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Avian-associated *Aspergillus fumigatus* displays broad phylogenetic distribution, no evidence for host specificity, and multiple genotypes within epizootic events

Lotus A. Lofgren [1,2,3,*], Jeffrey M. Lorch [4], Robert A. Cramer [5], David S. Blehert [4], Brenda M. Berlowski-Zier [4], Megan E. Winzeler [4], Cecilia Gutierrez-Perez [5], Nicole E. Kordana [5], Jason E. Stajich [1,2,*]

[1]Department of Microbiology and Plant Pathology, University of California Riverside, Riverside, CA 92521, USA
[2]Institute for Integrative Genome Biology, University of California Riverside, Riverside, CA 92521, USA
[3]Department of Biology, Duke University, Durham, NC 27708, USA
[4]U.S. Geological Survey—National Wildlife Health Center, Madison, WI 53711, USA
[5]Department of Microbiology and Immunology, Geisel School of Medicine, Dartmouth College, Hanover, NH 03755, USA

*Corresponding author: Department of Biology, Duke University, Durham, NC 27708, USA. Email: lotuslofgren@gmail.com; *Corresponding author: Department of Microbiology and Plant Pathology, University of California Riverside, Riverside, CA 92521, USA. Email: jason.stajich@ucr.edu

Abstract

Birds are highly susceptible to aspergillosis, which can manifest as a primary infection in both domestic and wild birds. Aspergillosis in wild birds causes mortalities ranging in scale from single animals to large-scale epizootic events. However, pathogenicity factors associated with aspergillosis in wild birds have not been examined. Specifically, it is unknown whether wild bird-infecting strains are host-adapted (i.e. phylogenetically related). Similarly, it is unknown whether epizootics are driven by contact with clonal strains that possess unique pathogenic or virulence properties, or by distinct and equally pathogenic strains. Here, we use a diverse collection of *Aspergillus fumigatus* isolates taken from aspergillosis-associated avian carcasses, representing 24 bird species from a wide geographic range, and representing individual bird mortalities as well as epizootic events. These isolates were sequenced and analyzed along with 130 phylogenetically diverse human clinical isolates to investigate the genetic diversity and phylogenetic placement of avian-associated *A. fumigatus*, the geographic and host distribution of avian isolates, evidence for clonal outbreaks among wild birds, and the frequency of azole resistance in avian isolates. We found that avian isolates were phylogenetically diverse, with no clear distinction from human clinical isolates, and no sign of host or geographic specificity. Avian isolates from the same epizootic events were diverse and phylogenetically distant, suggesting that avian aspergillosis is not contagious among wild birds and that outbreaks are likely driven by environmental spore loads or host comorbidities. Finally, all avian isolates were susceptible to Voriconazole and none contained the canonical azole resistance gene variants.

Keywords: *Aspergillus fumigatus*; azole resistance; avian; bird; population genomics

Introduction

Aspergillus fumigatus is one of the most common causative agents of animal-associated fungal infections (Seyedmousavi *et al.* 2015). The fungus is capable of causing a suite of syndromes ranging from allergy to fatal invasive infections in hosts as phylogenetically diverse as humans, harbor seals, honey bees, sea fans, and birds (Tekaia and Latgé 2005; Seyedmousavi *et al.* 2015; Pang *et al.* 2021). Invasive *Aspergillus* infection (aspergillosis) in humans is a growing global health concern, effecting an estimated 300,000 people per year (Leading International Fungal Education 2021). Aspergillosis causes up to 90% mortality in individuals with compromised immune systems and comorbidities such as leukemia, lung or other organ transplants, obstructive pulmonary diseases, autoimmune disorders, cirrhosis of the liver, influenza, and COVID-19 (Vanderbeke *et al.* 2018; Latgé and Chamilos 2019; Koehler *et al.* 2020). Frontline treatment options are centered on the use of azole antifungals, but the effectiveness of these treatments is under threat by the increasing prevalence of triazole-resistant strains (Howard *et al.* 2006).

Unlike human aspergillosis, avian aspergillosis can manifest as a primary infection, mainly in the respiratory system, but systemic infections have also been documented in skin, eyes, liver, kidney, spleen, heart, brain, ovary, joints, and bone (Beernaert *et al.* 2010; Leishangthem *et al.* 2015). It has been proposed that birds are particularly susceptible to aspergillosis due to physiological and immunological differences such as limited capacity for mucociliary clearance (lack of ciliated epithelium), the presence of an oxygen-rich air sac system with scarce immune surveillance cells, and a lack of an epiglottis or diaphragm to block inhalation and expulsion of spores (Tell 2005; Tell *et al.* 2019). Avian aspergillosis includes both acute and chronic forms, which are thought to be driven by different underlying etiologies. Whereas acute infections primarily affect young birds and are thought to be associated with high levels of spore inhalation,

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chronic infection is associated with adult animals and various levels of immunosuppression (Cacciuttolo *et al.* 2009). Also unique among birds is the high prevalence of conidiation during infection (Tell *et al.* 2019), increasing the potential dissemination of infective strains via fecal matter and the decay of infected carcasses, particularly for migratory birds with wide geographic ranges (Melo *et al.* 2020b).

Avian aspergillosis is overwhelmingly caused by the species *A. fumigatus*, and infrequently by other *Aspergilli* including *Aspergilli flavus*, *Aspergilli niger*, *Aspergilli glaucus*, and *Aspergilli nidulans* (Beernaert *et al.* 2010; Sabino *et al.* 2019). Aspergillosis is known to affect both captive and wild birds (Talbot *et al.* 2018; Melo *et al.* 2020a), but has been most studied in captive populations, where it causes high morbidity and mortality, and significant economic losses (Arné *et al.* 2011). Poultry farms can provide ideal conditions for *Aspergillus* growth and infection, including the presence of cellulose-rich feedstuffs and bedding material, warmth, high humidity, high nutrient levels, and overcrowding that facilitates the transmission of disease and the inhalation of fungal spores (Cafarchia *et al.* 2014). In addition to providing a suitable environment for the growth of spores arriving from ambient air sources, high densities of spores may arrive in the form of contaminated bedding or feedstuffs (Arné *et al.* 2011; Aliyu *et al.* 2016).

In wild birds, aspergillosis is understudied and infection sources are less well understood. Aspergillosis deaths in wild birds range from isolated cases affecting a single individual to large epizootic events affecting thousands of individuals (Neff 1955; McDougle and Vaught 1968; Melo *et al.* 2020a). Previous work has shown that *A. fumigatus* is a common contaminant of animal feed such as silage (Seyedmousavi *et al.* 2015) and grain (Zulkifli and Zakaria 2017), and epizootic aspergillosis events in wild ducks and geese are suspected to result from exposure to these contaminated feed sources (Bellrose and Hanson 1945; Neff 1955). Community analysis of contaminated grain has shown the simultaneous cocolonization by multiple species of *Aspergillus* (Zulkifli and Zakaria 2017). However, it is unknown whether each fungal species is represented primarily by homogeneous (clonal) populations, or by more diverse isolate pools. Similarly, it is unknown whether epizootic infections are driven by contact with clonal *A. fumigatus* that possess unique pathogenesis or virulence properties, or if epizootic events are associated with diverse fungal populations and driven primarily by environmental and host life-history factors, such as high spore load or poor host immune status. In addition, stress is likely to play an important role in chronic aspergillosis infections, including stress induced by migration (Ewbank *et al.* 2021), pollution (Daoust *et al.* 1998; Jung *et al.* 2009), smoke inhalation (Kinne *et al.* 2010), and malnutrition (Redig *et al.* 1980; Deem 2003; Gulcubuk *et al.* 2018).

The increasing prevalence of azole-resistant strains of *A. fumigatus* is a growing global concern (Chowdhary *et al.* 2013; Sewell *et al.* 2019; Duong *et al.* 2021). Azoles are used in the poultry industry as a decontamination agent, occasionally as a prophylactic agent, and as a prophylactic or therapeutic agent for captive wild birds kept as pets, in zoos, or in wildlife rehabilitation centers (Bunting *et al.* 2009; Krautwald-Junghanns *et al.* 2015). Because long-term azole treatment has been associated with acquired *A. fumigatus* azole resistance in human patients (Dannaoui *et al.* 2001), the habitual use of azoles as prophylactic agents for avian species raises concerns that birds could play a role in both the acquisition of resistant variants and the dissemination of resistant strains (Beernaert *et al.* 2009; Melo *et al.* 2020b).

Here, we investigate 51 isolates of *A. fumigatus* isolated from avian carcasses with aspergillosis. These isolates were collected from 24 species of birds from across the United States (plus 1 isolate from New Zealand) and represented both individual bird mortalities and epizootic events. These 51 isolates were sequenced and analyzed along with 130 phylogenetically diverse human clinical isolates for which genome data was publicly available to assess: (1) the genetic diversity and phylogenetic placement of avian associated *A. fumigatus* relative to human clinical isolates, (2) whether there were any phylogenetic relationships based on geography or host species, (3) whether epizootics were associated with clonal strains among wild bird populations (with implications for a common inoculum source and differential virulence between strains), and (4) the presence of mutations in antifungal resistance genes, and the frequency of Voriconazole resistant phenotypes in avian isolates.

Methods

Isolate information

Fifty-one isolates of *A. fumigatus* were collected from postmortem avian sources between December 2014 and October 2019 from locations across the United States (Fig. 1a). One of the strains, ICMP_23421, was isolated from a kākāpō parrot [Strigops habroptila in New Zealand and was obtained from the National Center for Biotechnology Information (NCBI; BioProject no. PRJNA726267)]. Including the kākāpō strain, our avian dataset represented 24 bird species, classified into 4 host ecology categories, including freshwater birds ($n = 39$), sea birds ($n = 4$), raptors ($n = 7$), and parrot ($n = 1$), and included 16 isolates from cases of individual birds with aspergillosis, 28 isolates from epizootic events (10 events in total, and 7 events where multiple isolates were taken from the same event), and 3 isolates from potential epizootic events (3 separate events) (Fig. 1, Supplementary Table 1). Event size ranged from 2 to 300 individuals, with an average of 82 (Supplementary Table 2). Forty-eight of the 51 isolates originated from birds for which primary aspergillosis was diagnosed as the cause of death; the remaining 3 isolates represented suspected cases of secondary infections. Isolates were taken from a total of 5 tissue types including neck muscle ($n = 1$), heart ($n = 3$), liver ($n = 9$), lung ($n = 17$), and air sacs ($n = 18$). Two isolates were taken from pooled samples of lung and air sac tissue. In addition to the 51 avian-derived isolates noted above, sequence data for 130 publicly available *A. fumigatus* isolates were obtained from the NCBI's Sequence Read Archive (SRA) for population genomic comparison (Supplementary Table 3). These represented human clinical isolates from 8 distinct geographic origins including the United States, United Kingdom, Spain, India, The Netherlands, Canada, Japan, and Portugal.

Genome sequencing

Biomass was collected from cultures grown for 24 h in liquid LGMM + YE media, flash frozen in liquid nitrogen, and lyophilized for approximately 10 h. Lyophilized biomass was homogenized using glass beads, treated with RNAse, and extracted using Phenol: Chloroform: Isoamyl alcohol. DNA concentration was quantified using a Qubit 2.0 Fluorometer (Invitrogen, Waltham, MA, USA) with the Broad Range protocol and stored at −20°C until sequencing. Illumina paired-end DNA sequencing libraries were prepared at the UCR Institute for Integrative Genome Biology Genomics Core using the NEBNext Ultra II Library Prep Kit (New England Biolabs, Ipswich, MA, USA) following manufacturer recommendations for paired-end library construction and barcoding for multiplexing. Genome sequencing was carried out on an Illumina NovoSeq 6000 machine at the University

Fig. 1. Source information for avian-derived *A. fumigatus* isolates. a) State of collection for the 50 U.S. isolates used in this study. Location mapping represents centroid coordinates for each state, jittered to avoid overlap. Note, isolate ICMP-22421 collected from a kākāpō parrot in New Zealand is not depicted on the map. b) Phylogenetic distribution of the 51 avian-derived isolates used in this study, highlighting the lack of correlation between host group or year of collection and phylogenetic placement.

of California Berkeley Vincent J. Coates Genomics Sequencing Laboratory. All new genome data generated as part of this project were deposited into the NCBI SRA under BioProject no. PRJNA773725.

Population genomics

Sequence reads for each strain were aligned to the *A. fumigatus* Af293 reference genome downloaded from FungiDB v.39 (Basenko *et al.* 2018), using BWA v0.7.17 (Li and Durbin 2009) with the "mem" aligner. The alignments were processed with samtools v1.10 (Li *et al.* 2009) using the fixmate and sort commands to store alignments in the CRAM format. Duplicate reads were removed using MarkDuplicates from the picard toolkit v2.18.3 (http://broadinstitute.github.io/picard, accessed Aug 5, 2021). Alignments were further processed to realign reads around insertion/deletions to reduce false-positive variant calls using RealignerTargetCreator and IndelRealigner in GATK v3.7 (Van der Auwera *et al.* 2013). Single Nucleotide Polymorphisms (SNP) and short Insertion/Deletion (INDEL) variants were genotyped using HaplotypeCaller in GATK v4.0, to create a GVCF file for each strain, which were later combined with the GenotypeGVCFs tool to produce a Variant Call Format (VCF) file. A subsequent VCF of high quality variants was selected by filtering variants using GATK's SelectVariants and parameters indicated for GATK Best Practices (window-size = 10, QualByDept <2.0, MapQual <40.0, QScore <100, MapQualityRankSum <-12.5, StrandOddsRatio > 3.0, FisherStrandBias >60.0, ReadPosRankSum <-8.0. for SNPs, and window-size = 10, QualByDepth <2.0, MapQualityRankSum <-12.5, StrandOddsRatio >4.0, FisherStrandBias >200.0, ReadPosRank <-20.0, InbreedingCoeff <-0.8 for INDELs). These variants were further trimmed by intersecting the VCF with locations of transposable elements (TEs) annotated in FungiDB to remove those overlapping TEs using BEDtools subtract (Quinlan and Hall 2010). The effect of variants overlapping genes were annotated with snpEff (Cingolani *et al.* 2012). The resulting VCF file of variants were analyzed using a set of custom scripts in the R programing language. Pipelines for VCF production and all scripts used in data analysis are available from the Git repository for this project.

Phylogenomics

Aligned SNPs from all isolates were used to construct a phylogeny using the Maximum Likelihood algorithm in IQ-TREE v2.1.1 (Nguyen *et al.* 2015), with a +ASC to use the ascertainment bias correction model for SNP data. The GTR+F+ASC nucleotide substitution model was chosen based on Bayesian information criteria (BIC) tested by the ModelFinder function in IQ-TREE. Confidence in the phylogenetic tree was assessed by 1000 bootstraps using UFBoot ultrafast bootstrapping approximation (Hoang *et al.* 2018) on the 94,704 parsimony informative sites. Tree visualization and variant mapping were carried out using the R packages *ggtree* (Yu 2020), *ape* (Paradis and Schliep 2019), and *phytools* (Revell 2012).

Antifungal resistance

We screened avian isolates of *A. fumigatus* for previously characterized antifungal resistance genes mentioned in the literature and MARDy database for this species (http://mardy.dide.ic.ac.uk) (13 genes in total, Supplementary Table 4). Specific variant scans were conducted on 6 genes containing characterized amino acid

changes known to facilitate resistance, as well as whole gene analysis of all amino acid changing variants across these genes and an additional 7 genes associated with resistance but lacking a characterized mechanism. Variant scans were accomplished using a set of custom scripts in R and mapped onto the phylogeny with the packages *ggplot2* and *ggtree*.

Voriconazole susceptibility was performed as described in the CLSI M38 microbroth dilution method (Clinical and Laboratory Standards Institute 2017). Briefly, 1×10^4 conidia per well were incubated in a 96-well plate using RPMI 1640 culture medium (with glutamine, without bicarbonate, and with phenol red as a pH indicator). Spores were treated with a serial dilution of Voriconazole (1–0 μg/ml) and plates were incubated at 37°C and 5% CO_2 for 48 h before being assessed for the presence or absence of visible growth. This process was repeated a total of 4 times across 23 avian isolates, representing different combinations of the unique variants occurring in resistance genes identified above, plus the control strains Af293 and CEA10 (Supplementary Table 5).

Results

The phylogeny of *A. fumigatus* avian isolates demonstrated no clustering by avian host species or year of collection (Fig. 1b). The 181-strain phylogeny showed that avian isolates were broadly distributed across the *A. fumigatus* phylogeny, and routinely interleaved with human clinical isolates (Fig. 2). Confidence in strain placement for most isolates was high, with most bipartitions representing >95% bootstrap support. Avian isolates also displayed no obvious geospatial relationships, with isolates from the same state falling in disparate locations across the tree. Seven of the 10 epizootic events were represented by multiple isolates. Isolates from the same event clustered together in only 2 of the 7 cases, and in both cases the clusters contained only some of the isolates in each event (Fig. 2).

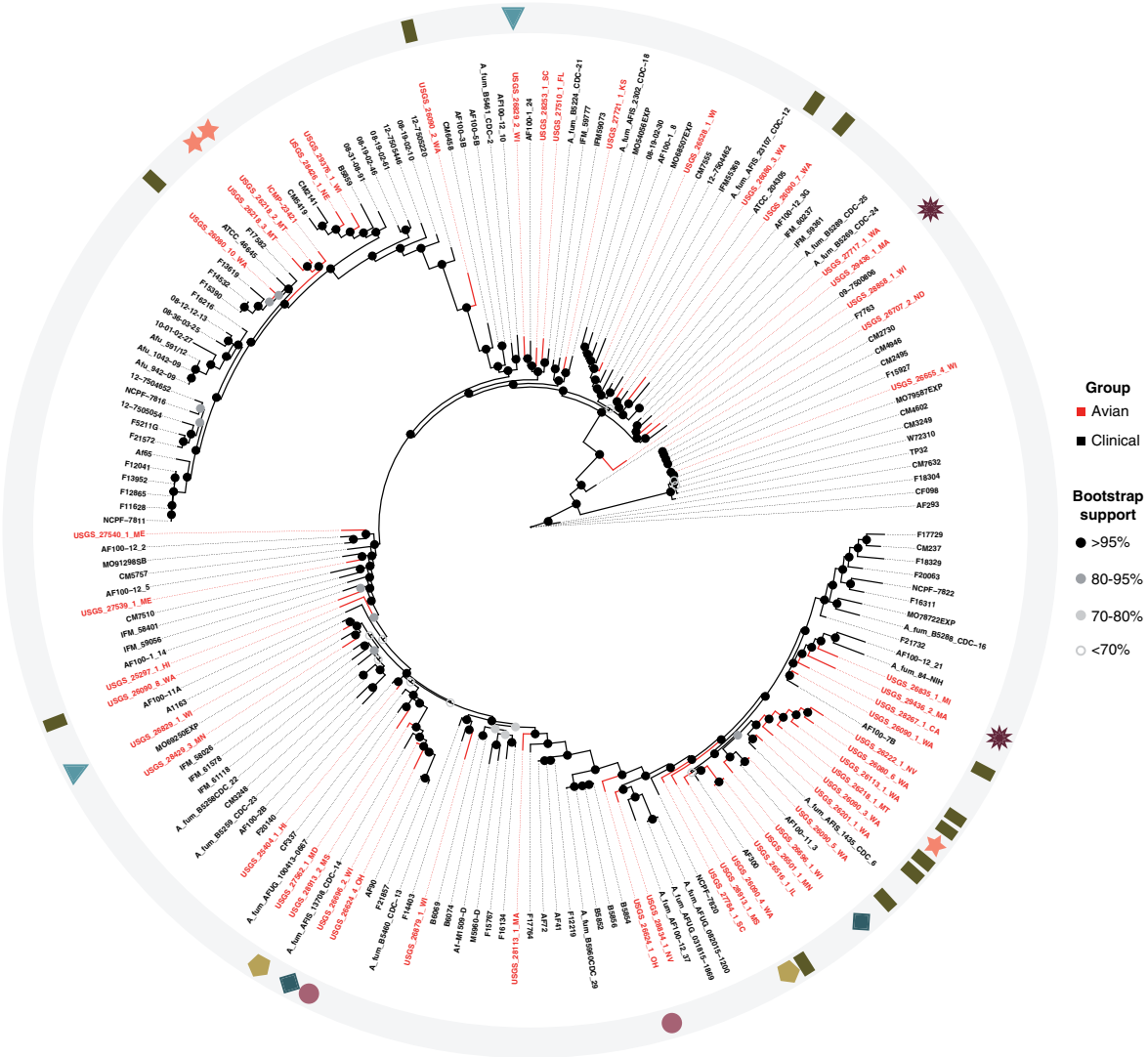

Fig. 2. Phylogenetic diversity of avian derived *A. fumigatus* isolates. Avian-derived *A. fumigatus* isolates represented phylogenetically diverse strains, with distribution interleaved with human clinical strains, and no relationship to geography (collection location is noted by the state suffix on each strain). Single epizootic events represented diverse *A. fumigatus* genotypes. Colored shapes around the parameter represent the 7 epizootic events where multiple isolates were available from the same event. Isolates with the same shape and color represent the same event. Circles at nodes represent branch support values with most bipartitions representing >95% bootstrap support.

To search for signatures of specialization in avian infecting isolates, we performed comprehensive variant scans across all avian derived and human clinical isolates. In total, we identified 53,187 missense variants in avian isolates across 8,862 unique genes. Compared to the full set of 181 isolates, which contained 76,125 missense variants across 9,306 genes. Four hundred and forty-four genes contained variants in some clinical isolates but in none of the avian isolates, and 91 genes were mutated in some of the avian isolates but in none of clinical isolates. However, the distribution of these variants was unstructured by host-source, with no variant appearing in all isolates of either the avian or human clinical strain sets.

Variant scans of 13 genes associated with antifungal drug resistance revealed amino acid changing variants in all 13 genes, including AFUA_7G01960, *atrF*, *cdr1B/abcC*, *cox10*, *Cyp51A*, *Cyp51B*, *fks1*, *hapE*, *hmg1*, *mdr1*, *mdr2*, *mdr3*, and *mdr4*. However, all variants represented uncharacterized amino acid changes, of unknown consequence. None of our avian-derived isolates contained the canonical *Cyp51A* variants representing the TR34/Leu98His or TR46/Tyr121Phe/Thr289Ala genotypes that are commonly associated with azole resistance. Similarly, Voriconazole susceptibility testing revealed no resistant phenotypes among the 23 avian isolates assessed (Supplementary Table 5).

Discussion

Using a 51-strain collection of *A. fumigatus* isolates from 24 species of wild birds with aspergillosis, paired with 130 publicly available human clinical isolates, we found that avian associated *A. fumigatus* isolates were genotypically diverse, displayed no phylogenetic distinction from human clinical isolates, no sign of host or geographic specificity, high strain diversity within single epizootic events, none of the characterized variants currently associated with canonical azole resistance, and no indication of azole resistance via other mechanisms.

Avian aspergillosis associated *A. fumigatus* isolates displayed high genotypic diversity with strain placement on disparate locations across the phylogeny. No variants were universally associated with avian isolates, and avian isolates were routinely interleaved with human clinical isolates, as well as with other avian isolates taken from unique host species, locations, and dates (Figs. 1 and 2). Similarly, previous work using microsatellite markers to genotype 65 avian aspergillosis isolates compared to a large database of mostly human clinical isolates found no association between isolate origin and genotype (Van Waeyenberghe *et al.* 2011). Interestingly, the high genotypic diversity observed across all avian isolates was mirrored within individual epizootic events, where all 7 of the events with multiple representative isolates included phylogenetically distant isolates (Fig. 2). Multiple genotypes of *A. fumigatus* have been previously isolated from domestic chickens (Spanamberg *et al.* 2016), and multiple genotypes (polyclonal infections) have been isolated from the same individuals in captive penguins (Alvarez-Perez *et al.* 2010) and wild storks (Olias *et al.* 2011). In domestic turkeys, acute aspergillosis infections have been associated with singular genotypes, and chronic infections with diverse genotypes (Lair-Fulleringer *et al.* 2003), but this result may also be associated with exposure to high spore density of a single genotype, rather than specific virulence traits present in the infective genotype. Taken together, these results support the conclusion that *A. fumigatus* isolates are broadly capable of causing infection in birds and that aspergillosis is contracted primarily from environmental sources rather than being a contagious disease in wild birds. Assuming that isolates are equally infectious, and given that wild birds are constantly encountering *A. fumigatus* spores in the environment, environmental and life-history factors, such as host exposure to unusually high spore density of heterogenous *A. fumigatus* populations, host stress, and host comorbidities, are likely to be the primary drivers of both individual bird mortalities and aspergillosis epizootics in wild birds. The influence of spore density on aspergillosis is well known to the poultry industry where conidial abundance has been converted to an LD50 of 5×10^5/g lung tissue in domestic turkeys (Richard *et al.* 1981). It has been suggested that spore density is also likely to play an important role in aspergillosis in wild birds, and would serve as an explanation for the higher frequency of the disease in species such as geese and ducks (Arné *et al.* 2021) which often forage in fields containing crop residues potentially contaminated with high numbers of *A. fumigatus* spores.

Because acquired azole resistance in *A. fumigatus* has been previously associated with long-term azole exposure (Dannaoui *et al.* 2001), the common use of azoles for crop protection and as a decontamination and prophylactic agent for poultry and captive wild birds (Chowdhary *et al.* 2013) raises concerns that birds could play a role in both the evolution and spread of azole resistant strains (Beernaert *et al.* 2009; Melo *et al.* 2020b). Here, avian-derived *A. fumigatus* isolates harbored none of the canonical (characterized) variants facilitating azole resistance. However, in avian isolates genes associated with specific resistance mechanisms contained many amino acid changing variants that have yet to be characterized, and which have unknown impacts on azole resistance (Fig. 3). To address the potential of these variants to influence drug resistance, we assessed the minimum inhibitory concentration (MIC) of the triazole antifungal agent Voriconazole on 23 avian strains representing the unique variants identified above. We found that all representative strains were Voriconazole susceptible, indicating that the uncharacterized variants in resistance-associated genes identified here do not influence Voriconazole susceptibility (Supplementary Table 5). However, it should be noted that Voriconazole susceptibility is not consistently indicative of susceptibility to other triazole antifungals not tested here (Zhou *et al.* 2021). To date, azole-resistant isolates of *A. fumigatus* have been identified from both wild and domestic birds, but generally at a low frequency (Beernaert *et al.* 2009; Barber *et al.* 2020) and are sometimes absent from the population entirely (Sabino *et al.* 2019). Azole-resistant avian isolates are not consistently associated with the *Cyp51A* resistance mutations canonically associated with azole resistance (Melo *et al.* 2021). Although *Cyp51A* mutations are by far the most well-studied azole resistance mechanism in *A. fumigatus*, these mutations are only found in an estimated 20–43% of azole-resistant isolates (Bueid *et al.* 2010; Zhou *et al.* 2021) highlighting the importance of further investigation into the mechanisms of noncanonical azole resistance. This includes inventories of variants that occur in genes associated with drug resistance but that do not result in resistant phenotypes, such as those presented here.

Data availability

Pipelines used for VCF generation can be accessed at https://github.com/stajichlab/PopGenomics_Afumigatus_Global. The specific release used for this project, along with all relevant code used for analysis and figure generation are available at https://github.com/MycoPunk/Afum_avian (DOI: 10.5281/zenodo.5592827). Sequencing data are available from NCBI's SRA under BioProject no. PRJNA773725.

Supplemental material is available at G3 online.

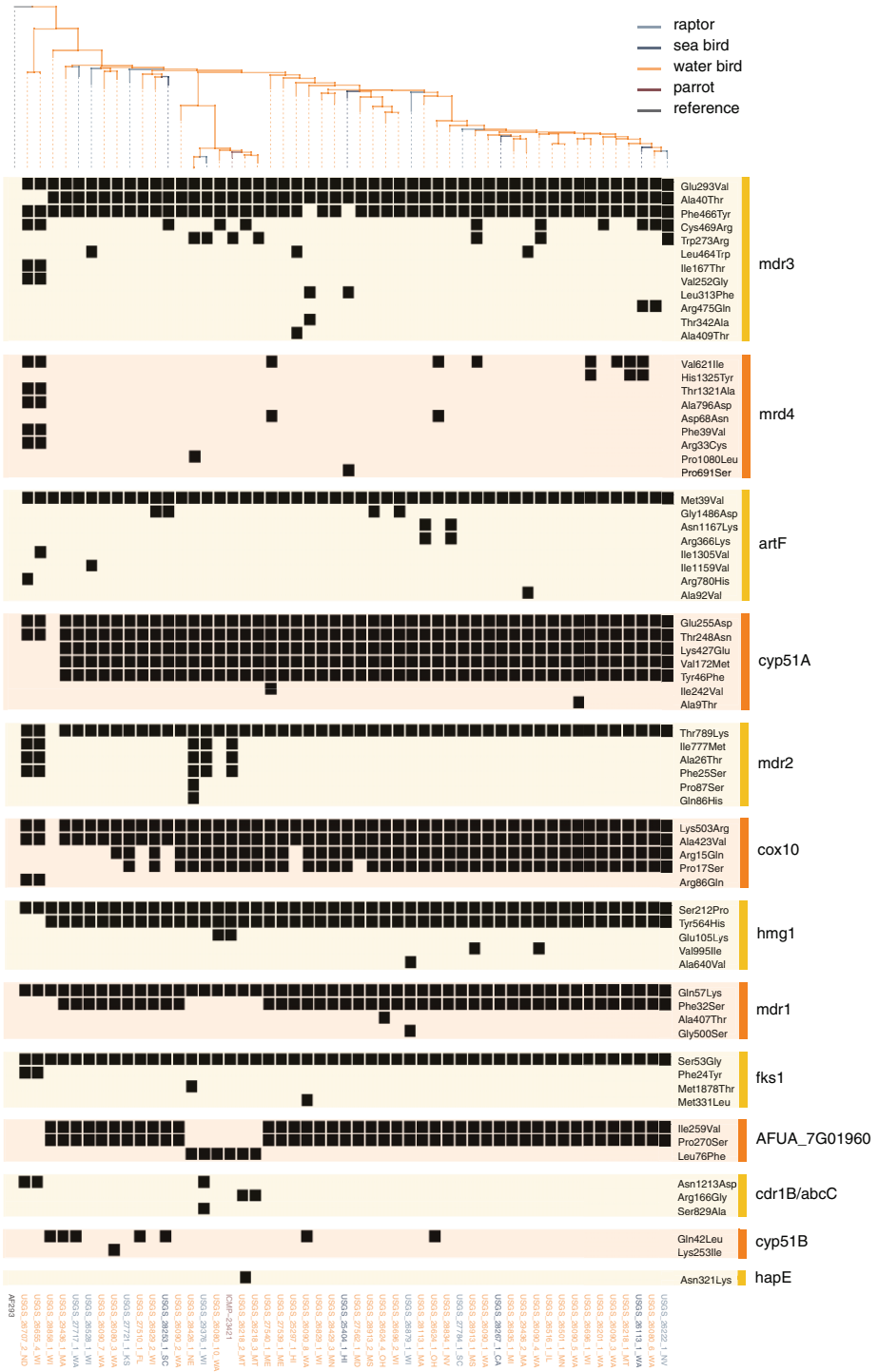

Fig. 3. Nonsynonymous variants in genes associated with azole resistance. A database of 13 genes known to be associated with antifungal drug resistance was scanned for nonsynonymous variants across all avian-derived *A. fumigatus* isolates. Variants were present in all 13 genes, representing uncharacterized amino acid changes, several of which demonstrated phylogenetic structure. No canonical *Cyp51A* resistance variants representing the TR34/Leu98His or TR46/Tyr121Phe/Thr289Ala genotypes were found.

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Conflicts of interest

None declared.

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