and the presence of itraconazole (0.5 mg/mL) at 37 °C with shaking. The drug-treated mycelia were then collected by filtration, immediately frozen with liquid nitrogen and kept at −80 °C until use.	ITC	PAIRED	Stranded	(Furukawa et al. 2020)	PRJNA551498 (ITC2)	A1160_control (A1C), A1160_ITC (A1I)

A1160	MM liquid media for 16 h and then exposed to 1 μg ml–1 itraconazole for an additional 1 h before RNA extraction versus control, 17 h in MM liquid media)	ITC	PAIRED	Stranded	(M. Chen et al. 2020)	PRJNA633308 (ITC1)	A1160ITC_exp2 (AI2), A1160ctl_exp2 (AC2)
Ku80	Spore suspension was grown for 22 h in the absence of any drug and voriconazole was then added at the concentration of 2 μg/ml for an additional 2 h of incubation (versus control, 24 h without drug)	VCZ	SINGLE	Stranded	(Marion Aruanno et al. 2021)	PRJNA631905 (VCZ)	KU80_VCZ (KUV), KU80 (KU8), KU80R_VCZ, KU80R

To identify *A. fumigatus* lncRNAs, we trained RNAsamba, a machine learning software recently developed by our research group (Camargo et al. 2020), designed to classify sequences into coding and noncoding. The RNAsamba model we created was trained with Ensembl Fungi v.47 (comprising ascomycete fungi; details of generation of the training dataset is detailed in the methods section). In order to compare the performance of this newly created model with the original RNAsamba model, which employed human complete and truncated sequences, we compared commonly used Machine Learning statistics, including precision and recall values (sensitivity/specificity) (https://rnasamba.lge.ibi.unicamp.br/; Google Colab digital notebook). We showed that our newly created model was more appropriate, allowing a more accurate identification of lncRNAs in representatives of ascomycete and basidiomycete fungi, using our model to classify sequences of *Fusarium graminearum* (W. Kim et al. 2018) and *Serpula lacrymans* S7.9 (Borgognone et al. 2019), respectively.

In order to identify novel noncoding sequences in *A. fumigatus*, we downloaded sequencing reads from publicly available RNA-Seq experiments in contrasting conditions, to maximize the identification of populations of transcripts (**Table 5**). In summary, we cleaned and mapped RNA-Seq reads to the *A. fumigatus* Af293 reference genome (FungiDB v.47), considering all library specificities (e.g. single- or paired-end; strand specificity). Given that not all RNA-Seq experiments were strand-specific, we focused on the identification of potentially novel long noncoding RNAs (lncRNAs) in intergenic regions, based on the current available annotation of protein-coding genes. Several criteria were established to assign transcripts to lncRNA class: i) transcripts must have at least 200bp, ii) they must not match Uniprot proteins, iii) they must be assigned to class ‘noncoding’ by our RNAsamba constructed model, and iv) they must be located apart from any annotated protein-coding gene, to avoid false positives that were actually parts of gene UTRs. Regarding the latter, we first calculated the summary statistics of annotated 5’ and 3’ prime UTRs available on FungiDB (**Figure 11**). Since we analyzed the transcripts employing two different assembling strategies in StringTie2 (see methods) and both were consistent and provided very similar results, we used the overlap of these StringTie2 runs in subsequent analyses. In total, we identified 924 intergenic and 56 intronic transcripts predicted to be putative lncRNAs.

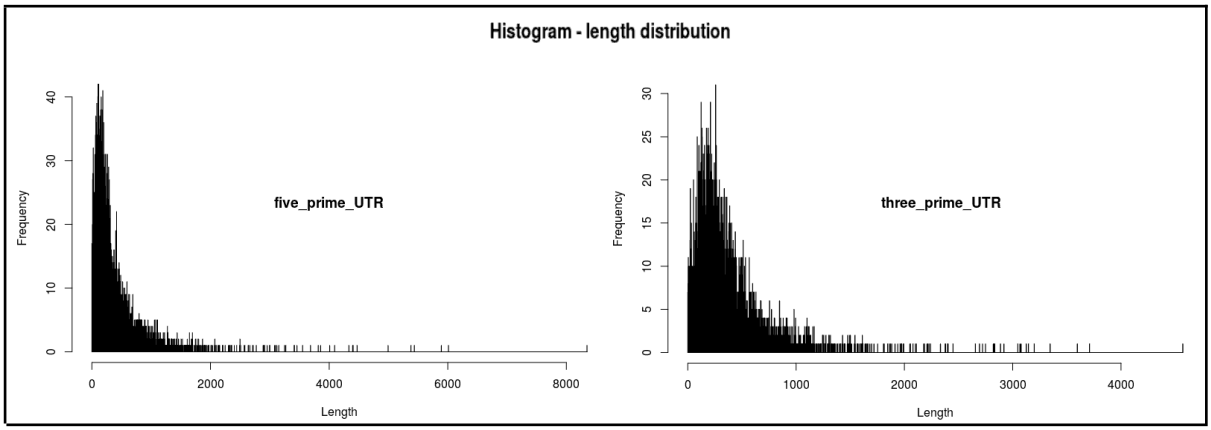

Figure 11 - Length distribution of 5' (left) and 5' (right) UTRs in *A. fumigatus* Af293, as annotated in FungiDB.

5.3.2 Several genes previously studied in azole response are mostly upregulated across different azole experiments with *Aspergillus fumigatus* strains

In order to identify genes that were differentially expressed under azole exposure, we analyzed three RNA-Seq datasets from previous studies that investigated *A. fumigatus* exposed to itraconazole (ITC) and voriconazole (VCZ). Employing the Walt Test as implemented in Sleuth under azole exposure, we obtained 699 and 807 genes that were down- and up-regulated, respectively (**Table 6**).

Table 6 - Number of differentially expressed genes (coding and putative lncRNAs) in all the compared conditions (azoles).

Gene type	**Up-regulated**	**Down-regulated**	**Azole**	**Reference / BIOPROJECT**
Coding	3,018	3,058	ITC	(Furukawa et al. 2020) / PRJNA551498
lncRNAs	154	110		
Total	3,172	3,168		
Coding	2,449	2,706	ITC	(M. Chen et al. 2020) / PRJNA633308
lncRNAs	146	18		
Total	2,595	2,724		
Coding	1,527	1,567	VCZ	(Marion Aruanno et al. 2021) / PRJNA631905
lncRNAs	60	15		
Total	1,587	1,582		

To study the putative roles of identified lncRNAs in azole response, we

first looked at a selected set of genes that were previously studied in azole response and resistance, comprising 86 genes. We selected genes that were considered significantly differentially expressed and had TMM fold changes (FCs) of at least 2 in VCZ and in at least one experiment with ITC. As expected, most differentially expressed genes were upregulated (12), whereas only two were downregulated.

To our knowledge, no previous work analyzed the intersection of differentially expressed genes in experiments comparing untreated and azole treatment. Therefore, in addition to these previously known genes, we generated a TMM matrix and recovered genes that were either turned on or off in all azoles (two experiments with ITC and one with VCZ) based on differential expression analysis and in the difference between average TMM expression between treatment and control conditions. We identified three genes consistently overexpressed across all azole experiments and one downregulated (**Table 7**).

Table 7 - Coding genes related to antifungal resistance and response that were differentially expressed in at least one experiment with ITC and in VCZ (fold change > 2) and genes turned on or off on azoles.

Venn Info	Gene name and Identifier Af293 / A1163	Description	TMM fold change			Importance
			ITC1	ITC2	VCZ	
Up	*atrR* (Afu2g02690 / AFUB_019790)	Zn_2Cys_6 Transcription Factor *atrR*	3.73	2.75	2.01	Literature
	abcC (Afu1g14330 / AFUB_013880)	ABC Transporter *abcG1* / *cdr1B* / *abcC*	2.99	2.79	2.71	Literature
	erg3 (Afu2g00320 / AFUB_017380)	C5 sterol desaturase *erg3*	1.66	2.46	8.51	Literature
	abcA (Afu2g15130 / AFUB_030790)	ABC Transporter *abcA*	1.58	17.88	11.08	Literature
	mdr1 (Afu5g06070 / AFUB_053630)	Transporter *mdr1*	4.17	3.34	4.99	Literature
	erg6 (Afu4g03630 / AFUB_099400)	Putative sterol 24-C-methyltransferase *erg6*	3.51	9.65	3.16	Literature
	atrI (Afu3g07300 / AFUB_041770)	Has domain(s) with predicted ATP binding, ATPase activity, ATPase activity, coupled to transmembrane movement of substances, nucleoside-triphosphatase activity, nucleotide binding activity and role in transport	2.58	3.65	2.31	Literature
	mfsC (Afu1g03200 / AFUB_003610)	MFS Transporter *mfsC*	3.58	4.08	8	Literature
	erg24 / *erg24A* (Afu1g03150 / AFUB_003560)	Putative C-14 sterol reductase *erg24*	2.14	2.57	6.92	Literature
	No gene name (Afu8g02110 /	No InterProScan matches; no significant Pfam matches; BLAST match only in *A. fumigatus* Af293, with low percent identity and coverage in	30.27	27.66	16	This work (on/off)

	AFUB_084500)	other few strains				
	No gene name (Afu3g00480 / AFUB_047980)	Has domain(s) with predicted role in response to stress and integral component of membrane localization (FungiDB). Pfam domain: RTA1 like protein (RTA1) This family is comprised of fungal proteins with multiple transmembrane regions. RTA1 (P53047) is involved in resistance to 7-aminocholesterol [1] while RTM1 (P40113) confers resistance to an an unknown toxic chemical in molasses [2]. These proteins may bind to the toxic substance, and thus prevent toxicity. They are not thought to be involved in the efflux of xenobiotics [1].	17.15	10.63	13	This work (on/off)
	No gene name (Afu1g17620 / AFUB_017000)	IPR018704 The tetratrico peptide repeat region (TPR) is a structural motif present in a wide range of proteins. It mediates protein-protein interactions and the assembly of multiprotein complexes. The TPR motif consists of 3-16 tandem-repeats of 34 amino acids residues, although individual TPR motifs can be dispersed in the protein sequence. Sequence alignment of the TPR domains reveals a consensus sequence defined by a pattern of small and large amino acids. TPR motifs have been identified in various different organisms, ranging from bacteria to humans. Proteins containing TPRs are involved in a variety of biological processes, such as cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, neurogenesis and protein folding. Pfam domain: Tetratricopeptide repeat (TPR_12)	6.73	9.19	3.71	This work (on/off)
Down	*mfsB* (Afu1g15490 / AFUB_015030)	Putative MFS Transporter	-2.10	-1.65	-4.14	Literature
	No gene name (Afu4g13090 / AFUB_069990)	Pfam domain: Major Facilitator Superfamily (MFS_1)	-3.92	-4.14	-3.43	This work (on/off)

To recover genes that showed highest differences in expression, we further filtered genes based on the FC and selected those in which at least one ITC and the VCZ experiment. Among these genes, we identified one overexpressed transcription factor (*atrR*), four transporters (*abcC, abcA, mdr1,* and *mfsC*), three genes in the ergosterol biosynthesis pathway (*erg3*, *erg6*, and *erg24*). Among the most relevant downregulated genes, we identified one transporter.

Concerning genes that were “turned on” on azoles, we identified Afu8g02110, Afu3g00480, and Afu1g17620. Curiously, a BLASTp search with Afu8g02110 sequence against GenBank identified a homolog with similar sequence only in *A. fumigatus* CNM-CM8714, recently sequenced by our group (Renato A. C. Dos Santos et al. 2020). Afu3g00480 is described in the FungiDB as related to stress response and encoding an integral membrane component. According to Pfam, a RTA1 domain (PF04479) is present in the protein and is related to resistance to chemicals. In Afu1g17620, InterProScan identified tetratrico peptide repeats (Pfam TPR_12). Proteins with this domain are usually associated with protein-protein interactions and assembly of multiprotein complexes. Among genes that were “turned down”, we identified one putative transporter (MFS domain).

5.3.3 Some of the identified lncRNAs are probably involved in *A. fumigatus* regulation during azole exposure

We next aimed to identify lncRNAs that were involved in response to azole antifungals. Among the differentially expressed genes, we didn’t find any intronic lncRNAs differentially expressed. Among intergenic lncRNAs, we identified 15 that were up-regulated in all azole experiments and only one was down-regulated (**Table 8**). Since most lncRNAs in fungi are known to be involved in *cis* regulatory mechanisms (J. Li et al. 2021), we identified genes that were nearby. Interestingly, these lncRNAs were usually located near other genes involved in different cellular functions.

Table 8. Putative lncRNAs that were differentially expressed in at least one experiment with ITC and in VCZ (fold change > 2).

DE	lncRNA	Chromosome	Coordinates and strand[a]	Genes located close to lncRNAs	Length	Differential expression - fold change (mean TMM drug/control)		
						ITC1	*ITC2*	*VCZ*
Up	MSTRG.51	Chr1	826145-827485(+)	Afu1g02870 (Ortholog(s) have role in negative regulation of cellular response to oxidative stress), Afu1g02880	1,340	4.38	6.53	7.22
	MSTRG.105		2952905-2953938(-)	Afu1g11210, Afu1g11220 (Predicted adhesin-like protein)	1,033	2.30	2	2.81
	MSTRG.121		3518467-3520085(+)	Afu1g13240 (Ortholog(s) have adenine deaminase activity), Afu1g13250 (Putative phospholipase C)	1,618	2.99	2.51	3.27
	MSTRG.129		3779715-3780727(-)	Afu1g14140 (Has domain(s) with predicted dolichyl-phosphate-mannose-glycolipid alpha-mannosyltransferase activity and role in GPI anchor biosynthetic process), Afu1g14150	1,012	1.37	4.44	2.81
	MSTRG.195	Chr2	1339554-1341065(-)	Afu2g04880, Afu2g04890	1,511	1.57	3.03	2.06
	MSTRG.408	Chr3	2557740-2559460(+)	Afu3g09960 (Putative aureobasidin resistance protein), Afu3g09970 (Ortholog(s) have ferrous iron transmembrane transporter activity, manganese ion transmembrane transporter activity)	1,720	1.61	3.39	5.94
	MSTRG.449		3760078-3761857(-)	Afu3g14140 (Predicted metacaspase, an aspartate-specific cysteine protease involved in apoptosis), Afu3g14150 (F-box protein 15)	1,779	3.10	2.43	2.50
	MSTRG.528	Chr4	3022788-3026044(-)	Afu4g11480 (Ortholog(s) have role in cellular potassium ion homeostasis), Afu4g11490 (Ortholog of *Aspergillus fumigatus* A1163 : AFUB_068510)	3,256	2.39	2.95	2.85
	MSTRG.754	Chr6	1967680-1968658(+)	Afu6g08400 (Ortholog of Aspergillus fumigatus A1163	978	11.23	2.33	2.62

				: AFUB_074360), Afu6g08410 (Has domain(s) with predicted nucleus localization)				
	MSTRG.805		3584785-3585211(+)	Afu6g14050 (Has domain(s) with predicted UDP-N-acetylmuramate dehydrogenase activity, flavin adenine dinucleotide binding activity and role in oxidation-reduction process), Afu6g14060 (protein of unknown function)	426	3.68	2.36	3.46
	MSTRG.829	Chr7	393862-394921(-)	Afu7g01500 (Ortholog of Aspergillus fumigatus A1163 : AFUB_088070), Afu7g01510 (Ortholog(s) have role in ascospore-type prospore membrane assembly, vesicle-mediated transport and Golgi apparatus, cell division site, cell tip, endosome, hyphal tip, plasma membrane raft, prospore membrane leading edge localization)	1,059	7.84	1.70	2.26
	MSTRG.830		239268-240480(+)	Afu7g00900 (protein of unknown function), Afu7g00910 (Putative oligopeptide transporter)	1,212	1.92	6.84	4.74
	MSTRG.843		876129-876898(-)	-	769	1.46	2.99	2.25
	MSTRG.908	Chr8	295875-297544(-)	Afu8g01260 (protein of unknown function), Afu8g01265 (protein of unknown function)	1,669	34.77	3.07	4.76
	MSTRG.951		1508639-1509397(-)	Afu8g06300 (Ortholog of Aspergillus fumigatus A1163 : AFUB_078930), Afu8g06310 (Has domain(s) with predicted zinc ion binding activity and role in lipid metabolic process)	758	4.20	4.96	2.28
Down	MSTRG.247	Chr2	2730768-2731775(-)	Afu2g10670 (Has domain(s) with predicted plasma membrane localization), Afu2g10680 (Ortholog of *Aspergillus fumigatus* A1163 : AFUB_026460)	1,007	-6.02	-2.19	-2.46

[a]genomic coordinates are based on the *A. fumigatus* Af293 assembly used for analyses of mapping and transcript assembly (FungiDB v.47).

However, due to the difficulty of assigning functions to the identified lncRNAs, we decided to analyze patterns of gene co-expression. For this purpose, we employed the clust software, which clusters genes that present similar patterns of gene expression. Genes that are grouped within the same clusters tend to have functional relationships (e.g., transcription factors and gene targets). In this sense, clust was able to identify lncRNAs that were possibly involved in positive regulation of target genes.

We identified ten clusters of co-expressed genes (**Figure 12**). Among them, eight presented either lncRNAs differentially expressed in all azole comparisons (four clusters), a gene that was turned off (Afu4g13090, in cluster C4), or genes relevant for azole response or resistance (five clusters). Interestingly, the C8 cluster presented a larger number of lncRNAs (70) relative to the number of other genes (455). Among them, four putative lncRNAs differentially expressed in all azole experiments were present (MSTRG.129, MSTRG.408, MSTRG.449, MSTRG.843). This cluster also had genes related to azole response / resistance.

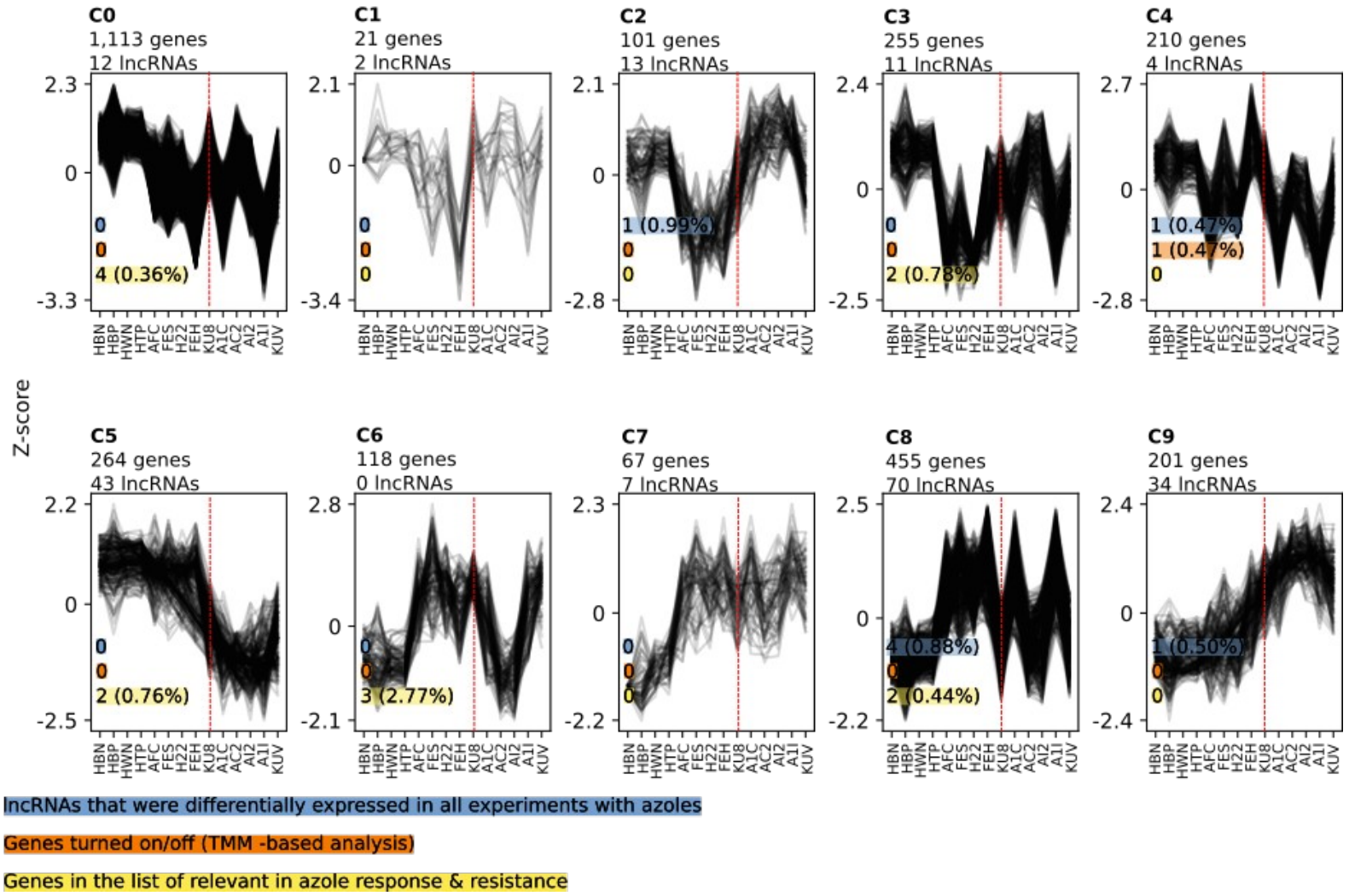

Figure 12 - Coexpression clusters generated with the clust software. TMM values were used as input to the algorithm and genes with similar patterns were grouped in ten clusters. Different categories are highlighted below (blue, orange, and yellow).

5.4 Discussion

Aspergillus fumigatus is an important opportunistic pathogen that infects millions of people annually (David W. Denning et al. 2016; David W. Denning, Pleuvry, and Cole 2013). The main treatment for Invasive Aspergillosis, caused by *A. fumigatus*, including closely related cryptic species and species in other sections, is the therapy with antifungals. The primary class comprises the azoles (Pérez-Cantero et al. 2020). Understanding the molecular mechanisms involved in response to azoles is important to comprehension of resistance to azoles, development of novel drugs. Previous works with antifungals analyzed the gene expression, but none of previous research has explored the long noncoding RNAs in response to azoles. This class of ncRNAs is involved in different processes, in particular in regulatory mechanisms in gene expression involving histone modification, chromatin remodeling, transcriptional interference, nucleosome repositioning, promoter occlusion, among others, with most lncRNAs known to act via *cis* regulation (J. Li et al. 2021). Only a few lncRNAs have been functionally characterized in fungi, and most of our knowledge about the role of these elements in drug susceptibility come from two studies in *Schizosacharomyces pombe* (Ard, Tong, and Allshire 2014) and in *Candida auris* (Gao et al. 2021).

In the present study, we searched the literature for RNA-Seq experiments of growth of *A. fumigatus* on azoles for which at least three replicates were available. For instance, even though (Hagiwara et al. 2017) ran transcriptome experiments of *A. fumigatus* Af293 responses to fluconazole and miconazole, they did not include replicates. Similarly, (Hokken, Zoll, et al. 2019) studied time points over which gene expression was analyzed in response to itraconazole; however, they only included duplicates. We also avoided experiments that tested the response to azoles using mutants. In Hagiwara et al. (2017), they generated null mutants for both *srbA* and *atrR*, and they were not considered in this work because we were interested in the response to azoles in wild type *A. fumigatus* strains. One study that we included that involves mutants is (Marion Aruanno et al. 2021); however, we only used their

analyses which included the wild-type strains under azole (or untreated). Recently, (Du et al. 2021) identified a C2H2 transcription factor, *SltA*, which is involved in regulation of genes in response to itraconazole in *A. fumigatus*, including *cyp51A*, *erg13A*, and *erg24A* (ergosterol biosynthesis) and *mdr1*, *mfsC*, and *abcE* (transporters). Even though this study involved RNA-Seq, we had already concluded our analyses (this dataset might be included in future analyses).

As expected several genes involved in ergosterol biosynthesis, transporters, and transcription factors were differentially expressed and showed a significant change in gene expression. For instance, *erg3* (Afu2g00320), *erg24* (Afu1g03150), *abcA* (Afu2g15130), *mdr1* (Afu5g06070), *mfsC* (Afu1g03200) were previously identified as overexpressed on voriconazole in *A. fumigatus* (Marion Aruanno et al. 2021). Similarly, the analysis of exposure of *A. fumigatus* to itraconazole in (M. Chen et al. 2020) revealed genes such as *erg3*, *atrR* (Afu2g02690), *abcC* (Afu1g14330), *abcA*, and *mdr1* as overexpressed. Our analyses are in agreement with previous studies that employed these datasets. Therefore, we were able to ask novel questions to these RNA-Seq experiments.

First, we identified genes that were either turned on or off on azoles. Interestingly, we identified three genes that were turned off on. One of the genes, Afu8g02110, encodes a protein of unknown function. According to FungiDB (Basenko et al. 2018), no ortholog has been identified in other fungal species, and searches against Pfam and InterProScan results no conserved domains; moreover, a BLASTp search against NCBI identifies a few *A. fumigatus* strains with homologs, including the cellulolytic strain Z5 (D. Liu et al. 2013) and CNM-CM8714, which has been recently sequenced by our research group and showed increased resistance to different azoles (Renato A. C. Dos Santos et al. 2020). Afu3g00480 encodes a protein with a RTA1 domain, which has been involved in resistance to 7-aminocholesterol (an antifungal that inhibits sterol biosynthesis) in the budding yeast *Saccharomyces cerevisiae*. During evaluation of gene expression of *A. fumigatus* over time points in response to itraconazole, (Hokken, Zoll, et al. 2019) identified two other genes with RTA1 domain that were strongly overexpressed. Another gene

turned was Afu1g17620, which contains tetratrico peptide repeats. Interestingly, this gene was recently identified with a significant SNP in a treeWAS analysis including populations of *A. fumigatus* in a study involving evolution of azoles in patients in the environment (Rhodes et al. 2021). Only one coding gene was turned down. Afu4g13090 (AFUB_069990), which contains a MFS transporte, for our knowledge has not been studied yet in the context of antifungal response .

To identify possible functions of the lncRNAs identified in *A. fumigatus*, we employed the software clust (Abu-Jamous and Kelly 2018) to identify patterns of co-expression. Interestingly, we identified four lncRNAs that were co-expressed with various other genes, including two which may be involved in response or resistance to azoles. It is important to note that this method is able to identify positive correlation in co-expression patterns, and therefore raises putative candidates of positive regulators (of elements in the same cluster). However, it is important to emphasize that several lncRNAs are known to be involved in negative regulatio. Future analyses with softwares such as WCGNA (Langfelder and Horvath 2008) and Conekt (Proost and Mutwil 2018) will be able to identify coexpressed genes that also present negative correlation (putative negative regulation).

It is important to note that RNA-Seq data is increasingly available. Besides the reported RNA-Seq projects in *A. fumigatus* that showed clear application in the discovery of novel possible functional elements, a recent study also described the comparative genomics of several species in section *Fumigati*, including the transcriptomic analysis of some cryptic species such as *A. lentulus* and *A. udagawae* (Takahashi et al. 2021). Future work will be able to use these available datasets in comparative transcriptomic analysis (e.g., evolutionary conservation of lncRNAs in section *Fumigati*).

6 Conclusion and Perspectives

This thesis covered several topics around aspergillosis diseases and addressed research questions concerning *A. fumigatus* and closely related species, employing genomics and transcriptomics. All five chapters approached antifungal susceptibility in *Aspergillus* of section *Fumigati*, in particular due to the concerns about antifungal resistance that have been increasing worldwide. By sequencing the genomes and phenotyping several Spanish clinical strains (from different host diseases and conditions) of *A. fumigatus* and the cryptic species *A. lentulus* and *A. fumigatiaffinis*, all in series *Fumigati*, we addressed the question of whether genomic and phenotypic heterogeneity of antifungal susceptibility and virulence existed intra- and interspecifically (Renato A. C. Dos Santos et al. 2020). These results have implications in both clinical identification and in our understanding about drug susceptibility and evolution. For instance, the first published manuscript (chapter 2) had a great impact and was greatly summarized by Dr. Bridget Barker in the editorial article describing manuscripts submitted to the Frontiers in Genetics special edition ("Research Topic") (Barker, Cuomo, and Govender 2021). Moreover, the genomes described in our manuscripts (chapters 2 and 3) were recently employed in a comparative genomic analysis of *Aspergillus* section *Fumigati* that studied biosynthesis gene clusters, also contributing by sequencing novel genomes in *Fumigati* (Takahashi et al. 2021).

Following the analyses on genomic and phenotypic diversity with specific strains in Series *Fumigati* and looking at the drug targets for azoles and echinocandins, evolutionary questions about antifungal resistance were also explored (chapter 4). To answer questions concerning a broader timescale, strains of species in the whole section *Fumigati* were analyzed with codon-based models, and the results raised insights on possible selective pressures imposed by echinocandins used in clinics today and those naturally occurring in the environment, as well as of azoles, that are synthetic antifungals that have been used recently in agriculture and clinics.

As the main conclusions, the following topics are highlighted:

- The phenotypic analyses revealed a high heterogeneity in antifungal susceptibility and virulence across strains of *A. fumigatus, A. fumigatiaffinis*, and *A. lentulus*.
- Analysis of mutations in targets of antifungal, *cyp51A* and *fks1*, identified *A. fumigatus* strains with known important mutations concerning drug resistance, and mutations that were unique to cryptic species.
- Orthogroup analysis and phylogenetics of protein sequences revealed a third paralog in *A. fumigatiaffinis*, *cyp51C*, that is not found in other sequenced species in section *Fumigati*, that could be related to azole resistance.
- Signatures of natural selection across important genes in sequenced species of section *Fumigati* revealed sites that did not overlap known resistance mutations, including *cyp51A*, suggesting an ancient selective pressure that probably differs from those of current strains and species.
- Differently from *cyp51A*, *fks1* which is the main target of echinocandins showed sites under selection, suggesting the existence of an ancient selective pressure, supported by the presence of analogous natural compounds that could have existed in the past.
- Transcriptome analysis showed evidence of the presence of lncRNAs distributed in intergenic and intronic regions, compared to the currently available *A. fumigatus* genome annotation.
- Differential gene expression under exposure of *A. fumigatus* to different azoles revealed novel candidates involved in drug response including coding genes and lncRNAs.

These results improve our understanding of the biology and evolution of *A. fumigatus* with impact on the clinical context. The main perspectives for the transcriptomic analyses include:

- Validation of gene expression for candidates in both coding and noncoding

classes of genes differentially expressed, using RT-qPCR.

- Analysis of co-expression networks using methods that predict co-expression networks for identifying possible co-regulation relationships between lncRNAs and their possible targets.

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8 Attachments

8.1 Document for bioethics and/or biosecurity

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "**Análise genômica de espécies crípticas de *Aspergillus***", desenvolvida no Programa de Pós-Graduação em Biociências e Tecnologia de Produtos Bioativos do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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