POPULATION STRUCTURE AND MOLECULAR EPIDEMIOLOGY OF THE FUNGAL PATHOGEN *ASPERGILLUS fUMIGATUS* AT GLOBAL AND LOCAL SCALES

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Lay Abstract

Aspergillus fumigatus is a mold capable of causing severe infection in humans. Infections caused by *A. fumigatus* can often be treated using antifungals. However, there have been several reported cases of treatment failure around the world over the last two decades. Generally speaking, treatment failure in patients is often associated with antifungal resistance in *A. fumigatus.* My thesis aims at better understanding the distribution and investigating the origin of resistance in *A. fumigatus* at both global and local levels. Here, we analyze *A. fumigatus* strains from 15 countries, including strains from Hamilton, Ontario. Our findings will potentially contribute towards establishing effective long-term management strategies against *A. fumigatus* infections locally and globally.

Abstract

Aspergillus fumigatus is an opportunistic fungus known to cause a group of life-threatening infections collectively known as aspergillosis. In this thesis, multilocus sequence and microsatellite markers, among others, were used to study global and local *A. fumigatus* population structures. We examined the roles of sexual and asexual reproduction in the initiation of azole resistance globally. Furthermore, we investigated the origin of multi-triazole resistance in India and whether the use of fungicides on farms propagates resistance in environmental strains of clinical importance in Hamilton, Ontario. We characterized for the first time the *A. fumigatus* population in Cameroon while concomitantly screening for environmental resistance. Our results showed that sexual reproduction plays a key role in the development of triazole resistance globally. We found that multi-triazole resistance in India has multiple origins, which include mutational, recombinational and exotic origins. Our results provided little to no evidence that azole fungicides are the origin, or increase the frequency of triazole resistance in clinical *A. fumigatus* in Hamilton. Additionally, we identified a significantly unique *A. fumigatus* population in Cameroon. Our findings will potentially contribute towards developing effective long-term management strategies against aspergillosis.

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Abbreviations

Acronyms

AMOVA analysis of molecular variance

BIC Bayesian information criterion

CPA chronic pulmonary aspergillosis

DAPC discriminant analysis of principal components

IA index of association

MIC minimum inhibitory concentration

MLST multilocus sequence typing

MTR multiple triazole resistance/resistant

SDA Sabouraud dextrose agar

ST sequence type

STR short tandem repeats

VPCI Vallabhbhai Patel Chest Institute

WNS white nose syndrome

WGS whole genome sequencing

Chapter 1: Introduction

1.1 Aspergillosis

Aspergillius fumigatus is a filamentous sac-fungus that belongs to the Trichocomaceae family. Due to its role in carbon and nitrogen recycling, *A. fumigatus* is predominantly found in soil and decaying matter. Its non-fastidious growth requirements, volatile spores and thermotolerance have made *A. fumigatus* an important opportunistic human fungal pathogen (Rhodes, 2006). *A. fumigatus* can be distinguished from other *Aspergillus* species belonging to section Fumigati by its ability to grow at high temepratures (50^{o}C). *Aspergillus* species, including *A. fumigatus*, can infect humans causing a group of infections collectively known as aspergillosis (Denning & Perlin, 2011). Invasive, allergic bronchopulmonary and chronic pulmonary aspergillosis are the three most frequently diagnosed types of aspergillosis. Aspergillosis is a major cause of morbidity and mortality in immunocompromised patients worldwide. At least 5 million people world-wide are estimated to be at risk of contracting aspergillosis (Denning & Perlin, 2011). *A. fumigatus* causes approximately 4 million cases of allergic bronchopulmonary aspergillosis globally, of which approximately 400,000 become chronic (Denning, Pleuvry, & Cole, 2013) (Fig 1.1). Invasive aspergillosis (IA) is the most severe form of aspergillosis: it is estimated to have a global annual incidence of up to 10% and mortality rates as high as 90% in high-risk groups (Chowdhary, Kathuria, Xu, & Meis, 2013; Dagenais & Keller, 2009).

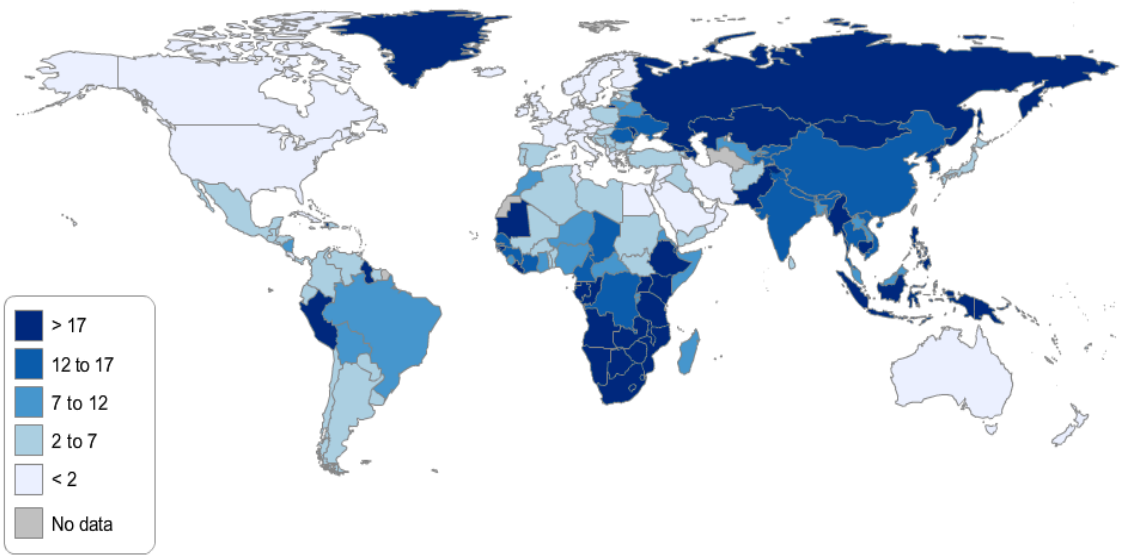

Figure 1.1. Global prevalence of chronic pulmonary aspergillosis (CPA). CPA 5 year prevalence rate, key numbers represent rates per 100,000 (Global Action Fund for Fungal Infections, n.d.).

1.2 Global and local scale population structure and molecular epidemiology

Effective global and local prevention and control measures against aspergillosis require a better understanding of the population structure and molecular epidemiology of *A. fumigatus*. Targeting the highly conserved fungal ergosterol biosynthesis pathway, triazoles are the most common and effective drug used in the first line treatment of aspergillosis. However, due to the increasing frequency of azole-resistance in *A. fumigatus*, aspergillosis has become a significant global health issue (Denning & Perlin, 2011). Indeed, the medical significance of aspergillosis and triazole resistance has recently attracted significant attention from public health agencies and mycologists globally. On a global scale, there have been quite a few studies on population genetic patterns of *A. fumigatus*, most of which made contrasting conclusions on how the global *A. fumigatus* population is structured (Balajee, Tay, Lasker, Hurst, & Rooney, 2007; Debeaupuis, Sarfati, Chazalet, & Latgé, 1997; Rydholm, Szakacs, & Lutzoni, 2006; Pringle et al., 2005; Duarte-Escalante et al., 2009). These contrasting results are likely due to differing sample sizes and/or types of markers used. It has been previously suggested that using more informative markers and analyzing more diverse and larger sample sizes would likely

produce more consistent results (Klaassen, Gibbons, Fedorova, Meis, & Rokas, 2012). To date, microsatellite markers have emerged as the best typing method in terms of reproducibility, cost, and discriminatory power (Klaassen, 2009). Although *A. fumigatus* microsatellite markers are known to be neutral and have a high genotyping resolution they have not been widely used in characterizing global and local *A. fumigatus* populations.

Thus far, the fine-scale population structure of *A. fumigatus* is known only for a few countries. For instance, the Dutch population of *A. fumigatus* is known to show evidence of genetic differentiation and recombination (Klaassen et al., 2012). Similarly, the clonal expansion of a resistant isolate is known to shape the Indian population of *A. fumigatus* (Chowdhary, Kathuria, Xu, et al., 2012). Despite these significant efforts to understand the population structure of this medically important fungus, its population genetic patterns remain poorly studied in most countries. Case in point, little to nothing is known about West African populations of *A. fumigatus*. Likewise, the fine-scale population structure of *A. fumigatus* in Canada is still to be described. Furthermore, there is need for continuous surveillance as *A. fumigatus* continues to evolve and adapt in response to local biotic and abiotic pressures.

1.3 Origins of resistance to triazoles

Over the last few decades aspergillosis has steadily grown into a significant health menace making it the second leading cause of nosocomial fungal infections (Perlroth, Choi, & Spellberg, 2007). A previously published literature review pointed out that there have been at least 53 documented aspergillosis outbreaks between January 1966 and August 2005 (Vonberg & Gastmeier, 2006), after which, many more aspergillosis outbreaks have been reported (Balajee et al., 2007; Chang et al., 2008; Peláez et al., 2012; Pettit et al., 2012). In addition to frequent outbreaks, the rapid local and global emergence of triazole resistance has been observed over the last two decades. For instance, resistance to intraconazole in the United Kingdom went from ~5% between 2002–2004 to ~20% in 2009. Similarly, in the Netherlands, triazole resistance has risen from ~10% to ~20% (Chowdhary et al., 2013; Fuhren et al., 2015). The most dominant resistance mechanism is characterized by an amino acid substitution (L98H) in the CYP51A protein and a tandem repeat of a 34-base pair sequence (TR34) found

in the promoter region of the CYP51A protein coding gene. Comprehensive studies have been done in the Netherlands and India to elucidate the origin of triazole resistance (Klaassen et al., 2012; Chowdhary, Kathuria, Xu, et al., 2012). However, the local and global origins and migration patterns of resistance, more specifically for TR 34 /L98H isolates, are still poorly understood.

There are two major explanations for triazole resistance outbreaks: chronic exposure to triazole therapy and extensive use of triazole-fungicides on agricultural sites. Although chronic exposure to triazole therapy is a very plausible explanation for recent rises in triazole resistance, triazole resistant strains have been reportedly isolated from azole-naïve patients in Netherlands, India, and United Kingdom (Van der Linden et al., 2011; Chowdhary, Kathuria, Randhawa, et al., 2012; Howard et al., 2009), hinting at an environmental origin for resistance. It has been hypothesized that dispersal of airborne conidia from triazole-treated farms facilitates the spread of antifungal resistance (Enserink, 2009). In support of this hypothesis, it has been shown that clinical resistant strains of *A. fumigatus* exhibit cross-resistance to agricultural triazoles. This is largely due to the fact that both agricultural and clinical triazoles have similar molecular structures and adopt similar conformations while docking to their target protein, CYP51A (Snelders et al., 2012; Chowdhary, Kathuria, Xu, et al., 2012). The mycology community is still debating the validity of the fungicide-driven resistance hypothesis. Until now, 3 major field studies have tested this hypothesis. One of these field studies was carried out in the United Kingdom and showed evidence supporting the fungicide-driven resistance hypothesis (Bromley et al., 2014). The other two studies, carried out in Japan, rejected this hypothesis (Kano et al., 2014; Toyotome et al., 2016). There is a pressing need for more field studies, in different settings/countries in order to determine whether the fungicide-driven resistance hypothesis is geography specific.

1.4 Objectives

The overall objective of my thesis was to further current knowledge on the population structure and molecular epidemiology of *A. fumigatus* at global and local scales. More specifically, my thesis had five main objectives which are described in detail in

chapters 2 to 6 of this thesis. In chapter 2, by means of a literature review on fungal infectious disease outbreaks—aspergillosis included, I investigated the roles of sexual and asexual reproduction in the origin and dissemination of fungal strains causing infectious disease outbreaks. In the next chapter, I analyzed over 2000 isolates collected from 13 countries on 5 continents by means of 9 microsatellites (Valk et al., 2005) in order to examine the genetic relationships within and among isolates from diverse geographic populations. In chapter 4, I elucidated the diversity and origin of India's multi-triazole resistant strains using 7 multi-locus and 9 microsatellite markers (Bain et al., 2007; Valk et al., 2005). $(GACA)_4$ and M13 primers were also used for genotyping, PCR reactions were modified from the protocol suggested by Meyer and Mitchell (1995). Next, by means of the 9 aforementioned microsatellite markers I investigated whether triazole fungicide use leads to triazole resistance in environmental *A. fumigatus* strains of clinical importance in Hamilton. Finally, I characterized for the first time the Cameroonian *A. fumigatus* population while simultaneously screening for environmental resistance following the Clinical and Laboratory Standard Institute's M38-A2 guideline (Clinical and Laboratory Standards Institute, 2002).

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Chapter 2: The roles of sexual and asexual reproduction in the origin and dissemination of strains causing fungal infectious disease outbreaks

2.1 Preface

Several factors can lead to the emergence of fungal outbreaks, including changes to host populations, pathogen population, and the environments from which host-pathogen interact. In this review, we focus on changes to pathogen population. In contrast to the majority of plants and animals, most fungi species including *A.fumigatus* can reproduce both sexually and asexually. Although most pathogenic fungi are known to reproduce both sexually and asexually, the roles of sexual and clonal reproduction in the origin and propagation of strains responsible for fungal infectious disease outbreaks are still poorly understood. Through a review of current literature, we found that the roles sexual and asexual reproduction often differ between the initiation and expansion of disease outbreaks. We proposed a framework that exemplifies how sexual and asexual reproduction contributes to the origin and propagation of fungal disease outbreaks. This review is now published in Infection, Genetics and Evolution, volume 36, pages 109-209. References in this chapter appear as they are in the originally published manuscript. Authors of this article are Eta Ashu and Jianping Xu. I conceived and wrote the first draft of the manuscript. JX significantly edited subsequent versions of the manuscript. This article is reprinted in accordance with ELSEVIER author's right to share their articles for personal use.

2.2 Abstract

Sexual reproduction commonly refers to the reproductive process in which genomes from two sources are combined into a single cell through mating and then the zygote genomes are partitioned to progeny cells through meiosis. Reproduction in the absence of mating and meiosis is referred to as asexual or clonal reproduction. One major advantage of sexual reproduction is that it generates genetic variation among progeny which may allow for faster adaptation of a population to novel and/or stressful environments. However, adaptation to stressful or new environments can still occur through mutation, in the absence of sex. In this review, we analyzed the relative contributions of sexual and asexual reproduction in the origin and spread of strains causing fungal infectious diseases outbreaks**.** The necessity of sex and the ability of asexual fungi to initiate outbreaks are discussed. We propose a framework that relates modes of reproduction to the origin and

propagation of fungal disease outbreaks. Our analyses suggest that both sexual and asexual reproduction can play critical roles in the origin of outbreak strains and that the rapid spread of outbreak strains is often accomplished through asexual expansion.

2.3 Introduction

Fungi are heterotrophic eukaryotic microorganisms with thick cell walls made of chitin and cellulose. Fungi show great diversity in morphology, lifecycle and ecology, and are commonly known as molds, mushrooms, yeasts, rusts, etc. Based on the estimated fungi to plant ratio in Britain and assuming that such a ratio holds in the rest of the world, it was estimated that globally, there are about 1.5 million fungal species (Hawksworth, 2001). However, direct analyses of environmental DNA suggested that there could be as many as 5.1 million fungal species but only about 100,000 of them have been described so far (Blackwell, 2011). A large number of known fungal species can cause diseases to plants and animals and this ability is widely distributed in all major phylogenetic groups (James et al., 2006). These pathogenic fungi can cause a wide array of diseases ranging from rust in plants (Saunders et al., 2012) to aspergillosis in humans (Lin et al., 2001) and chytridiomycosis in amphibians (Berger et al., 1998).

For fungi to cause damage to host tissue they need to be able to do the following: (i) adhere to host external surfaces, (ii) penetrate host external surfaces and migrate to targeted tissue and/or organ, (iii) circumvent host defense system and multiply (Richardson, 1991). Pathogenic fungal species have evolved through different genetic pathways to acquire genes associated with host adherence, penetration, and survival; a process that is largely driven by gene duplication, mutation, and selection (Moran et al., 2011). Over the past several decades, because of the increasing number of immunocompromised hosts, increased global travel, and rapidly changing environmental conditions due to anthropogenic activities, pathogenic fungi have become a significant worldwide threat to plant and animal health. Indeed, the threat posed by emerging infectious diseases, including those caused by pathogenic fungi, has been rising over the last two decades (Fisher et al., 2012; Jones et al., 2008). Some of these outbreaks have resulted in grave consequences (Chiller et al., 2013; Kentish, 2015; Malkin, 2014; Moskin, 2009; Peláez et al., 2012; Skerratt et al., 2007a). To make matters worse, fungal

species that were previously obscure are now known to be at the root of very deadly outbreaks. For example, since February 2006, an emerging cutaneous fungal disease has ravaged bat populations in Northeastern United States and Eastern Canada (Blehert et al., 2009; Blehert, 2012; Leopardi et al., 2015). It is estimated that the white-nose syndrome has killed over 5 million bats and spread to 30 US states and 5 Canadian provinces. The emergence and re-emergence of such fungal diseases have grave consequences on food security and ecosystems health. It was estimated that crop losses caused by low-level persistent plant fungal diseases could feed about 600 million people if averted (Fisher et al., 2012). Consequently, understanding the origin and the mechanism by which these outbreaks are disseminated is paramount.

Several factors can lead to the emergence of fungal outbreaks, including changes in host populations, in pathogen population, in the environment from which host-pathogen interact, or a combination of all three factors. In this review, we focus on the fungal factor. Specifically, we are interested in the roles of sexual and asexual reproduction in the emergence and spread of fungal outbreak strains. Different from the majority of plants and animals, about 20% of all described fungal species are known to predominantly or exclusively reproduce asexually, the remaining 80% or so can reproduce both sexually and asexually (Lee et al., 2010; Xu, 2004). Such characteristics make pathogenic fungi excellent model organisms for studying interplays between sexuality and clonality in outbreak initiation and propagation.

2.4 Search criteria

Electronic bibliographic databases were the main sources for our literature review. We searched databases such as ProMed, PubMed, and Google Scholar for fungal infectious disease outbreaks. Searches were conducted very broadly and included terms “outbreaks,” “molecular epidemiology,” “population structure,” “sexual,” “asexual,” “reproduction,” “role,” “epidemic,” “fungi,” and “infectious diseases.” Articles that contained at least 50% of a combination of search terms were further reviewed. Authors and co-authors of relevant articles were profiled and references of relevant articles were also reviewed.

2.5 Tradeoffs between sexual and asexual reproduction

A broad definition of sex that accommodates both conventional sex in eukaryotes and lateral gene transfer is used here to refer to any natural process that amalgamates genes from two or more sources in a single cell (Xu, 2004). Reproduction in the absence of gene amalgamation from two or more sources is referred to as asexual. Most pathogenic fungi are known reproduce both sexually and asexually (Feretzaki and Heitman, 2013; Tibayrenc and Ayala, 2012; Xu, 2004, 2010). However, the roles of sexual and clonal reproduction in the origin and propagation of strains responsible for fungal infectious disease outbreaks are still poorly understood. In addition, recent genomic and population genetic analyses have shown that many so called "asexual" fungi actually possess intact and functioning genes related to sexual reproduction or have been shown to recombine in nature (Kück and Pöggeler, 2009). These recent findings have prompted biologists to seek an understanding of underlying conditions where one reproductive form may be favored over another.

Life-history theory hypothesizes that evolution in reproductive development is shaped by a need to achieve optimal population fitness (Nespolo et al., 2009). For example, a study published by Liu et al., (2008) showed that available nutrient levels could influence the trade-offs between sexual reproduction, clonal propagation and vegetative growth in the aquatic plant *Sagittaria pygmaea*. Similarly, a handful of other studies have revealed rather unique situations in which the selection for asexual or sexual reproduction in a population is often accompanied by trade-offs in non-selected traits to maintain optimal population fitness (Nespolo et al., 2009, Ronsheim and Bever, 2000, Sutherland and Vickery, 1988). In fungi, external factors such as temperature, light, quality and quantity of nutrients, water availability, aeration, and pH are known to influence the interplay between asexual and sexual reproduction (Chamberlain and Ingram, 1997; Xu, 2005).

Generally speaking, high and/or novel virulence trait(s) is a characteristic commonly associated with outbreak strains of most microorganisms (Baldwin et al., 2004; Taha et al., 2002; Valway et al., 1998; Warny et al., 2005). For example, adaptations to host defense by changing virulence and transmissibility are vital mechanisms by which pathogens initiate outbreaks (Deitsch et al., 1997; Finlay and

Falkow, 199; Subramanian et al., 2001). Over the last few decades several hypotheses have been put forth to explain why sexual reproduction is ubiquitous even though it can be costly. For example, the reproductive output per individual in asexual reproduction is twice that for sexual reproduction (Otto and Lenormand, 2002). Nonetheless, most pathogenic fungi continue to reproduce sexually, at least occasionally (Lee et al., 2010). One hypothesis states that sexual reproduction within populations is maintained to remove deleterious recessive mutations accumulated as part of the DNA replication process, through a process known as Muller's Ratchet (Kondrashov, 1988). An alternative hypothesis, also known as the Red Queen Hypothesis, states that the maintenance of sexual reproduction within populations promotes adaptation in ever-changing environments (Hamilton and Zuk, 1982; Lively et al., 1990; Morran et al., 2011). The environmental changes refer to not only those of abiotic factors but also biotic factors, including interacting species.

Although sexual reproduction can accelerate adaptation to novel or changing ecological niches, adaptation to stressful or new environments can still occur in the absence of sex. For example, a study on a group of asexual rotifers showed genetic diversification that was approximately equivalent to that found in related sexual groups (Fontaneto et al., 2007). More interestingly the study demonstrated differentiation in feeding morphology among these asexual aquatic animals. Indeed, among the ancient asexual bdelloid rotifers, no male rotifers or traces of meiosis have ever been reported. The study concludes that sex is not necessary for adaptation and genetic differentiation in these rotifers.

In the following sections, we review some of the major contemporary fungal infectious disease outbreaks and analyze the relative roles of sexual and asexual reproduction in the origin and spread of these outbreaks. Specifically, we sought to answer the following question: (i) can asexual fungi initiate outbreaks? (ii) Is sex absolutely necessary for the initiation of outbreaks in sexual fungi? (iii) Are there trade-offs between sexual and asexual reproduction during outbreak initiation and expansion? (iv) What is the role of sexual and asexual reproduction in the expansion of outbreak populations?

2.6 Emerging modes of fungal infectious disease outbreaks

Table 2.1 summarizes the major fungal infectious disease outbreaks from our survey. To help discern the relative roles of sexual and asexual reproduction in these outbreaks, only outbreaks containing molecular epidemiological information are presented here. Thus, these outbreaks are only part of the many fungal infectious disease outbreaks reported in the literature. Nonetheless, our review included a diverse group of fungi belonging to four major phylogenetic groups: Chytridiomycota, Ascomycota, Zygomycota and Basidiomycota. Furthermore, we reviewed fungi with diverse life cycles, ranging from asexual to homothallic and heterothallic mating systems. Both obligate and opportunistic pathogenic fungi were reviewed.

Table 2.1. Summary table of fungal pathogens that have caused recent infectious disease outbreaks.

Fungal pathogen	Infection	Mode of reproduction	Most plausible cause of outbreaks	Population structure	References
Apophysomyces trapeziformis	Invasive mucormycosis	Asexual —sexual reproduction has not been described in Apophysomyces	Changes to host populations or host-pathogen interaction environment — traumatic inoculation/natural disasters	Evidence of recombination, distinct and diverse clonal clusters among the Joplin outbreak population— very little is known about the global population genetics of this fungus	(Etienne et al., 2012)
Aspergillus fumigatus	Aspergillosis	Sexual and asexual— recent identification of a supermater pair	Changes to host population, host-pathogen interaction environment and/or sexual recombination	High genetic diversity, lack of linkage disequilibria, and little phylogenetic structure	(Guinea et al.,2011) (Sugui et al., 2011)
Aspergillus. sydowii	Aspergillosis	Asexual — sexual cycle is currently undescribed	Changes to host population, host-pathogen interaction environment and/or recombination	High genetic diversity, lack of linkage disequilibria, and little phylogenetic structure	(Rypien et al., 2008)
Batrachochytrium dendrobatidis	Chytridiomycosis	Asexual—sexual cycle has never been observed	Cryptic sex	Three deeply diverged lineages were uncovered—the most widely distributed lineage being hyper virulent	(Farrer et al., 2011)
Candida albicans	Candidiasis	Asexual and parasexual	Changes to host population and/or host-pathogen interaction environment	Genetically diverse and in linkage equilibrium — no significant association between identified clades and virulence or body sites	(Odds et al., 2007) (Ene and Bennett, 2014)
Coccidioides immitis	Valley fever	Asexual— recent identification of functional heterothallic mating type loci	Changes to host population and/or host-pathogen interaction environment	High genetic diversity, lack of linkage disequilibria, and little phylogenetic structure	(Fisher et al., 2000) (Flynn et al., 1979) (Mandel et al., 2007)

Cryphonectria parasitica	Chestnut blight	Sexual and asexual	Exotic introduction, sexual recombination	High genetic diversity, evidence of recombination, differentiated — population structure is consistent with sexual reproduction at epidemic sites	(Springer et al., 2013) (Dutech et al., 2010) (Ježić et al., 2012) (Milgroom et al., 1996)
Cryptococcus gattii	Cryptoco-ccosis	Sexual and asexual	Cryptic same sex/mutator alleles or exotic introduction	Evidence of sexual recombination—the majority of outbreak lineages exhibit increased virulence and are differentiated	(Billmyre et al.2014) (Fraser et al., 2005)
Exserohilum rostratum	Phaeo-hyphomycosis	Sexual (*Setosphaeria rostrate*) and asexual	Changes to host population and host-pathogen interaction environment: exposure to infected equipment and immunosuppression of host	United States outbreak population is clonal, genetically differentiated from control strains. However, relative virulence of the outbreak strain over control strains is unknown.	(Litvintseva et al., 2014) (Ritter et al., 2013) (Saint-Jean et al., 2007)
Fusarium oxysporum f. sp. cubense	Fusarium wilt	Asexual— teleomorph is unknown	Mutation events and /or cryptic sex, horizontal gene transfer, exotic introduction	Diverse, little evidence of recombination, distinct phylogenetic lineages — there is a moderate correlation between clusters (races) and virulence; however, this classification varies by genetic markers and pathogenicity test.	(Costa et al., 2015) (Fourie et al., 2009) (Guo et al., 2014) (Sutherland et al., 2013) (Thangavelu et al., 2012)
Fusarium pseudogramine arum	Fusarium crown rot (FCR) of wheat and barley	Sexual (*Gibberella coronicola*) and asexual	Introduction of toxigenic genes into native population through horizontal gene transfer and/or changes to host populations	High genetic diversity, evidence of recombination, and frequent gene flow— strong association between aggressiveness and Amplified Fragment Length Polymorphism (AFLP) clusters	(Scott and Chakraborty, 2006) (Mishra et al., 2006) (Gardiner et al., 2012) (Akinsanmi et al., 2006)
Hemileia vastatrix	Coffee rust	Asexual— recent evidence for an ancestral or cryptic sexual stage	Changes to host population and/or host-pathogen interaction environment	Moderately low genetic diversity — unstructured variability with regard to physiological race, geographical origin or host	(Rozo et al., 2012) (Carvalho et al., 2011) (Gouveia et al., 2005)

Magnaporthe oryzae	Rice blast disease	Sexual and asexual (*Pyricularia oryzae*)	Duplication/deletion events, horizontal gene transfer and/or exotic introduction	Clonal reproduction is predominant in most rice growing areas; however, sexual recombination has been reported in limited areas spread all over the south foothills of the Himalayas — unique genetic events engendering virulent genotypes followed by long distance clonal propagation.	(Tharreau et al., 2009) (Dong et al., 2015)
Nosema ceranae	Nosemosis	Asexual —sexual cycle is currently unknown	Exotic introduction	Moderate genetic diversity, evidence of recombination, and little phylogenetic structure	(Pelin et al., 2015) (Gómez-Moracho et al., 2015)
Pseudogymno-ascus destructans formerly *Geomyces destructans*	White nose syndrome	Asexual— recent identification of a functional heterothallic mating system	Exotic introduction/ sexual recombination	North American population are strikingly clonal— there is a 100% genetic similarity between the North American genotype and most widely distributed European genotype	(Khankhet et al. 2014) (Leopardi et al., 2015) (Palmer et al., 2014)
Puccinia graminis f.sp. tritici (pgt)	Cereal rusts	Sexual and asexual	Step-wise mutations and/or exotic introduction	High genetic differentiation among pgt races — TTKSK and variants are genetically differentiated from other races	(Visser et al., 2010) (Jin et al., 2008) (Schumann and Leonard, 2000)
Pyrenophora tritici-repentis (ptr)	Tan spot wheat disease	Sexual and asexual (*Dreshslera tritici-repentis*)	Changes to host populations, exotic introduction and/or horizontal gene transfer	Global population shows high genetic diversity and evidence of sexual recombination — Ptr ToxA non-producing isolates cluster together but are distantly related to Ptr ToxA-producing isolates	(Aboukhaddour et al., 2011) (Gurung et al., 2013) (Aboukhaddour et al., 2013) (Oliver et al., 2008)
Saprochaete clavata formerly Geotrichum clavatum,	Invasive fungal infections	Asexual — produces arthroconidia— no known teleomorph	Changes to host population and host-pathogen interaction environment — exposure to infected equipment and	French outbreak population is clonal —most outbreak strains differentiated into a single cluster (cluster A) that was different from control strains (strains from other European countries); however,	(Vaux et al., 2014) (Camus et al., 2014) (Kolecka et al., 2013)

			Immunosuppression of host	there was weak correlation between Clade A and pathogenic potential	
Sporothrix brasiliensis	Sporotrichosis	Asexual— recent identification of a functional heterothallic mating type loci	Clonal expansion of virulent clones /exotic introduction	Low level of genetic diversity, significant population differentiation and clonality	(Teixeira et al., 2015) (Rodrigues et al., 2013)
Verticillium dahliae	Verticillium wilt	Asexual — recent evidence for an ancestral or cryptic sexual stage	Chromosomal rearrangements or exotic introduction	High genetic diversity, lack of linkage disequilibria, differentiated — lineage-specific genomic regions that act as a source for genetic variation to mediate virulence	(Atallah et al., 2010) (de Jonge et al., 2013) (Short et al., 2014) (Jiménez-Díaz et al., 2006) (Short et al., 2015) (Maruthachalam et al., 2010)
Zymoseptoria tritici formerly *Mycosphaerella graminicola*	Septoria leaf blotch	Sexual and asexual (formerly *Septoria tritici*)	Sexual recombination	Global population shows high genetic diversity and gametic equilibrium—24% of all progeny in field experiment were estimated to be sexual recombinants	(Zhan et al.2007) (Zhan et al., 2003)

Engering et al. (2013) recently proposed a conceptual framework that integrates knowledge on all events related to emerging infectious disease events, including those for fungal, viral and bacterial diseases. Their framework integrated changes in the interplay of pathogens, hosts and environment. Building upon their proposed framework, we classify fungal infectious disease outbreak events into three major categories: (1) the introduction of novel strains, (2) significant changes to host populations and, (3) changes to the environment from which host-pathogen interact. Introduction of novel strains in the context of this review covers three different types of events: (i) the generation of novel fungal strains by sexual reproduction, including horizontal gene transfer, (ii) exotic introduction of existing or novel fungal strains from one geographic location to another, and (iii) the generation of novel fungal strains by asexual reproduction and mutation, including genetic rearrangements. Figure 2.1 shows a nonexclusive framework of the major events that can lead to the initiation and propagation of fungal disease outbreaks. The major focus of this review with regard to the framework is on how sexual and asexual reproduction shapes fungal populations to initiate and expand the outbreak strains.

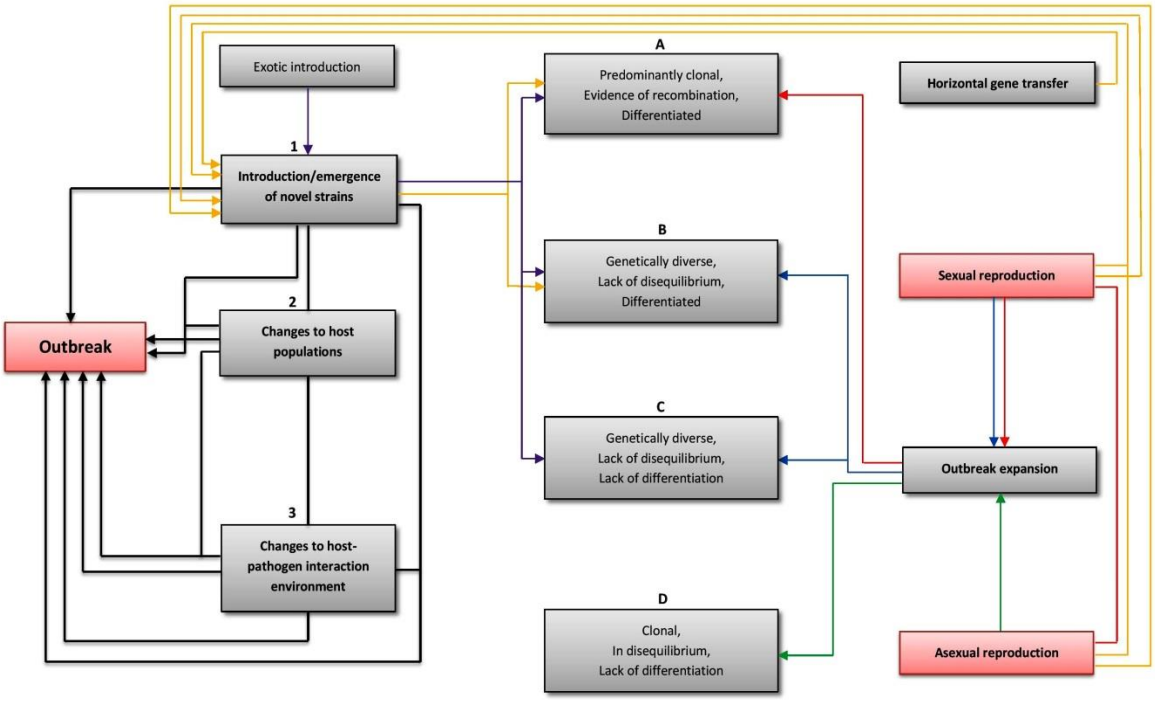

Figure 2.1. A conceptual framework of events that lead to the origin and propagation of fungal disease outbreaks. Letters A to D depict major fungal population structures noted

from our review of literature. Numbers 1-3 represent major events leading to fungal disease outbreaks. Yellow lines depict mechanisms that lead to introduction of novel strains and how fungal populations are structured after the introduction of novel strains. Purple lines depict mechanisms by which exotic introduction introduces novel strains and how fungal populations are structured after exotic introduction. Green lines depict mechanisms by which asexual reproduction shapes outbreak populations and how asexually expanding outbreak populations are structured. Blue lines depict mechanisms by which sexual reproduction shapes outbreak populations and how sexually expanding outbreak populations are structured. Red lines depict mechanisms by which both asexual and sexual reproduction synergistically shape outbreak population and how such populations are structured.

2.6.1 The role of sexual and asexual reproduction in the introduction of novel strains

Adaptation of pathogenic fungi to host environment by means of sexual recombination or mutation can lead to the generation of novel strains with high virulence. Chen and McDonald (1996) experimentally showed that the population structure of the plant fungal pathogen *Mycosphaerella graminicola* (Zymoseptoria tritici) is consistent with random-mating over the course of an epidemic cycle. Furthermore, Zhan et al., (2007) experimentally demonstrated that sexual reproduction facilitated pathogen evolution to overcome host defense, which is a key factor in the initiation of certain outbreaks. Among the surveyed human fungal disease outbreaks, the Vancouver Island outbreak is suggested to have been initiated by the emergence of a novel hyper-virulent Cryptococcus offspring generated through recombination in Canada (Fraser et al., 2005) or in the Brazilian Amazon rainforest (Hagen et al., 2013). Likewise, despite the fact that no sexual form of *Batrachochytrium dendrobatidis* has been reported, cryptic sex is allegedly reported to have played a critical role in its emergence as a dominant amphibian pathogen (Table 2.1).

It has also been suggested that asexual reproduction through a transient mutator phenotype could have contributed to the evolution of virulence and outbreak of *Cryptococcus gattii* in the Pacific Northwest (Billmyre et al., 2014). Similarly, six variant races of a virulent lineage of *Puccinia graminis f.sp. tritici* (pgt) have been identified in South Africa. The virulent lineage emerged in Uganda in 1998 (Pretorius et al., 2000)

and it has been hypothesized that step-wise mutations and exotic introductions contributed to the observed genetic diversity in the South African *P. graminis f.sp. tritici* population as no sexual form of this organism has ever been identified in South Africa (Visser et al., 2009, 2010). Hitchhiking of mutator alleles with favorable mutations conferring fitness advantages have been experimentally demonstrated in asexual bacterial and fungi. In response to new environments, asexual fungi can accumulate a large number of mutations which eventually lead to the fixation of mutants that are better adapted to their new environmental (Raynes et al., 2011, Shaver et al., 2002, Tenaillon et al., 1999). Furthermore, asexual reproduction can initiate fungal infectious disease outbreaks by means of complex duplication and deletion events or chromosomal rearrangements (Table 2.1). For example, extensive chromosomal rearrangements are known to drive the evolution of virulence in the asexual fungus *Verticillium dahliae*.

2.6.2 The role of horizontal gene transfer in the origin of outbreak strains

Horizontal gene transfer (HGT) is the transmission of genetic material between organisms by means other than typical sexual or asexual reproduction, specifically across distinct reproductively isolated genomes (Richards et al., 2011). Often overlooked, HGT is an outbreak initiation mechanism worth noting. The transfer of entire chromosomes, gene clusters or even individual genes can often lead to the introduction of novel strains with novel traits(Tharreau et al., 2009; Gardiner et al., 2012). In the context of outbreak initiation, two categories of HGT are of prime importance: (i) gene transfers from prokaryotic to fungal genomes and, (ii) transfers between distinct fungal species. There is growing evidence that both categories of HGT significantly change fungal genomes, making them more infectious or hyper virulent (Table 2.1). When HGT orchestrates the introduction of novel strains, fungal populations tend to adopt structures comparable to those of A or B in Fig 2.1. *Fusarium pseudograminearum* and *Pyrenophora tritici-repentis* are two well-known fungal pathogens wherein HGT has led to the introduction of novel hyper-virulent strains (Table 2.1). *Tox A* gene is a gene encoding a critical virulence factor in *P. tritici-repentis* and is known to have been horizontally transferred from *Stagonospora nodorum* just before 1941 (Friesen et al., 2006). The global

population structure of *P. tritici-repentis* shows high genetic diversity, evidence of recombination and reveals that the ToxA producing isolates are significantly differentiated from non-producing isolates (Table 2.1). The broad distribution of *ToxA* genes among diverse genotypes suggested the important role of sexual recombination in its expansion through natural populations of P. tritici-repentis.

2.6.3 The role of sexual and asexual reproduction in outbreak expansion

Consistent with the expectations of the life-history theory, there is evidence of trade-offs between sexual and asexual reproduction during the expansion of infectious disease outbreaks. When outbreak populations are predominantly shaped by sexual reproduction, such populations tend to be very diverse, with evidence of linkage equilibrium (B or C, Fig 2.1). However, this is a rare occurrence as most fungal outbreaks are expanded asexually, with evidence of linkage disequilibrium and many strains sharing the same genotypes (D, Fig 2.1). Limitation of recombination is generally associated to the acquisition of the most successful traits required for environmental adaptation (Tibayrenc and Ayala, 2012). Consistent with concept of restrained recombination, Saleh et al. (2012) demonstrated *in vitro* how the evolution of asexual reproduction can induce a rapid and permanent loss of sexuality in the fungal pathogen *Magnaporthe oryzae*. Indeed, it has been reported that pathogenic fungi capable of reproducing sexually start evolving clonally after adaptation to a new environment (Teixeira et al., 2015; Tharreau et al., 2009). In an outbreak context, this explains why asexual reproduction tends to be a more efficient and less costly way to disseminate novel hyper virulent strains. For example, even though the Vancouver Island outbreak was allegedly initiated by sexual recombination, outbreak lineages have shown evidence of clonal expansion (Billmyre et al., 2014; Byrnes et al., 2010; Hagen et al., 2013; Kidd et al., 2004). Furthermore, it is possible that sexual and asexual reproduction both act synergistically to expand outbreak populations, in which case, fungal populations would adopt structures similar to that of A in Fig 2.1.

2.6.4 The role of sexual and asexual reproduction in outbreaks caused by changes to host populations and/or to the environment from which host-pathogen interact

Significant physiological changes to host populations and/or to the environment from which host-pathogen interact are often known to cause outbreaks. For example, although there is evidence of sex in source populations, sexual reproduction plays little or no role in the initiation of most aspergillosis outbreaks (Guinea et al., 2011). It is more plausible that physiological changes to host, such as host immuno-incompetency, play greater roles in the initiating apsergillosis outbreak than changes in pathogens. The same can be said about outbreaks caused by the apparently asexual fungus *Candida albicans*, given that most *C. albicans* diploid sequence types (DST) are known to have similar chances of causing life-threatening diseases in immunocompromised humans (Odds et al., 2007). Thus, host frailty appears to be more important than fungal genotypes and virulence in the initiation of apsergillosis and candidiasis outbreaks.

Similarly, fungal infectious disease outbreaks are known to have followed many natural disasters such as earthquakes, tornados, hurricanes, tsunamis, dust storms, heavy rainfalls and unusual temperature spikes (Benedict and Park, 2014). Such events can disrupt the normal equilibrium in the interactions between hosts and pathogens, increase pathogen populations, and lead to greater exposure of hosts to pathogens. For example, the Columbia coffee rust outbreak in 2008 was initiated by excessive rainfall— a major ecological change in Columbia between 2006-2011— not by a change in virulence or exotic introduction (Rozo et al., 2012).

2.7 Case examples of contemporary outbreaks

Below we describe four representative outbreaks in detail. We describe outbreaks that represent all three major types of events that lead to fungal infectious disease outbreaks. The four chosen case studies represent outbreaks caused by both obligate and opportunistic pathogenic fungi belonging to 3 of the 4 major fungi phyla. Furthermore, these case studies represent outbreaks caused by pathogenic fungi with different reproductive systems.

2.7.1 *Cryptococcus gattii*

Every year, about one million people worldwide are affected by cryptococcosis. *Cryptococcus neoformans* and *Cryptococcus gattii* are the main etiologic agents of cryptococcosis (Cogliati, 2013). A member of the phylum Basidiomycota, *C. gattii* is a pathogenic fungus which can cause cryptococcosis both in humans and animals. *C. gattii* is heterothallic with a bipolar mating system consisting of mating types α and a, and is capable of completing either heterosexual or unisexual sexual cycles (Lin et al., 2005). C. gatti can also reproduce asexually by means of mitosis and budding. Supported by a variety of molecular typing techniques, *C. gattii* has been grouped into five major lineages: AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV and AFLP10/VGIV (Hagen et al., 2015; Kidd et al., 2005).

C. gatti emerged on Vancouver Island in Canada in 1999. Since then, the outbreak has expanded from Vancouver Island to other parts of Canada and to the Pacific Northwest of the USA. This outbreak is considered the largest ever life-threatening fungal outbreak in a healthy human population (Voelz et al., 2013). Several hypotheses have been proposed for the origin of the outbreak strain(s). The more recent hypothesis is that the outbreak lineage (AFLP6/VGII) was introduced from a highly-recombining *C. gattii* population in the native rainforest of Northern Brazil. Specifically, using coalescence gene genealogical approach, Hagen et al. (2013) showed that the global *C. gattii* AFLP6/VGII population originated in South America and that the North American *C. gattii* AFLP6/VGII population was derived from South America. Consistent with the rainforest origin theory, the South American populations were found to have a nearly equal proportions of mating-types a and α. Various studies have suggested that the outbreak lineage likely existed on Vancouver Island in Canada several decades before the emergence the *C. gattii* outbreak. However, it is still unclear why it took until the end of the 20th century before the outbreaks emerged, or why the clinical strain CBS6956 (H01) isolated from the sputum of a patient in Seattle in the 1970s is less virulent than the AFLP6A/VGIIa outbreak strains.

Other hypotheses for the origin of the major outbreak *C. gattii* genotype have also been proposed. One hypothesis states that fertile MATα cells of the VGII lineage may be particularly well adapted for alpha-alpha unisexual mating (Fraser et al.

2005; Kidd et al., 2005). In a bid to identify the origin of the Vancouver Island outbreak, Fraser et al.(2005) conducted a global genealogical analysis. They identified two distinct genotypes of the AFLP6/VGII lineage to be at the root of the outbreak, a major genotype (AFLP6A/VGIIa) commonly found in the environment and clinics, and a minor genotype (AFLP6A/VGIIb) represented by a single clinical and several environmental samples. The major genotype was identified as being significantly more virulent than the minor genotype. They demonstrated that the hyper-virulent genotype was then unique to Canada and was a predicted offspring of a hypothetical α–α sexual cycle. Their results were supported by another study which experimentally demonstrated that sexual reproduction can transmit hyper-virulence within the AFLP6/VGII lineage and between AFLP6/VGII and AFLP5/VGIII lineages (Voelz et al., 2013).

On the other hand, Billmyre et al.(2014) highlighted the role of asexual reproduction in the initiation of the outbreak. They showed that one of the virulent outbreak subpopulations (AFLP6/VGIIa) potentially arose mitotically from a less virulent clonal population that contained a mutation in the DNA mismatch repair gene (MSH2) orthologue. Furthermore, previous studies have shown evidence of both sexual and asexual evolution within the VGII lineage. Bovers et al. (2009) and Xu et al. (2009) identified the mitochondrial population structure of the VGII lineage as being predominantly clonal; however, they highlighted clear signatures of hybridization and recombination among mitochondrial genes within the AFLP6/VGII lineage and between the AFLP6/VGII and AFLP5/VGIII lineages. Indeed, the Vancouver outbreak is an excellent example of the importance of mutation, selection, and sexual and asexual reproduction during outbreak initiation and expansion.

2.7.2 *Batrachochytrium dendrobatidis*

Frist described in 1999, a member of the phylum Chytridiomycota, *Batrachochytrium dendrobatidis* is an aquatic fungus which infects the skin of amphibians and has been implicated in the decline and extinction of about 200 species (Berger et al., 1998; Longcore et al., 1999; Skerratt et al., 2007). Due to the large number of species impacted and its high mortality rate, chytridiomycosis has been described as the worst ever recorded infectious disease outbreaks among vertebrates (Gascon, 2007). Similarly, the emergence of *Batrachochytrium salamandrivorans*, a sister species of *B. dendrobatidis*,

has resulted in rapid declines of European populations of fire salamanders (Martel et al., 2014). *Batrachochytrium* spp is suggested to have likely spread by means of imported amphibians (Martel et al., 2014; Weldon et al., 2004).

Since its emergence in 1999, *B. dendrobatidis* has been reported in every continent except Antarctica. There are two hypotheses for the origin of the chytrid fungal disease outbreak. One suggests that *B. dendrobatidis* is an emerging novel pathogen, while another argues that *B. dendrobatidis* is an endemic pathogen which has become more virulent due to changes in selective pressures (Fisher et al., 2009). The later school of thought benefits from evidence demonstrating that the presence of *B. dendrobatidis* in amphibian populations goes way back to the early 1900s (Ouellet et al., 2005; Weldon et al., 2004). However, after a critical examination of the population structure of *B. dendrobatidis*, Morgan et al. (2007) showed that *B. dendrobatidis* had no amphibian-host specificity, little population differentiation by geography, low diversity and little evidence of human-assisted fungal migration; all of which supported the emerging novel pathogen hypothesis. Nonetheless, two recombining Californian populations have been previously reported, consistent with the endemic hypothesis. Though there is no known *B. dendrobatidis* sexual cycle; evidence of recombination suggested the possibility of cryptic sex. A follow up study by James et al. (2009) demonstrated high levels of genotypic diversity among 59 global *B. dendrobatidis* strains. They attributed this genetic variation to the loss of heterozygosity through recent mitotic recombination, not cryptic sexual recombination, providing support for the emerging pathogen hypothesis. Different from that of James et al. (2009) and consistent with growing evidence demonstrating differences in virulence levels among *B. dendrobatidis* strains (Fisher et al., 2009), another study implicated cryptic sex in the evolution of virulence and outbreak origin (Farrer et al., 2011).

2.7.3 Pseudogymnoascus destructans

White-nose syndrome (WNS) is another recently occurring pathogenic fungal outbreak that has led to drastic declines (>75%) in North American bat populations. In the winter of 2006, WNS began devastating colonies of hibernating bats in Albany, New York. Since then, it has spread to about 30 US states and 5 Canadian provinces. A member of the phylum Ascomycota, *Pseudogymnoascus destructans* is the etiologic agent of WNS.

It was recently discovered that the emergence of WNS in North America most likely resulted from an intercontinental transfer of a prevalent genotype from Western Europe. Compared to the diverse genotypes among European strains, North American strains all shared the same multilocus sequence type (Khankhet et al., 2014; Leopardi et al., 2015). Results from inoculum experiments showed no significant difference in virulence between strains from North America and those from Europe, thereby rejecting the hypothesis that underling selective pressures may have spawned the emergence of more virulent strains in North America (Warnecke et al., 2012). Palmer et al. (2014) recently identified a heterothallic mating system in isolates of *P. destructans* from Europe. Their findings are in line with previously described higher levels of genetic diversity in Europe. In contrast, North American strains are strikingly clonal and of a single mating type. These results are consistent with the hypothesis that exotic introduction and host susceptibility are the most plausible explanations to the WNS outbreak in North America (Puechmaille et al., 2011). However, since the European *P. destructans* population can reproduce sexually and has been shown to be the source population for the North American outbreak, sex cannot totally be ruled out in the origin of the North American genotype in Europe. Interesting, PCR fingerprinting analyses of North American isolates also identified signatures of mutation accumulation and phenotypic diversification, suggesting that this genotype is evolving rapidly through asexual reproduction (Khankhet et al. 2014).

2.7.4 Aspergillus fumigatus

A member of the phylum Ascomycota, *Aspergillus fumigatus* is one of the most prevalent opportunistic pathogens of humans. *A. fumigatus* is heterothallic with two mating types, MAT1-1 and MAT1-2, respectively. Its sexual cycle is characterized by the mating of haploid hyphae to form cleistothecia which contain numerous ascospores produced via meiosis. It can also reproduce asexually via mitotic division of haploid hyphae to form conidiospores. *A. fumigatus* causes a group of infections collectively known as aspergillosis. Invasive, allergic bronchopulmonary and chronic pulmonary aspergillosis are the three major manifestation of aspergillosis. Invasive aspergillosis (IA) is the most severe form, it is estimated to affect about 300,000–500,000 patients globally and has a mortality rate as high as 90% in high risk groups

(Dagenais and Keller, 2009; Denning and Perlin, 2011). Over the years aspergillosis has steadily grown into a significant health menace making it the second leading cause of fungal nosocomial infection (Perlroth et al., 2007). *A. fumigatus* is the most frequent species of the genus causing aspergillosis; however, *A. flavus* is known to be more frequent in tropical climates (Hadrich et al., 2012). Another species of this genus, *Aspergillus sydowii* is also of significant concern, causing large-scale outbreaks to sea fan corals in the Caribbean and resulting in significant declines of the corals (Rypien et al., 2008). For *A. fumigatus,* a literature review in 2006 pointed out that there had been at least 53 documented aspergillosis outbreaks between January 1966 and August 2005 (Vonberg and Gastmeier, 2006). Since then, many more aspergillosis outbreaks have continued to emerge (Balajee et al., 2007; Chang et al., 2008; Peláez et al., 2012; Pettit et al., 2012).

About 50% of all nosocomial aspergillosis outbreaks are associated with demolition or construction work; furthermore, among patients with aspergillosis, those with severe immune deficiency have significantly higher fatality rates than those with milder immune deficiency (R-P. Vonberg and Gastmeier, 2006). Guinea et al. (2011) did a detailed molecular epidemiology study of a recent outbreak in which they compared outbreak strains of *A. fumigatus* to a presumed source population. They showed an overall genotypic diversity of 0.984 in all studied isolates. They identified only 3 genotypes in the outbreak hospital that were common to those isolated from other hospitals in the area. Interestingly, the outbreak strains showed a significant amount of genetic diversity, which is an indicator of population genetic heterogeneity and sexual reproduction. Similar results were reported by other studies which used different typing methods (Leenders et al., 1996; Mellado et al., 2000).It has been difficult to pinpoint the source of aspergillosis outbreaks and most studies concluded that aspergillosis outbreaks did not have single-sources and consisted of a series of complex events that were most probably unrelated. Although these studies did not show any significant association between sexual recombination and increased pathogenicity, sexual reproduction has been proposed to generate a multi-azole resistant genotype in India, followed by clonal expansion across many ecological niches throughout India to cause outbreaks (Chowdhary et al., 2012). Indeed,

aspergillosis outbreaks exemplify the complexity of fungal infectious disease outbreaks that are contributed by a diversity of factors including (i) the introduction of novel resistant strains, (ii) significant changes to host immuno-competency and/ or, (iii) changes to the environment from which host-pathogen interact.

2.8 Conclusions and perspectives

This review shows that, (i) both asexual and sexual pathogenic fungi can initiate outbreaks, (ii) sex is not absolutely necessary for the initiation of outbreaks in sexual fungi, (iii) there is evidence of trade-offs between sexual and asexual reproduction during the initiation and expansion of certain outbreaks and, (iv) asexual reproduction is common during the expansion of outbreak populations. Despite significant efforts to understand the role of sexual and asexual reproduction in the origin and dissemination of pathogenic fungi outbreaks, many important questions remain. For example, in apparently asexual fungi, what is the role of cryptic sexual cycles in causing infectious diseases outbreaks? Second, how common and important are lateral gene transfers in strains causing fungal disease outbreaks? Also, exotic strains causing fungal disease outbreaks seem to be a growing trend at both local and global levels. What are the best strategies to prevent and control such exotic introductions? We believe better diagnosis, real-time tracking and effective treatments are all needed in order to prevent and control such outbreaks.

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Chapter 3: A global population genetic analysis of *Aspergillus fumigatus*

3.1 Preface

The genetic diversity and geographic structure of the human fungal pathogen *A. fumigatus* has been the subject of many studies. However, most previous studies had relatively limited sample ranges and sizes and/or used genetic markers with low-level polymorphisms. In this paper we characterize a global collection of strains of *A. fumigatus* using a panel of 9 highly polymorphic microsatellite markers. Using these markers, we analyze over 2000 isolates, which is ~3 times the number of isolates reported so far in previous studies. Our analyses suggest that *A. fumigatus* contains historically differentiated genetic populations that are continuously being impacted by contemporary evolutionary forces. In the wake of a global rise in resistance to azoles in fungal pathogens, our findings should aid in developing management strategies to mitigate current increases to azole-resistance. This study is now published in mSphere, volume 2, issue 1, e00019-17. The referencing style in this chapter is as in the originally published manuscript. Authors of this article are Eta E. Ashu, Ferry Hagen, Anuradha Chowdhary, Jacques F. Meis, and Jianping Xu. This study was conceived by EA and JX. FH, AC, and JFM obtained the genotype data used for this work; EA analyzed the data and wrote the first draft of the manuscript.

3.2 Abstract

Aspergillus fumigatus is a ubiquitous opportunistic fungal pathogen capable of causing invasive aspergillosis, a globally distributed disease with a mortality rate of up to 90% in high-risk populations. Effective control and prevention of this disease require a thorough understanding of its epidemiology. However, despite significant efforts, the global molecular epidemiology of *A. fumigatus* remains poorly understood. In this study, we analyzed 2026 *A. fumigatus* isolates from 13 countries in four continents using nine highly polymorphic microsatellite markers. Genetic cluster analyses suggest our global sample of *A. fumigatus* isolates belonged to eight genetic clusters with seven of the eight clusters showing broad geographic distributions. We found common signatures of sexual recombination within individual genetic clusters and clear evidence of hybridization between several clusters. Limited but statistically

significant genetic differentiations were found among geographic and ecological populations. However, there was abundant evidence for gene flow at the local, regional and global scales. Interestingly, the triazole-susceptible and triazole-resistant populations showed different population structures, consistent with antifungal drug pressure playing a significant role in local adaptation. Our results suggest that global populations of *A. fumigatus* are shaped by historical differentiation, contemporary gene flow, sexual reproduction, and localized antifungal drug selection that is driving clonal expansion of genotypes resistant to multiple triazole drugs.

3.3 Introduction

Aspergillus fumigatus is a globally distributed opportunistic human fungal pathogen. Whilst its primary ecological niche is decomposing organic matter, *A. fumigatus* is ubiquitous in the environment and can be found in a broad range of ecological niches. In humans, it can colonize the lung and several other body sites, causing infections collectively known as aspergillosis. *A. fumigatus* is a major source of morbidity and mortality in immunocompromised patients causing approximately 4-5 million cases of allergic bronchopulmonary aspergillosis globally, of which, approximately 10% become chronic (1). Invasive aspergillosis is the most severe form of aspergillosis, it is estimated to have a global annual incidence of up to 10% and a mortality rate as high as 90% in high-risk groups (2,3).

Compounding the increasing incidences of aspergillosis is antifungal drug resistance associated with such infections. Targeting the highly conserved fungal ergosterol biosynthesis pathway, triazoles are the most common and effective drug used in the first line treatment of aspergillosis. However, due to the increasing frequency of azole-resistance in *A. fumigatus*, treatment failures are increasingly common and drug-resistant aspergillosis has become a significant global health issue (4,5). The medical significance of aspergillosis and azole-resistance has attracted significant attention from microbiologists, healthcare workers, and public health agencies worldwide. Indeed, several studies have examined the genetic relationships among *A. fumigatus* strains from different geographic areas (6-10). For instance, Debeaupuis et al. (1997) examined 879 isolates from five countries using restriction fragment length polymorphisms (RFLP) based on the Southern hybridization pattern

of a retrotransposon-like element and reported no evidence of genetic differentiation between environmental and clinical isolates. Similarly, Rydholm et al. (2006) examined patterns of genetic variation at three intergenic loci for 70 isolates from 22 countries using multi-locus sequence typing (MLST) and obtained similar results. Interestingly, Pringle et al. (2005) examined 63 isolates collected from 14 countries based on DNA sequence data at five loci and identified two globally distributed and genetically differentiated clusters. More recently in 2009, another study analyzed 55 isolates from four countries using amplified fragment length polymorphisms (AFLP) and showed some evidence of differentiation by geographic and ecological origins (9). Klaassen et al. (2012) analyzed 255 isolates from the Netherlands using 20 molecular markers, including nine highly polymorphic microsatellite markers, and found no evidence of differentiation among geographic populations within the Netherlands (10). However, they found that samples from the Netherland could be grouped into five genetic populations and that all the isolates containing the multiple-triazole-resistance allele at the *CYP51A* gene belonged to only one of the five genetic populations (10). Discrepancies in results obtained by these studies were likely due to the use of relatively small sample sizes and/or different markers. It has been previously suggested that using markers with higher discriminatory power and analyzing more diverse and larger sample sizes would likely produce more robust and consistent results for *A. fumigatus* (10).

Over the last few decades several molecular methods have been used for genotyping *A. fumigatus* including MLST, microsatellite markers, randomly amplified polymorphic DNA (RAPD) typing, PCR-RFLP, RFLP detected through Southern hybridization, and AFLP (11,12). However, some of these typing methods such as RAPD and AFLP have limited reproducibility, and are difficult to interpret or compare among laboratories. Among these methods, microsatellite markers have emerged as the best typing method in terms of reproducibility, costs, and discriminatory power (13). For example, Klaassen et al. showed that of the 225 *A. fumigatus* genotypes revealed by 20 combined markers — nine microsatellite, one indel, and ten sequence/PCR-typing markers — 224 could be recognized by the nine microsatellites alone (10). Although this set of nine microsatellite markers are likely

neutral and suitable for population genetic and epidemiological studies, they have not been used to characterize the global population of *A. fumigatus* (14,10).

Using the aforementioned nine microsatellite markers, we analyzed 2026 *A. fumigatus* isolates from 13 countries in 4 continents. We aimed to: (i) critically examine the genetic relationships of the isolates within and among geographic populations of *A. fumigatus,* (ii) investigate if antifungal drug susceptibility pattern is related to genetic variations, and (iii) evaluate the roles of sexual and asexual reproduction in shaping the evolution of azole resistance globally. We tested the Baas-Becking hypothesis that "everything is everywhere, the environment selects" (15). Due to its close associations with humans and human activities, both intrinsic natural factors as well as anthropogenic factors are expected to influence the genetic structure of *A. fumigatus* populations. For example, diverse microbial populations can often be structured by environmental factors (16-20), including the use of antifungal drugs. Thus, we hypothesize that there should be some genetic differentiation among samples of *A. fumigatus* with regard to their geographic origin, triazole resistance and other ecologic factors. Indeed, recent investigations identified that local clonal expansion played a significant role in the spread of triazole resistant genotypes in the Netherlands and India (10, 21-23). However, given the abundance of asexual spores in *A. fumigatus*, their ability for long-distance dispersal, and their non-fastidious requirements for growth and reproduction, gene flow was expected to be common among geographic populations of *A. fumigatus,* potentially obscuring the geographic and ecological patterns of genetic variations. Below we describe the relative roles of various factors that impact the global *A. fumigatus* population structure.

3.4 Results

3.4.1 Limited but statistically significant geographic and ecological niche contributions to genetic variation

Analysis of molecular variance (AMOVA) showed that 6% of total genetic variation was contributed by geographic separation, with the majority of genetic variation found within individual geographic populations (P=0.001) (Fig S3.1). Several pairwise geographic differentiations were significant, with the biggest differentiations found

between India and other countries, including that between India and China (PhiPT=0.375, P=0.001), India and Italy (PhiPT=0.375, P=0.001), and India and Australia (PhiPT=0.357, P=0.001). Similarly, we observed relatively limited but statistically significant genetic differentiations based on ecological niches. Among the 2026 isolates, 434 had unspecified ecological origins and were excluded from this analysis. The remaining 1592 isolates were classified into four ecological niches (clinical, air, water, and soil) and only 3% of the total genetic variation was contributed by such ecological niche separation (P=0.001) (Fig S3.2).

3.4.2 Distinct genetic clusters and evidence of historical differentiation

Given the diverse geographic and ecological niches that the samples came from, the relatively limited contributions of geography and ecological niche to the total genetic variations were surprising. One reason contributing to the limited geographic and ecological differentiations was the high levels of allelic and genotypic diversities within most geographic and ecological niche populations (Fig S3.3). In total, 1230 multi-locus microsatellite genotypes were found among the 2026 analyzed isolates. In order to examine potentially divergent genetic clusters, the Bayesian algorithm as implemented by STRUCTURE software was used. However, since STRUCTURE was unable to analyze all 2026 isolates at the same time to infer an optimal number of genetic clusters from our set of isolates, a clone-corrected sample of 1230 genotypes was used instead. Here, one randomly selected isolate was picked to represent each individual multilocus genotype. STRUCTURE analyses separated the 1230 genotypes into eight genetic clusters, Pop #1 to Pop #8 (Fig 3.1). Less than one percent of all 1230 genotypes were assigned to Pop #1 (8/1230), whereas 18.7% (230/1230), 7.7% (95/1230), 13.4% (165/1230), 16.7% (206/1230), 7.3% (90/1230), 9.8 % (120/1230) and 25.7% (316/1230) were assigned to Pops #2-8, respectively. AMOVA analysis on the eight STRUCTURE-inferred genetic clusters showed that 18% of the total genetic variance was found among the eight clusters (P=0.001) (Fig S3.4). One of the eight clusters (Pop #1) was found in only one country, Belgium, suggesting Belgium was most likely the geographic origin for this cluster. However, all other clusters contained

isolates from at least nine of the 13 countries analyzed here (Table 3.1). The eight genetic clusters inferred based on STRUCTURE analyses is supported by results from the discriminant analysis of principal components (DAPC), a non-model based approach implemented in R. We note that although the Bayesian Information Criterion (BIC) was still decreasing after separating the genotypes to eight genetic populations, the rate of change slowed considerably after that point (Fig 3.1).The existence of distinct genetic clusters is consistent with historical differentiation within *A. fumigatus.*

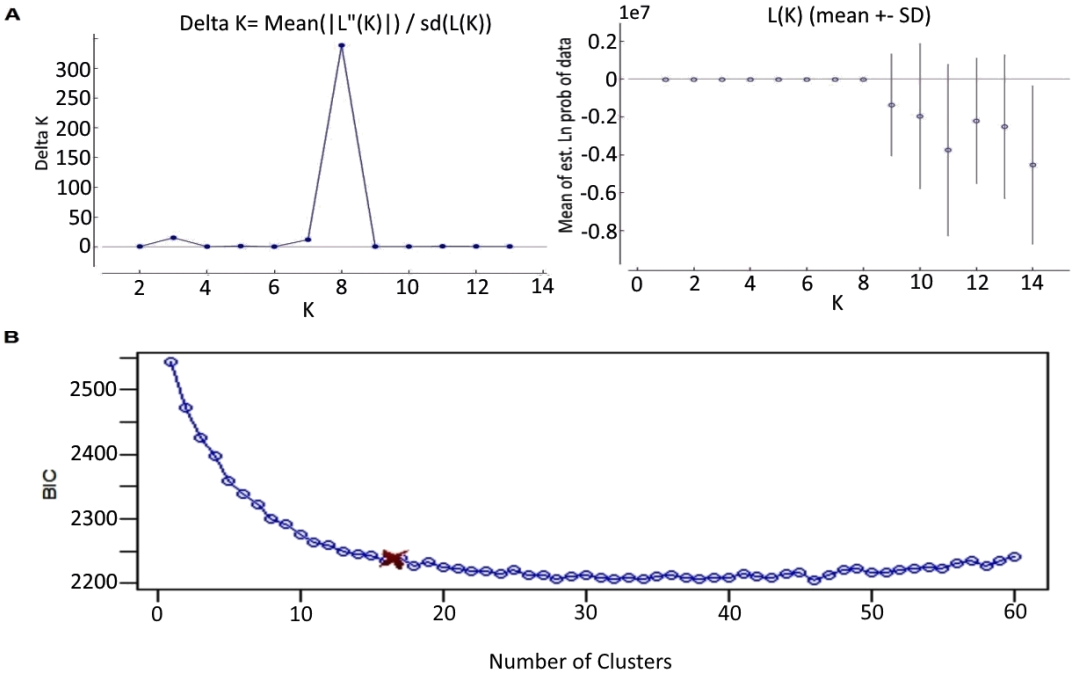


Figure 3.1. The optimal number of genetic clusters inferred by STRUCTURE and DAPC for our dataset. A) Rate of change in the log probability of data between successive runs of K (ΔK) (24) and the average posterior probability (ln K) for each K (1-14). The optimal predicted number of populations (K) for our set of isolates is eight. B) A plot of the optimal number of cluster (K) versus the Bayesian Information Criterion (BIC). BIC rate of change slows down considerably after 8 clusters and flattens out after ~14 clusters.

Table 3.1. Global distribution of the 8 inferred genetic clusters. Numbers in brackets are the number of genotypes in individual genetic clusters and in geographic sub-populations of the said genetic clusters.

Genetic Clusters (number of genotypes)	Country												
	Australia	Belgium	China	Cuba	France	Germany	India	Italy	Netherlands	Norway	Spain	Switzerland	USA
Pop #1 (8)	--	100%(8)	--	--	--	--	--	--	--	--	--	--	--
Pop #2 (230)	--	4%(9)	2%(5)	2%(4)	1%(3)	5%(12)	4%(10)	--	43%(99)	8%(18)	10%(24)	6%(14)	14%(32)
Pop #3 (95)	--	7%(7)	1%(1)	1%(1)	7%(7)	2% (2)	1% (1)	--	66%(63)	4%(4)	2%(2)	7%(7)	--
Pop #4 (165)	1%(2)	5%(9)	--	1%(1)	5%(9)	7%(11)	1%(1)	1%(2)	61%(101)	8%(13)	5%(8)	2%(3)	3%(5)
Pop #5 (206)	1%(2)	6%(12)	--	--	3%(7)	2%(5)	2%(4)	--	50%(102)	8%(17)	14%(29)	8%(16)	6%(12)
Pop #6 (90)	--	6%(5)	--	--	3%(3)	19%(17)	3%(3)	--	47%(42)	7%(6)	9%(8)	6%(5)	1%(1)
Pop #7 (120)	1%(1)	3%(4)	--	--	--	3%(3)	1%(1)	--	41%(49)	44%(53)	3%(4)	3%(4)	1%(1)
Pop #8 (316)	--	6%(19)	1%(2)	1%(4)	6%(20)	1%(4)	2%(5)	1%(2)	56%(178)	13%(42)	7%(21)	4%(12)	2%(7)

While STRUCTURE analysis is based on allelic association patterns to separate reproductively isolated strains into genetic populations/clusters and can be used to indicate historical differentiations, other indicators can also be used to support the hypothesis of historical differentiation in *A. fumigatus*. Here in this study, six of the eight clusters had at least five private alleles each at the nine analyzed loci. The percentage of strains with private alleles by genetic cluster was as follows: Pop #2, 23% (53/230); Pop #3, 5.3% (5/95); Pop #4, 15.8% (26/165); Pop #5, 12.6% (26/206); Pop #6, 25.6% (23/90); Pop #8, 32.9 % (104/316); Pops #1 and #7 had no private alleles.

3.4.3 Evidence for recombination within genetic and geographic populations and hybridization among genetic clusters.

We found varying levels of linkage equilibrium and evidence of recombination within each of the eight *A. fumigatus* genetic clusters (Table 3.2). For example, all eight genetic clusters had phylogenetically incompatible pairs of loci (Table 3.2). However, as expected, evidence for clonal reproduction was also found and all eight genetic clusters strongly rejected the null hypothesis of random recombination ($P<0.01$). Similarly, evidence for both clonality and recombination was also found within individual geographic populations of *A. fumigatus* (Table 3.2). Specifically, phylogenetic incompatibility was found in each of the 13 geographic populations. We further tested the effects of clone correction on the Index of Association (IA), and our analyses showed clonally corrected geographic samples all had reduced levels of linkage disequilibrium but still deviated from random recombination. Populations from four countries (Australia, China, Cuba and Italy) were each represented by less than ten isolates after clonal correction were excluded from this analysis.

Table 3.2. Indices of association and phylogenetic compatibility within individual genetic clusters and geographic populations. The eight genetic clusters are represented by Pop #1 to Pop #8. The IA values in parenthesis represent those based on clone-corrected samples.

Populations	Index of Association	Phylogenetic Compatibility
Pop 1	1.18	0.97
Pop 2	0.30	0.00
Pop 3	0.38	0.06
Pop 4	0.67	0.00
Pop 5	0.78	0.00
Pop 6	0.40	0.20
Pop 7	0.37	0.06
Pop 8	0.34	0.00
Belgium	2.24 (0.73)	0.00
France	1.72 (0.95)	0.00
Germany	1.38 (0.88)	0.00
India	6.66 (0.30)	0.30
Netherlands	0.75 (0.69)	0.00
Norway	0.89 (0.80)	0.00
Spain	1.72 (0.48)	0.00
Switzerland	0.74 (0.55)	0.00
USA	0.88 (0.84)	0.00

Some of the phylogenetic incompatibilities observed within individual geographic populations may represent evidence of hybridization and genetic recombination between different genetic clusters. Specifically, a number of microsatellite genotypes within the eight genetic populations showed evidence of ancestry belonging to more than one genetic cluster. For example, certain strains within Pop #1 had mixtures of alleles from Pops #2, #3, #4 and #8 in Belgium. Specifically, loci 2A and 2B of Pop #1 had allelic matches with those in Pop #2; locus 2C had an allelic match with that in Pop #4; loci 3A and 3B had allelic matches with that in Pop #3; locus 4A had an allelic match in Pops #2 and #4; and locus 4C had a match in Pop #8. Locus 4B did not have any exact matches with other genetic clusters within Belgium and was significantly differentiated from all other genetic clusters in Belgium. While these results are consistent with potential recent hybrid origins of certain strains in Pop #1, there is another possibility. That is, the sharing of alleles among genetic clusters might represent the results of recent mutational convergence from different populations. Furthermore, interestingly, for some strains with evidence

of multiple ancestries, their putative ancestral populations were often closely related. Thus, for these strains, there is a third possibility, i.e. their mixed ancestries were due to incomplete lineage sorting among the ancestral polymorphisms.

3.4.4 Evidence of contemporary gene flow

Our results showed abundant evidence of gene flow among ecological and geographic populations of *A. fumigatus* (Figs S3.1 & S3.2). For example, of the total 625 environmental (air, soil, and water) and 582 clinical *A. fumigatus* genotypes, 90 were shared between the two ecological types (Table S3.1). However, the number of shared genotypes between clinical and environmental samples varied from country to country, with some countries having as many as 50 shared genotypes between the clinical and environmental sources.

Similarly, there is abundant evidence for gene flow among geographic populations. First, pair-wise PhiPT values between most geographic populations were low (Fig S3.1). Second, the allelic distribution patterns across geographic populations revealed that many alleles were shared by countries located far away from each other (File S3.3). Third, certain genotypes were shared by isolates from long distances. For example, genotype 115 has microsatellite allelic combination of 13-10-9-10-11-9-8-9-19 at loci 2A-2B-2C-3A-3B-3C-4A-4B-4C respectively with the allelic numbers representing the number of di- or tri- nucleotide repeats at each of the microsatellite loci. This genotype was isolated from air in Belgium and a patient in the USA, while genotype 1153 with allelic combination 25-19-19-26-19-17-10-16-8 was isolated from air in Norway and a patient in India. Genotype 356 with allelic combination 18-12-8-28-10-20-9-9-5 was isolated both from air and a patient in the Netherlands and was also isolated from a patient in the USA. Furthermore, cluster analysis by means of the minimum spanning network identified evidence of dispersal for clonal complexes across countries that are up to 1800km apart from each other (Fig 3.2). Indeed, the Mantel test revealed no significant correlation between genetic distance and geographic distance (r^2=0.002) at a global scale.

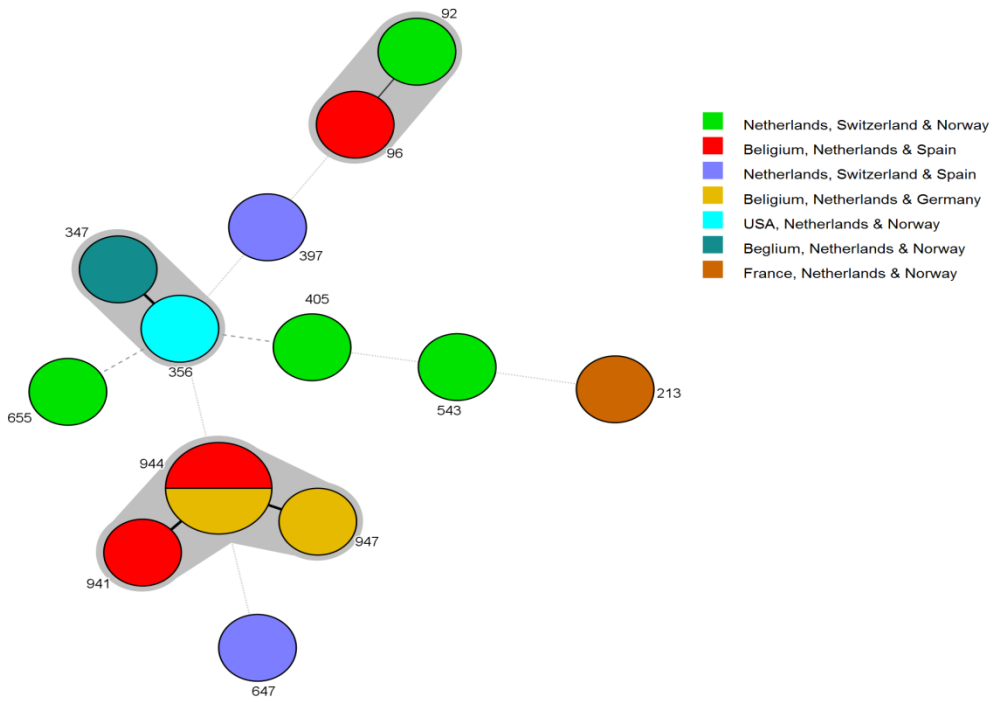


Figure 3.2 Minimum spanning tree of all genotypes identified in at least three countries. Each circle represents a genotype. Thick, short, solid lines connect variants that differed at one of the nine loci; thick, longer, solid lines connect variants with different alleles at two loci; dash and dotted lines connect variants at four and more loci, respectively. Unique colours were assigned to represent countries in which genotypes were identified. Grey partition depicts genotypes belonging to the same clonal complex

While the allele and genotype sharing between geographically far-apart countries such as the above represents long-distance gene flow, evidence for gene flow among regions within individual countries is also abundant. For example, the samples from within India were obtained from diverse locations stretching hundreds of kilometers and several genotypes were found to be widely distributed across several locations. Taken together, our results suggest frequent gene flows among regional and continental populations of *A. fumigatus*.

3.4.5 Evidence of local contemporary drug selection followed by clonal expansion

About 6% of the isolates in our dataset showed resistance to triazole antifungal drugs. Overall, there was a small but statistically significant genetic difference between the triazole-susceptible and resistant samples (PhiPT=0.063, P=0.001). Interestingly, while triazole-susceptible populations showed relatively little genetic difference among geographic populations, triazole-resistant isolates separated based on geographic origins showed a large amount of genetic differentiation (PhiPT=0.57, P=0.001) (Fig S3.5). We further tested the hypothesis that the differences between the triazole-susceptible and triazole-resistant samples were due to local clonal expansion. Indeed, after clonal correction, the level of differentiation among geographic populations of the triazole-resistant samples reduced significantly (PhiPT=0.23, P=0.001). Together, the results from these analyses suggest that clonal expansion of triazole resistant genotypes plays a significant role in geographic structuring of *A. fumigatus*.

3.5 Discussion

We investigated the global population structure using nine highly informative microsatellite markers to analyze a comprehensive set of isolates from four continents. Our analyses showed the existence of eight significantly differentiated genetic clusters within the global sample of *A. fumigatus,* a result consistent with historical differentiation. We however noted low differentiation levels and frequent sharing of alleles and genotypes among geographic and ecological populations, suggesting that contemporary gene flow is prevalent. Our analyses also identified abundant signatures of both sexual recombination and local clonal expansion. Local clonal expansions were most evident in geographic populations where resistance to triazole antifungal drugs was prevalent. Taken together, our results suggest that both historical and contemporary factors have played a significant role in shaping the global population structure of *A. fumigatus*.

STRUCTURE analyses separated all 1230 genotypes into eight genetic clusters, 3-6 more than previously identified (8, 10). In 2005, Pringle and colleagues

reported the presence of two globally distributed *A. fumigatus* genetic clusters and suggested that one of these clusters could be a cryptic *Aspergillus* species. In 2012, Klaassen et al. (10) identified five genetic clusters in the Netherlands based on the analyses of 255 isolates representing 225 multilocus genotypes. In our study, there were 1081 isolates representing 637 multilocus genotypes from the Netherlands, revealing two additional genetic clusters in this country (now seven genetic populations in total in the Netherlands, Table 3.1). The identification of additional clusters over previous studies suggests that there are likely more genetic clusters from both the analyzed geographic regions as well as those not analyzed in this study. While the Klaassen et al. (10) study and our current study used the same genetic markers and the same assumptions in inferring genetic clusters, we should note that our current inferred "optimal cluster number (K)" "8" represent only the samples we have and is based on a series of assumptions that might not be entirely valid in nature (25). Indeed, the multivariate clustering approach inferred a less clear-cut solution (Fig 3.1). However, there is some consistency between results obtained from the multivariate and the Bayesian clustering methods. Interestingly, most inferred genetic clusters by both methods were globally distributed, and the majority of triazole-resistant genotypes clustered into a single genetic population.

Comparable to results published previously, our analyses reveal that geographic populations often contain strains of divergent genotypes from different genetic clusters. At present, the putative geographic origin(s) for each of the eight genetic clusters is unresolved. Generally, the geographic center of origin should contain the highest allelic and genotypic diversities. In fungi, abundant evidence of sexual recombination has also been used as a signature for ancestral populations. However, uneven sample sizes and incomplete sampling in many countries make any conclusions at present extremely tentative (Table 3.1). Additional sequence data from multiple gene loci as well as more samples could help resolve this issue.

Cosistent with evidence of both local and long distance dispersals, limited but statistically significant population differentiations were observed among geographical or ecological populations. Finlay et al. argued that microbes smaller than about 2 µm in size are unlikely to be restricted by geographical barriers in their dispersals (26). However, it is not uncommon to see geographical differentiation in

microbial eukaryotes (27,28). For example, using DNA sequence variation in four protein-coding genes, Kasuga et al. showed population differentiation by geographic origins in the human pathogenic fungus *Histoplasma capsulatum* (27). In contrast, the global population structure of the wheat fungal pathogen *Mycosphaerella graminicola* is characterized by frequent gene flow (29). In *A. fumigatus*, the low but statistically significant differentiations were likely due to historical separations; however, these genetic differentiations are being broken down by contemporary gene flow.

Our evidence for gene flow among regional populations at the global scale extends observations reported earlier based on smaller sample sizes and/or geographically limited samples (6-10). Gene flow within and between geographic populations of *A. fumigatus* can be accomplished by wind-aided spore dispersal or by anthropogenic activities. *A. fumigatus* can reproduce both sexually and asexually and can produce abundant ascospores and conidiospores respectively in the process. These hydrophobic spores can become readily airborne and disperse by wind. The emission rate of *A. fumigatus* conidia from an undisturbed compost pile is estimated to be 8–11×10^3 cfu/m^2/s at the mean wind speed of 1 m/s (30). Our data suggested two major dispersal patterns. The first and most observed dispersal involved intermediate and short distance dispersals (SDD and IDD). For example, it was common to find several isolates from diverse locations and ecological niches within the same country sharing the same genotype. Similarly, despite relatively limited genotype sharing, allele frequencies among local populations within the Netherlands were very similar (10). Frequent gene flow can mask population differentiation and represent a major challenge for molecular epidemiologists in their efforts to track and contain fungal strains of public health interest.

The second dispersal pattern is long distance dispersal (LDD), which can be assisted or unassisted by humans. Assisted LDD refers to dispersal involving humans or associated with human artifacts whereby travelers can carry conidia on/in their bodies or on infested inanimate objects from one region to another far away. In contrast, unassisted LDD involves dispersals without human interventions, e.g. by air currents. Our data here show that certain microsatellite genotypes are found in countries up to 7500km apart from each other. For example, genotype 115 was isolated from air in Belgium and from patients in the USA. However, whether this and

other shared genotypes of *A. fumigatus* were dispersed assisted or unassisted by humans is currently unknown. Documenting and understanding the frequency and mechanism(s) for such long distance dispersals are of utmost importance in containment and eradication of pathogens. LDD events have been previously reported to play a crucial role in fungal pathogen recolonization, invasion and adaptation to host resistance (31). For example, it was suggested that cyclonic winds introduced *Puccinia melanocephala* uredospores into the Dominican Republic from Cameroon, in early June of 1978 (32). Similarly, *Cryptococcus deuterogattii* (previously *C. gattii* AFLP6/VGII) was likely spread from Vancouver Island to mainland British Columbia, Canada, and the Pacific Northwest of the United States in dust on cars and under shoes of travelers that took the ferry to Vancouver City (33). Owing to the fact that *A. fumigatus* is ubiquitous, LDD likely plays a limited role in future pathogen invasion or recolonization. However, in view of adaptation to host resistance and recent rises in azole-resistance, our finding of single event long distance dispersal would be of great significance to epidemiologists, although all identified cases of LDD in this study so far involved only triazole-susceptible isolates.

Gene flow can have both advantageous and disadvantageous consequences on the selective maintenance of genetic variation within and between microbial populations. For instance, gene flow is thought to limit population divergence and hence local selection. Gene swamping into naïve populations favors fixing of alleles with the best average reproductive success thereby counteracting the stability of local selection (34). However, the concept of gene flow moderating population divergence has been contested by observations that several species, including fungal species, are held together even though they lack sufficient gene flow to counteract divergence (35). On the other hand, gene flow is thought to facilitate adaptation by disseminating beneficial alleles. However, dissemination of highly beneficial alleles does not necessarily require high levels of gene flow (35). In *A. fumigatus,* frequent gene flow, which is known to facilitate dissemination of highly beneficial alleles such as triazole-resistance, can be attributed to the abundance of asexual spores, their ability for long-distance dispersal, and their non-fastidious requirements for growth and reproduction. The limited geographic differentiation, multiple private alleles within individual geographic and genetic populations, and the

evidence for gene flow among global populations of *A. fumigatus* indicate that the evolution of *A. fumigatus* will be continuously impacted by both local and global factors.

Our analyses suggest that genetic Pops #2 and #8 are the most widely distributed and recombining. They also contained the most private alleles. The previous study by Klaassen et al. (10) revealed that all multi-triazole resistant strains with the TR34/L98H mutations at the *CYP51A* gene from the Netherlands belonged to one genetic cluster (Pop 3 in their study and Pop #8 here in our study). Interestingly, while Pop #8 contained 80% (35/44) of all triazole-resistant genotypes, triazole resistant genotypes were also found in other genetic clusters. However, our results do indicate that the frequency of triazole resistance in Pop #8 (11%, 35/316) was at least four times higher than any other genetic population, suggesting that special attention should be placed in understanding this genetic cluster. Since the gene(s) related to resistance are not known to be tightly linked to the analyzed nine microstatellite loci, the tight clustering of most triazole-resistant isolates in Pop #8 suggest one of three possibilities. The first possibility is that Pop #8 is more frequently distributed in geographic regions where triazole drug use is very common. It has been previously hypothesized that extensive agricultural use of azole fungicides leads to resistance in *A. fumigatus* in India and elsewhere (2,36,37). Although azoles are used worldwide, the absolute amounts used differ among countries (38). The differences in the type and absolute amounts of azole fungicides used by individual regions and countries could act as distinctive selective pressures to generate the different frequencies of triazole-resistant strains. This hypothesis was supported by evidence that resistant isolates grouped by geographic origin were genetically highly differentiated (P=0.001). Using *Escherichia coli* as a model organism, it has been experimentally demonstrated that the strength of antibiotic exposure plays a significant role in the evolution of antibiotic resistance (39). The second possibility is that when exposed to triazole drugs, strains in this genetic cluster are more likely than those in other genetic clusters to develop triazole resistance. If so, when treating infections caused by strains in this genetic cluster, caution should be taken in using triazole drugs. Similar clade-specific patterns have also been shown in the plant pathogenic fungi *Puccinia graminis f. sp. tritici* and *Pyrenophora tritici-repentis* (40,41). The third possibility is that strains in Pop #8 are

more receptive than those in other clusters in accepting triazole-resistant genes via mating and recombination. Indeed, even though the early study by Klaassen et al. (10) did not show evidence of recombination for this cluster in the Netherlands, our analysis of the expanded samples in Pop #8 showed the most evidence of recombination, where all pairs of loci among the nine microsatellite markers showed phylogenetic incompatibility. The occurrence of recombination within and between *A. fumigatus* genetic populations could have significant implications in the initiation and dissemination of resistant/virulent strains capable of causing aspergillosis outbreaks (42).

Although sexual reproduction can generate genotypic variation among *A. fumigatus* populations which may allow for faster adaptation to host resistance, it can however be costly in terms of energy, time, and may have no measurable advantage in new ecological niches (43). This could possibly explain why the other genetic populations (Pops #1 & #3-7) evolved predominantly clonally and contain significantly fewer resistant isolates. Furthermore, although most triazole-resistant genotypes belonged to a highly recombinant genetic cluster, our data as well as previous studies showed that the expansion of triazole-resistant strains at the local (country level) level is predominantly clonal (10, 21-23). Such localized clonal expansion is a significant factor that shapes the current *A. fumigatus* population structure. Indeed, the clonal spread of an adaptive recombinant progeny resistant to multiple triazole drugs in India was the main reason causing the significant genetic differentiations between the Indian *A. fumigatus* population from those isolated from elsewhere in the world.

In conclusion, using a large number of isolates from geographically and ecologically diverse regions allowed us to address several fundamental questions about the global population of *A. fumigatus*. Our findings go beyond those previously identified by other studies. We report limited but statistically significant genetic differentiations among geographic and ecological populations of *A. fumigatus*. The identification of eight genetically differentiated clusters are consistent with historical differentiation but contemporary gene flows are blurring the historical patterns. Interestingly, unlike the triazole-susceptible samples where geographic populations were largely undifferentiated, triazole-resistant samples were significantly

differentiated according to geographic regions. The differences in triazole usage among the countries have likely contributed to the genetic differences among the triazole-resistant samples. Though evidence for clonality was found in all geographic, ecological, and genetic populations, we also found evidence for recombination in all analyzed populations, a result different from those reported in earlier studies (6-10). This difference is especially noteworthy for Pop #8, the dominant genetic cluster containing most of the triazole-resistant strains where no evidence of recombination was found in an earlier study (10), but abundant evidence for recombination was found in our current study. However, despite the large sample size and broad geographic and ecological representations in our samples, additional genetic diversities and other types of genetic relationships among samples could exist when additional samples are analyzed. Our results provide important data for future assessment of *A. fumigatus* migration patterns. From a practical perspective, our findings should aid in better tracking and management of aspergillosis outbreaks.

3.6 Material and methods

3.6.1 Isolates used for analyses and genotyping

Samples used in this study were obtained as part of collaborative studies between Canisius Wilhelmina Hospital and research centers in several countries (4,10,14, 44-50). A total of 2026 isolates from 13 countries in 4 continents were genotyped and included in our analyses. Genotyping was performed with a panel of nine short tandem repeats (STRAf 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C) as previously described (14).

3.6.2 Identification of genetic clusters

In order to investigate the existence of distinct genetic clusters, we used both multivariate and model-based Bayesian clustering as implement by ADEGENET package in R version 3.0 and STRUCTURE software version 2.3 (51-52). Multivariate clustering was used to compliment Bayesian clustering bearing in mind that this approach assumes linkage equilibrium within clusters (51). However,

multivariate clustering cannot be solely used for clustering analysis as its efficiency is limited by correlations between variables (alleles). Moreover, the contravention of the assumption of uncorrelated alleles is further emphasized in the presence of linkage disequilibrium. When multivariate clustering was used, the optimal number of clusters (K) was inferred based on BIC (51). For model-based Bayesian clustering, we chose the admixture and the correlated allele frequencies between population options as our ancestry and frequency models, respectively. Markov Chain Monte Carlo (MCMC) simulations were run for K=1–14. However, given the huge data set, the MCMC sampling scheme was run for only 9,000 iterations with a 1,000 burn-in period. Notwithstanding, summary statistics values (Alpha, Fst and Likelihood) seemed to have converged at 10^4 iterations, thereby validating the run length. Two approaches were used to identify the optimal number of clusters. The ad-hoc statistic DK, based on the rate of change in the log probability of data between successive runs of K, was calculated as previously recommended (24). The second approach recommended by Pritchard et al (2000) calculates the average log probability (LnP (D)) of each K value (52).

3.6.3. Differentiations among samples separated by geographic, ecological, and triazole-susceptibility patterns

In order to identify the potential contributors to the observed genetic variation, the pairwise samples grouped based on different criteria were compared using the GENALEX version 6.5 (53). In these analyses, three criteria were used to group the samples: their triazole susceptibility status (triazole-susceptible vs. triazole resistant), their ecological niche (clinical, soil, air, and aquatic), and geographical origin (country). Cluster analysis was done using the minimum spanning tree algorithm as implemented by Bionumerics version 7.0 (Applied Maths, Saint-Martens-Latem, Belgium)

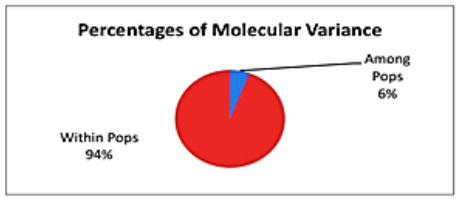
3.6.4. Analyses of genetic variability, allelic diversity and recombination

Nei's genetic diversity corrected for sample size was calculated for independent populations using GENALEX version 6.5 (53). The MULTILOCUS program version 1.3b was used to evaluate the presence and prevalence of linkage disequilibrium (LD) and phylogenetic compatibility as indicators of clonality and recombination (54). A diversity of samples and sub-samples were analyzed, including those representing individual genetic clusters and geographic populations. The statistical significance of each test was determined by 1000 permutations.

3.7 Supplementary material

Results of Analysis of Molecular Variance

Data Sheet	File S1
Data Title	Geo separation
No. Samples	2026
No. Pops	13
No. Permutations	999
N0	115.699
SSTOT	7892.766



Pop	Australia	Belgium	China	Cuba	France	Germany	India	Italy	Netherland	Norway	Spain	Switzerland	USA
n	7	108	8	10	66	85	94	7	1081	203	186	70	101
SSWP	20.000	397.843	21.625	34.600	248.515	313.235	222.117	20.286	4188.110	708.246	705.323	269.671	379.564

Summary AMOVA Table

Source	df	SS	MS	Est. Var.	%
Among Pops	12	363.630	30.303	0.230	6%
Within Pops	2013	7529.136	3.740	3.740	94%
Total	2025	7892.766		3.970	100%

Stat	Value	P(rand >= data) Probability, P(rand >= data), for PhiPT is based on standard permutation across the full data set.
PhiPT	0.058	0.001 PhiPT = AP / (WP + AP) = AP / TOT
PhiPT max	0.178	
Phi'PT	0.325	
Nm (Haploid)	8.146	Nm (Haploid) = [(1 / PhiPT) - 1] / 2

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

Pairwise Population PhiPT Values

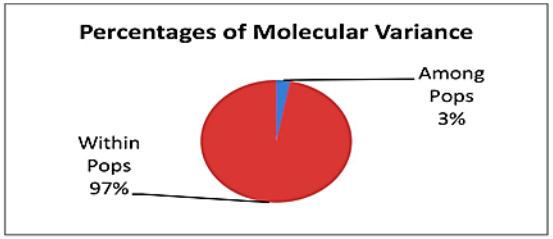
Australia	Belgium	China	Cuba	France	Germany	India	Italy	Netherland	Norway	Spain	Switzerland	USA	
	0.038	0.001	0.032	0.008	0.015	0.001	0.050	0.005	0.001	0.025	0.010	0.011	**Australia**
0.051		0.001	0.007	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**Belgium**
0.185	0.152		0.001	0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.001	**China**
0.068	0.049	0.163		0.030	0.021	0.001	0.113	0.027	0.001	0.019	0.024	0.014	**Cuba**
0.075	0.035	0.115	0.035		0.001	0.001	0.009	0.001	0.001	0.001	0.003	0.001	**France**
0.061	0.058	0.137	0.034	0.049		0.001	0.028	0.001	0.001	0.001	0.001	0.001	**Germay**
0.357	0.225	0.375	0.297	0.183	0.273		0.001	0.001	0.001	0.001	0.001	0.001	**India**
0.113	0.103	0.208	0.048	0.088	0.044	0.373		0.001	0.001	0.004	0.001	0.003	**Italy**
0.050	0.021	0.115	0.028	0.015	0.034	0.180	0.068		0.001	0.001	0.017	0.001	**Netherland**
0.095	0.051	0.162	0.082	0.063	0.083	0.230	0.138	0.032		0.001	0.001	0.001	**Norway**
0.051	0.034	0.110	0.038	0.031	0.041	0.208	0.103	0.023	0.050		0.002	0.001	**Spain**
0.046	0.021	0.107	0.029	0.017	0.036	0.218	0.081	0.005	0.033	0.012		0.001	**Switzerland**
0.068	0.060	0.120	0.044	0.053	0.054	0.257	0.105	0.044	0.074	0.037	0.035		**USA**

PhiPT Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

Figure S3.1. Contribution of geographic separation to genetic variation in the global sample of 2026 *Aspergillus fumigatus* isolates. Pie charts show percentages of among and within population variation that can be explained by grouping isolates by geographic origin. PhiPT values are in the lower diagonal of the pairwise comparison table. Probabilities based on 999 permutations are shown above the diagonal.

Results of Analysis of Molecular Variance

Data Sheet	File S2
Data Title	Eco separation
No. Samples	1592
No. Pops	4
No. Permutations	999
N0	313.448
SSTOT	6187.791



Pop	**Air**	**Clinical**	**Soil**	**Water**
n	529	858	117	88
SSWP	2060.386	3325.593	381.051	300.159

Summary AMOVA Table

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	3	120.602	40.201	0.116	3%
Within Pops	1588	6067.189	3.821	3.821	97%
Total	1591	6187.791		3.937	100%

Stat	**Value**	**P(rand >= data) Probability, P(rand >= data), for PhiPT is based on standard permutation across the full data set.**
PhiPT	0.029	0.001 **PhiPT = AP / (WP + AP) = AP / TOT**
PhiPT max	0.158	
Phi'PT	0.187	
Nm (Haploid)	16.459	**Nm (Haploid) = [(1 / PhiPT) - 1] / 2**

Key: AP = Est. Var. Among Pops, WP = Est. Var. Among Pops

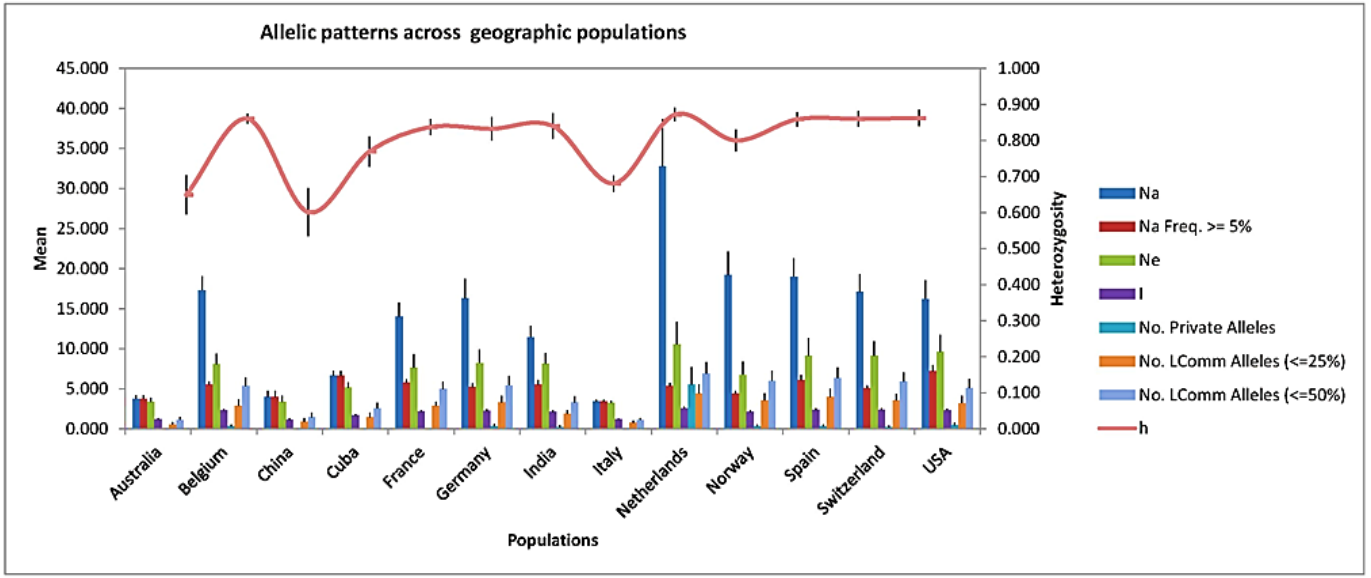
Pairwise Population PhiPT Values

Air	**Clinical**	**Soil**	**Water**	
	0.001	0.001	0.001	**Air**
0.005		0.001	0.001	**Clinical**
0.089	0.082		0.001	**Soil**
0.032	0.034	0.115		**Water**

PhiPT Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

Figure S3.2. Contribution of ecological niches to the total genetic variation. A total 1592 isolates from our data set had known ecological origins (environmental or clinical). Pie charts show percentages of among and within population variation that can be explained by grouping isolates by ecological origin. PhiPT values are in the lower diagonal of the pairwise comparison table. Probabilities based on 999 permutations are shown above the diagonal.

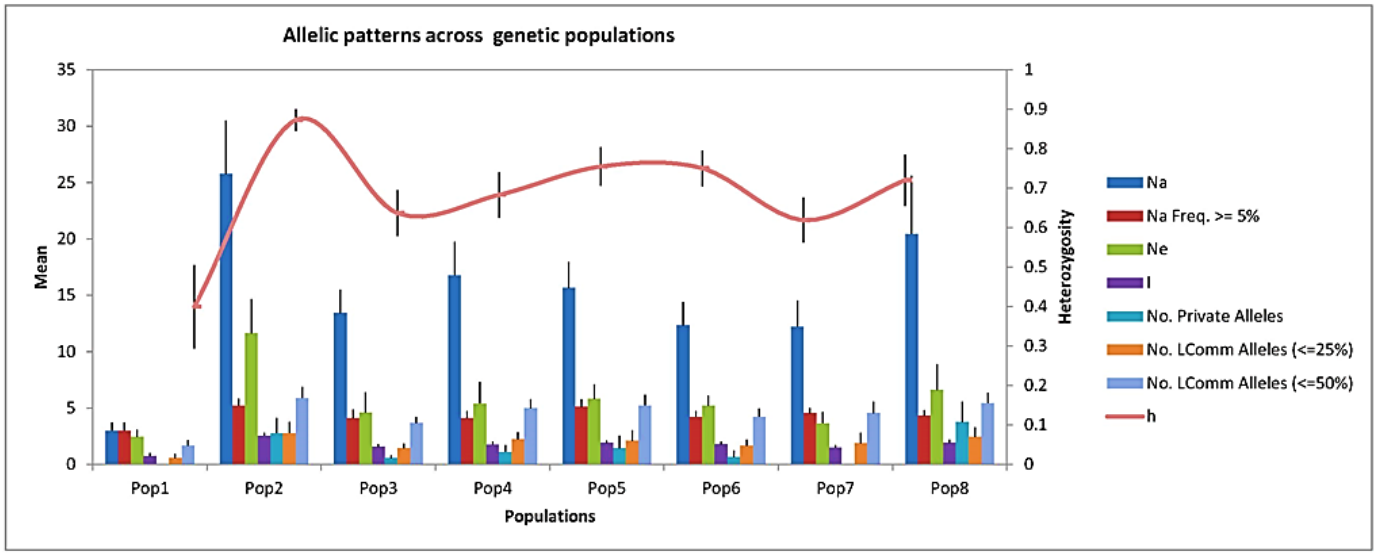
Data Sheet File S3
Data Title **Mean allelic patterns across geographic and genetic populations**
No. Loci 9



Mean values

Population	Australia	Belgium	China	Cuba	France	Germany	India	Italy	Netherlands	Norway	Spain	Switzerland	USA
Na	3.778	17.333	4.000	6.667	14.000	16.333	11.444	3.444	32.778	19.222	19.000	17.111	16.222
Na Freq. >= 5%	3.778	5.556	4.000	6.667	5.778	5.222	5.556	3.444	5.333	4.444	6.111	5.111	7.222
Ne	3.381	8.075	3.378	5.158	7.659	8.232	8.155	3.259	10.563	6.745	9.111	9.117	9.621
I	1.205	2.352	1.141	1.709	2.183	2.265	2.153	1.194	2.551	2.142	2.389	2.367	2.353
No. Private Alleles	0.000	0.333	0.000	0.000	0.000	0.333	0.222	0.000	5.556	0.333	0.333	0.222	0.444
No. LComm Alleles (<=25%)	0.556	2.889	0.889	1.444	2.889	3.333	1.889	0.778	4.444	3.556	4.000	3.556	3.222
No. LComm Alleles (<=50%)	1.111	5.333	1.444	2.556	5.000	5.444	3.333	1.111	6.889	6.000	6.333	5.889	5.111
h	0.649	0.860	0.601	0.769	0.837	0.832	0.840	0.681	0.872	0.800	0.858	0.860	0.862
uh	0.811	0.872	0.687	0.854	0.854	0.848	0.875	0.907	0.873	0.805	0.867	0.874	0.877

Standard Error (SE) values													
Population	Australia	Belgium	China	Cuba	France	Germany	India	Italy	Netherlands	Norway	Spain	Switzerland	USA
Na	0.364	1.675	0.707	0.553	1.732	2.392	1.365	0.176	5.883	2.900	2.255	2.163	2.332
Na Freq. >= 5%	0.364	0.338	0.707	0.553	0.401	0.434	0.530	0.176	0.408	0.294	0.588	0.309	0.722
Ne	0.461	1.282	0.758	0.640	1.627	1.628	1.308	0.234	2.775	1.657	2.162	1.776	2.125
I	0.130	0.111	0.184	0.131	0.146	0.198	0.167	0.061	0.188	0.192	0.160	0.163	0.174
No. Private Alleles	0.000	0.167	0.000	0.000	0.000	0.236	0.222	0.000	2.167	0.167	0.167	0.147	0.338
No. LComm Alleles (<=25%)	0.242	0.790	0.455	0.556	0.484	0.799	0.455	0.222	1.107	0.852	1.000	0.801	0.862
No. LComm Alleles (<=50%)	0.351	1.093	0.530	0.689	0.816	1.156	0.707	0.200	1.457	1.179	1.333	1.136	1.124
h	0.054	0.014	0.066	0.042	0.022	0.033	0.035	0.022	0.019	0.031	0.020	0.022	0.022
uh	0.068	0.014	0.076	0.047	0.023	0.034	0.037	0.029	0.019	0.031	0.021	0.022	0.023



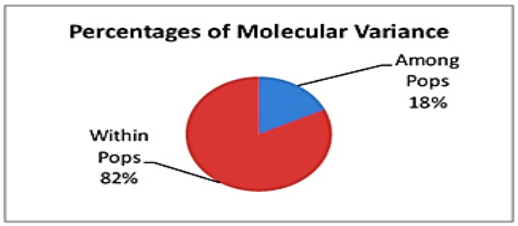
Mean values								
Population	**Pop1**	**Pop2**	**Pop3**	**Pop4**	**Pop5**	**Pop6**	**Pop7**	**Pop8**
Na	3.000	25.778	13.444	16.778	15.667	12.333	12.222	20.444
Na Freq. >= 5%	3.000	5.222	4.111	4.111	5.111	4.222	4.556	4.333
Ne	2.445	11.634	4.579	5.399	5.828	5.202	3.650	6.616
I	0.756	2.558	1.581	1.770	1.924	1.796	1.515	1.909
No. Private Alleles	0.000	2.778	0.556	1.111	1.444	0.667	0.000	3.778
No. LComm Alleles (<=25%)	0.556	2.778	1.444	2.222	2.111	1.667	1.889	2.444
No. LComm Alleles (<=50%)	1.667	5.889	3.667	5.000	5.222	4.222	4.556	5.444
h	0.399	0.872	0.637	0.683	0.754	0.750	0.619	0.720
uh	0.456	0.876	0.644	0.687	0.758	0.759	0.624	0.722

Standard Error (SE) values								
Population	**Pop1**	**Pop2**	**Pop3**	**Pop4**	**Pop5**	**Pop6**	**Pop7**	**Pop8**
Na	0.707	4.725	2.015	2.938	2.242	2.041	2.296	5.140
Na Freq. >= 5%	0.707	0.572	0.754	0.588	0.676	0.494	0.444	0.471
Ne	0.625	3.004	1.813	1.883	1.265	0.894	0.969	2.259
I	0.227	0.215	0.209	0.250	0.189	0.204	0.201	0.280
No. Private Alleles	0.000	1.310	0.242	0.588	1.082	0.553	0.000	1.762
No. LComm Alleles (<=25%)	0.377	0.969	0.377	0.619	0.920	0.527	0.904	0.835
No. LComm Alleles (<=50%)	0.471	0.992	0.527	0.782	0.940	0.722	0.973	0.884
h	0.105	0.027	0.059	0.058	0.049	0.045	0.057	0.065
uh	0.120	0.028	0.059	0.058	0.049	0.046	0.058	0.065

Figure S3.3. Mean allelic information patterns across geographical and genetic populations. Na=number of different alleles, Ne=No. of Effective Alleles=1/(Sum pi^2), I=Shannon's Information Index=-1*Sum (pi*Ln(pi)), h=Diversity=1-Sum pi^2, uh=Unbiased Diversity=(N/(N-1))*h. Where pi is the frequency of the ith allele for the population and Sum pi^2 is the sum of the squared population allele frequencies.

Results of Analysis of Molecular Variance

Data Sheet	File S4
Data Title	Gen Pop separation
No. Samples	1230
No. Pops	8
No. Permutations	999
N0	146.213
SSTOT	4830.342



Pop	**Pop1**	**Pop2**	**Pop3**	**Pop4**	**Pop5**	**Pop6**	**Pop7**	**Pop8**
n	8	230	95	165	206	90	120	316
SSWP	14.375	906.652	273.526	507.527	699.500	303.944	334.200	1023.630

Summary AMOVA Table

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	7	766.987	109.570	0.727	18%
Within Pops	1222	4063.355	3.325	3.325	82%
Total	1229	4830.342		4.052	100%

Stat	**Value**	**P(rand >= data) Probability, P(rand >= data), for PhiPT is based on standard permutation across the full data set.**
PhiPT	0.179	0.001 **PhiPT = AP / (WP + AP) = AP / TOT**
PhiPT max	0.263	
Phi'PT	0.683	
Nm (Haploid)	2.288	**Nm (Haploid) = [(1 / PhiPT) - 1] / 2**

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

Pairwise Population PhiPT Values

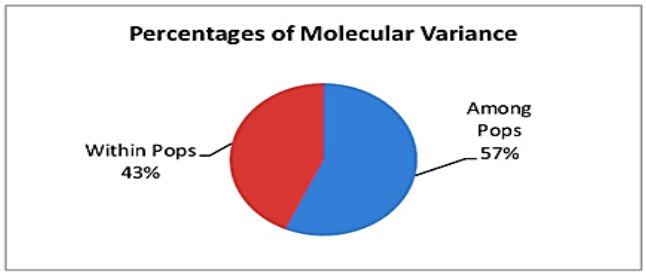
Pop1	**Pop2**	**Pop3**	**Pop4**	**Pop5**	**Pop6**	**Pop7**	**Pop8**	
	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**Pop1**
0.216		0.001	0.001	0.001	0.001	0.001	0.001	**Pop2**
0.373	0.135		0.001	0.001	0.001	0.001	0.001	**Pop3**
0.344	0.099	0.194		0.001	0.001	0.001	0.001	**Pop4**
0.247	0.104	0.220	0.180		0.001	0.001	0.001	**Pop5**
0.300	0.083	0.195	0.157	0.162		0.001	0.001	**Pop6**
0.365	0.152	0.257	0.244	0.144	0.202		0.001	**Pop7**
0.336	0.145	0.242	0.249	0.184	0.188	0.243		**Pop8**

PhiPT Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

Figure S3.4. Population differentiation among STRUCTURE-inferred genetic clusters. Our clonally corrected data set of 1230 genotypes was used to infer the number of genetic cluster. Pie charts show percentages of among and within population variation. PhiPT values are in the lower diagonal of the pairwise comparison table. Probabilities based on 999 permutations are shown above the diagonal.

Results of Analysis of Molecular Variance

Data Sheet	File S6
Data Title	Res seperation
No. Samples	102
No. Pops	3
No. Permutations	999
N0	27.108
SSTOT	219.902



Pop	**China**	**India**	**Netherlands**
n	8	61	33
SSWP	21.625	0.000	104.909

Summary AMOVA Table

Source	**df**	**SS**	**MS**	**Est. Var.**	**%**
Among Pops	2	93.368	46.684	1.675	57%
Within Pops	99	126.534	1.278	1.278	43%
Total	101	219.902		2.953	100%

Stat	**Value**	**P(rand >= data) Probability, P(rand >= data), for PhiPT is based on standard permutation across the full data set.**
PhiPT	0.567	0.001 **PhiPT = AP / (WP + AP) = AP / TOT**
PhiPT max	0.685	
Phi'PT	0.828	
Nm (Haploid)	0.382	**Nm (Haploid) = [(1 / PhiPT) - 1] / 2**

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops

China	**India**	**Netherlands**	
	0.001	0.001	**China**
0.897		0.001	**India**
0.232	0.566		**Netherlands**

PhiPT Values below diagonal. Probability, P(rand >= data) based on 999 permutations is shown above diagonal.

Figure S3.5. Contribution of geographic separation to genetic variation in the triazole-resistant sample of 102 isolates. Pie charts show percentages of among and within population variation that can be explained by grouping resistant isolates by geographic origin. A total of 106 resistant isolates from five countries were identified, however, countries with less than seven isolates were excluded from our analysis of molecular variance. PhiPT values are in the lower diagonal of the pairwise comparison table. Probabilities based on 999 permutations are shown above the diagonal.

Table S3.1. Genotype matches between environmental and clinical populations. Allelic profiles of all 90 genotypes with nine microsatellite loci matches between the two ecological populations. Population labeled Env had an environmental origin; however, its specific (air, water or soil) environmental niche was unknown.

Sample ID	Population	2A	2B	2C	3A	3B	3C	4A	4B	4C	No. Matches	Label
533	Clinical	10	14	10	17	13	14	7	5	5	0	A
1389	Env	10	14	10	17	13	14	7	5	5	2	A
738	Clinical	18	12	11	16	10	13	8	9	5	0	AA
186	Air	18	12	11	16	10	13	8	9	5	2	AA
957	Clinical	19	22	21	34	10	29	14	9	7	0	AAA
311	Air	19	22	21	34	10	29	14	9	7	2	AAA
1383	Clinical	25	19	19	26	19	17	10	16	8	0	AAAA
483	Air	25	19	19	26	19	17	10	16	8	2	AAAA
534	Clinical	10	14	10	17	13	14	7	5	6	0	B
10	Air	10	14	10	17	13	14	7	5	6	2	B
745	Clinical	18	12	11	26	11	20	8	8	5	0	BB
1460	Env	18	12	11	26	11	20	8	8	5	2	BB
994	Clinical	19	25	34	34	23	26	10	10	5	0	BBB
1519	Env	19	25	34	34	23	26	10	10	5	2	BBB
1268	Clinical	25	20	11	10	9	7	8	10	20	0	BBBB
492	Air	25	20	11	10	9	7	8	10	20	2	BBBB
536	Clinical	10	14	10	17	13	15	7	5	5	0	C
11	Air	10	14	10	17	13	15	7	5	5	2	C
750	Clinical	18	12	13	14	10	12	8	9	5	0	CC
1735	Water	18	12	13	14	10	12	8	9	5	2	CC
1021	Clinical	20	17	16	39	11	22	12	11	8	0	CCC
330	Air	20	17	16	39	11	22	12	11	8	2	CCC
1274	Clinical	25	20	13	37	9	10	8	10	8	0	CCCC
1575	Env	25	20	13	37	9	10	8	10	8	2	CCCC
538	Clinical	10	15	10	18	12	12	7	5	6	0	D
1592	Soil	10	15	10	18	12	12	7	5	6	2	D

756	Clinical	18	12	14	14	14	14	19	10	5	0	DD
197	Air	18	12	14	14	14	14	19	10	5	2	DD
1032	Clinical	20	19	15	39	12	7	10	11	12	0	DDD
333	Air	20	19	15	39	12	7	10	11	12	2	DDD
1256	Clinical	25	20	8	10	10	21	9	10	5	0	DDDD
1784	Water	25	20	8	10	10	21	9	10	5	2	DDDD
540	Clinical	10	16	10	17	13	19	7	5	6	0	E
20	Air	10	16	10	17	13	19	7	5	6	2	E
765	Clinical	18	12	14	27	11	14	9	11	7	0	EE
198	Air	18	12	14	27	11	14	9	11	7	2	EE
1035	Clinical	20	19	15	40	12	7	10	11	12	0	EEE
1529	Env	20	19	15	40	12	7	10	11	12	2	EEE
1257	Clinical	25	20	8	10	10	22	9	10	5	0	EEEE
486	Air	25	20	8	10	10	22	9	10	5	2	EEEE
542	Clinical	10	16	10	22	12	12	7	5	5	0	F
1595	Soil	10	16	10	22	12	12	7	5	5	2	F
784	Clinical	18	12	16	26	11	20	8	8	7	0	FF
206	Air	18	12	16	26	11	20	8	8	7	2	FF
1026	Clinical	20	19	8	31	14	20	9	9	5	0	FFF
1766	Water	20	19	8	31	14	20	9	9	5	2	FFF
1260	Clinical	25	20	9	10	10	7	8	10	10	0	FFFF
1785	Water	25	20	9	10	10	7	8	10	10	2	FFFF
545	Clinical	10	16	10	23	13	21	7	5	6	0	G
26	Air	10	16	10	23	13	21	7	5	6	2	G
785	Clinical	18	12	17	25	10	21	8	9	7	0	GG
1736	Water	18	12	17	25	10	21	8	9	7	2	GG
1061	Clinical	21	22	18	26	10	14	9	13	8	0	GGG
351	Air	21	22	18	26	10	14	9	13	8	2	GGG
1275	Clinical	25	21	8	29	9	6	8	10	20	0	GGGG
494	Air	25	21	8	29	9	6	8	10	20	2	GGGG
547	Clinical	10	16	10	24	11	8	7	5	5	0	H
30	Air	10	16	10	24	11	8	7	5	5	2	H

706	Clinical	18	12	8	27	10	19	9	9	5	0	HH
1730	Water	18	12	8	27	10	19	9	9	5	2	HH
1063	Clinical	21	22	18	26	10	15	9	13	8	0	HHH
1681	Soil	21	22	18	26	10	15	9	13	8	2	HHH
1295	Clinical	25	22	19	28	9	26	10	9	5	0	HHHH
1577	Env	25	22	19	28	9	26	10	9	5	2	HHHH
549	Clinical	10	16	10	25	11	8	7	5	6	0	I
1597	Soil	10	16	10	25	11	8	7	5	6	2	I
710	Clinical	18	12	8	27	10	20	9	9	5	0	II
1669	Soil	18	12	8	27	10	20	9	9	5	2	II
1070	Clinical	21	25	18	27	12	7	20.3	10	8	0	III
1682	Soil	21	25	18	27	12	7	20.3	10	8	2	III
1297	Clinical	25	22	20	27	9	26	10	9	5	0	IIII
508	Air	25	22	20	27	9	26	10	9	5	2	IIII
551	Clinical	10	16	10	25	12	8	7	5	6	0	J
33	Air	10	16	10	25	12	8	7	5	6	2	J
720	Clinical	18	12	8	28	10	20	9	9	5	0	JJ
1732	Water	18	12	8	28	10	20	9	9	5	2	JJ
1077	Clinical	21	25	19	27	12	7	20.3	10	8	0	JJJ
361	Air	21	25	19	27	12	7	20.3	10	8	2	JJJ
1300	Clinical	25	26	9	10	10	7	9	9	5	0	JJJJ
510	Air	25	26	9	10	10	7	9	9	5	2	JJJJ
554	Clinical	10	16	10	26	12	8	7	5	6	0	K
42	Air	10	16	10	26	12	8	7	5	6	2	K
721	Clinical	18	12	8	28	10	21	9	9	5	0	KK
170	Air	18	12	8	28	10	21	9	9	5	2	KK
1080	Clinical	21	25	19	27	12	7	21	10	8	0	KKK
363	Air	21	25	19	27	12	7	21	10	8	2	KKK
1313	Clinical	26	20	8	25	9	7	8	10	10	0	KKKK
517	Air	26	20	8	25	9	7	8	10	10	2	KKKK
556	Clinical	10	16	10	30	13	21	7	5	5	0	L
43	Air	10	16	10	30	13	21	7	5	5	2	L

730	Clinical	18	12	8	31	10	20	9	9	5	0	LL
178	Air	18	12	8	31	10	20	9	9	5	2	LL
1089	Clinical	22	19	15	37	11	21	10	26	5	0	LLL
369	Air	22	19	15	37	11	21	10	26	5	2	LLL
1317	Clinical	26	20	8	35	9	7	8	10	20	0	LLLL
518	Air	26	20	8	35	9	7	8	10	20	2	LLLL
557	Clinical	10	16	10	31	13	21	7	5	5	0	M
44	Air	10	16	10	31	13	21	7	5	5	2	M
816	Clinical	18	17	16	36	21	24	15.3	9	8	0	MM
1474	Env	18	17	16	36	21	24	15.3	9	8	2	MM
1101	Clinical	23	16	8	56	10	20	12.3	10	8	0	MMM
375	Air	23	16	8	56	10	20	12.3	10	8	2	MMM
575	Clinical	11	12	17	25	22	21	14	8	5	0	N
1706	Water	11	12	17	25	22	21	14	8	5	2	N
836	Clinical	18	19	11	27	10	34	20	11	5	0	NN
1748	Water	18	19	11	27	10	34	20	11	5	2	NN
1117	Clinical	23	19	15	47	11	7	13	9	5	0	NNN
388	Air	23	19	15	47	11	7	13	9	5	2	NNN
588	Clinical	11	14	14	27	11	5	15	8	7	0	O
1407	Env	11	14	14	27	11	5	15	8	7	2	O
843	Clinical	18	19	11	27	10	35	20	11	5	0	OO
244	Air	18	19	11	27	10	35	20	11	5	2	OO
1125	Clinical	23	19	15	48	11	7	13	9	5	0	OOO
394	Air	23	19	15	48	11	7	13	9	5	2	OOO
593	Clinical	13	10	9	10	11	9	8	9	19	0	P
1410	Env	13	10	9	10	11	9	8	9	19	2	P
819	Clinical	18	19	8	10	10	20	9	9	10	0	PP
224	Air	18	19	8	10	10	20	9	9	10	2	PP
1130	Clinical	23	19	15	49	11	7	13	9	5	0	PPP
1548	Env	23	19	15	49	11	7	13	9	5	2	PPP
600	Clinical	13	19	8	22	10	10	9	10	21	0	Q
1708	Water	13	19	8	22	10	10	9	10	21	2	Q

824	Clinical	18	19	8	25	23	20	15	9	5	0	QQ
228	Air	18	19	8	25	23	20	15	9	5	2	QQ
1136	Clinical	23	19	15	50	11	7	13	9	5	0	QQQ
405	Air	23	19	15	50	11	7	13	9	5	2	QQQ
622	Clinical	14	12	9	26	10	20	8	8	10	0	R
1417	Env	14	12	9	26	10	20	8	8	10	2	R
830	Clinical	18	19	8	28	14	21	9	9	5	0	RR
1742	Water	18	19	8	28	14	21	9	9	5	2	RR
1150	Clinical	23	19	16	29	17	7	10	26	5	0	RRR
1549	Env	23	19	16	29	17	7	10	26	5	2	RRR
646	Clinical	14	20	11	34	9	7	8	10	12	0	S
1427	Env	14	20	11	34	9	7	8	10	12	2	S
853	Clinical	18	20	9	10	10	12	8	10	5	0	SS
1749	Water	18	20	9	10	10	12	8	10	5	2	SS
1153	Clinical	23	19	16	30	17	7	10	26	5	0	SSS
411	Air	23	19	16	30	17	7	10	26	5	2	SSS
640	Clinical	14	20	8	36	13	10	8	10	5	0	T
118	Air	14	20	8	36	13	10	8	10	5	2	T
872	Clinical	18	21	19	22	11	31	10	8	8	0	TT
1751	Water	18	21	19	22	11	31	10	8	8	2	TT
1154	Clinical	23	19	16	44	20	7	13.3	9	7	0	TTT
415	Air	23	19	16	44	20	7	13.3	9	7	2	TTT
1350	Clinical	14	20	9	31	9	10	8	10	28	0	U
1658	Soil	14	20	9	31	9	10	8	10	28	2	U
876	Clinical	18	21	27	62	11	30	17.3	9	5	0	UU
1498	Env	18	21	27	62	11	30	17.3	9	5	2	UU
1177	Clinical	23	21	8	48	9	6	8	9	5	0	UUU
433	Air	23	21	8	48	9	6	8	9	5	2	UUU
666	Clinical	15	19	8	26	10	7	8	9	5	0	V
1720	Water	15	19	8	26	10	7	8	9	5	2	V
924	Clinical	18	25	19	28	11	7	17.3	10	8	0	VV
1511	Env	18	25	19	28	11	7	17.3	10	8	2	VV

1199	Clinical	23	23	15	38	11	49	10	26	8	0	VVV
444	Air	23	23	15	38	11	49	10	26	8	2	VVV
667	Clinical	15	19	8	33	15	20	9	10	5	0	W
136	Air	15	19	8	33	15	20	9	10	5	2	W
932	Clinical	18	28	8	16	10	18	8	9	5	0	WW
1755	Water	18	28	8	16	10	18	8	9	5	2	WW
1216	Clinical	23	24	15	48	13	7	10	9	5	0	WWW
1691	Soil	23	24	15	48	13	7	10	9	5	2	WWW
668	Clinical	15	19	8	34	15	20	9	10	5	0	X
1722	Water	15	19	8	34	15	20	9	10	5	2	X
943	Clinical	19	12	12	25	25	35	12	12	5	0	XX
295	Air	19	12	12	25	25	35	12	12	5	2	XX
1381	Clinical	25	10	8	86	9	12	8	7	5	0	XXX
1695	Soil	25	10	8	86	9	12	8	7	5	2	XXX
670	Clinical	15	19	9	10	10	13	9	10	5	0	Y
1723	Water	15	19	9	10	10	13	9	10	5	2	Y
944	Clinical	19	12	15	17	25	12	16	10	5	0	YY
297	Air	19	12	15	17	25	12	16	10	5	2	YY
1242	Clinical	25	16	23	22	11	20	12	11	8	0	YYY
476	Air	25	16	23	22	11	20	12	11	8	2	YYY
687	Clinical	17	12	13	16	10	22	8	8	8	0	Z
147	Air	17	12	13	16	10	22	8	8	8	2	Z
956	Clinical	19	22	21	33	10	29	14	9	7	0	ZZ
310	Air	19	22	21	33	10	29	14	9	7	2	ZZ
1250	Clinical	25	19	16	26	13	7	10	16	8	0	ZZZ
1700	Soil	25	19	16	26	13	7	10	16	8	2	ZZZ

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Chapter 4: Diversity and origins of Indian multi-triazole resistant strains of *Aspergillus fumigatus*

4.1 Preface

All multi-triazole resistant *A. fumigatus* strains in India were thought to have a single origin, a recombination event between a susceptible native strain and a resistant European strain. In this paper we further investigate the origin of multi-triazole resistance in India using a total of 37 loci. Our results suggest that multi-triazole resistance in India has multiple origins. Our results have significant implications in the management of aspergillosis in India. This study is now published in Mycoses, volume 59, issue 7, pages 450-466. References in this chapter appear as they are in the originally published manuscript. Authors of this article are Howard Chang, Eta Ashu, Cheshta Sharma, Shallu Kathuria, Anuradha Chowdhary, and Jianping Xu. I am the co-first author of this article. I genotyped 54 of the 89 isolates used in this study by means of $GACA_4$, M13, MAT1 and seven MLST loci. EA and HC analyzed genotype data. EA, HC and JX wrote and revised the manuscript. Strains used in this study were contributed by CS, SK and AC. They also tested the susceptibility of all the isolates against a host of azole antifungals. This article is reprinted in accordance with Wiley's sharing gudelines.

4.2 Abstract

Aspergillus fumigatus is a widespread opportunistic fungal pathogen causing an alarmingly high mortality rate in immunocompromised patients. Nosocomial infections by drug-resistant *A. fumigatus* strains are of particular concern, and there is a pressing need to understand the origin, dispersal, and long-term evolution of drug resistance in this organism. The objective of this study was to investigate the diversity and putative origins of triazole resistance in *A. fumigatus* from India. Eighty-nine isolates, including 51 multiple triazole resistant (MTR) isolates and 38 azole-susceptible isolates, were genotyped using multilocus-sequence typing (MLST), mating typing, and PCR fingerprinting. MLST resolved the 51 MTR isolates into three genotypes, two of which have susceptible counterparts, suggesting that MTR isolates originated multiple times in India. The multiple-origin hypothesis was further supported by the diversity of PCR fingerprints among the MTR isolates. Interestingly, there is abundant evidence for mating and recombination in natural population of *A.*

fumigatus in India, suggesting that sexual spread of TR_{34}/L98H, the dominant MTR allele, is possible. Our results call for greater attention to MTR in *A. fumigatus* and for better management of antifungal drug use.

4.3 Introduction

The mold *Aspergillus fumigatus* is among the most ubiquitous saprophytic fungi in natural environments [1]. It is found almost everywhere in the world, in part due to its prolific production of small airborne conidia, allowing it to readily disperse and remain airborne [1]. In nature, this filamentous ascomycete fungus plays an important role in nutrient cycling within the organic layer of soil [2]. Around residential and suburban regions, *A. fumigatus* is commonly found in composts and organic wastes, partly due to its ability to grow at temperatures of up to 60^{o}C [3]. However, over the last 30 years, due to the increasing number of immunocompromised hosts, *A. fumigatus* has become an important opportunistic pathogen causing significant morbidity and mortality.

A. fumigatus can cause many diseases, including allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis [2]. Most infected individuals are immunocompromised, among them individuals with AIDS, patients undergoing organ transplantation, and those undergoing chemotherapy. Indeed, *Aspergillus* species are now the second most common culprits of nosocomial fungal infections worldwide [4-6].

In addition to the increasing incidence of aspergillosis, there is growing concern over the past decade regarding the emergence of triazole resistant *A. fumigatus* strains around the world. Normally, *Aspergillus* infections are treated with triazoles such as voriconazole, itraconazole and posaconazole, which target the biosynthetic pathway for ergosterol by deactivating the 14-α-sterol demethylase enzyme (CYP51) [7]. However, resistance to azoles has now been reported in many countries in six continents, aside from Antarctica [8]. For example, around 17-20% of patients in the United Kingdom infected with *A. fumigatus* are burdened with strains that are triazole resistant [9], and in the Netherlands the number stands at about 20% [10].

Several different mutations related to triazole resistance have been characterized over the last few years [9, 11]. Among these mutations, one characterized by both an amino acid substitution (L98H) in the CYP51A protein and a tandem repeat of a 34-base pair sequence (TR34) found in the promoter region of the *CYP51A* protein coding gene [9, 12, 13] has attracted significant attention. Strains with the TR_{34}/L98H mutant allele are resistant to multiple triazoles used commonly not only in medicine but also in agriculture and animal husbandry. Isolates harboring the TR_{34}/L98H mutation and the recently described TR_{46}/Y121F/T289A mutations conferring high voriconazole resistance are also widespread in the environment and in certain hospitals [14-16]. Similarly, mutations related to triazole resistance in the CYP51A protein have also been described in other fungi, such as the maize pathogen *Colletotrichum graminicola* [17]. Because 64%-71% of multi-triazole resistant (MTR) strains have been reported in patients with invasive aspergillosis but who had never been exposed to triazole treatments, an environmental origin for many of these triazole resistant *A. fumigatus* strains has been proposed [9, 13-15]. Azole resistance complicates patient management and increases the probability of treatment failure. The broad distribution of the TR_{34}/L98H mutation in many parts of the world, coupled with its MTR feature, have led to the suggestion that environmental origin(s) of such mutations and long distance dispersal mechanisms played important roles for its emergence and distribution [9, 18]. This environmental origin(s) hypothesis is supported by the frequent use of 14-α-demethylase inhibitors in agriculture, which can put strong selection pressures on *A. fumigatus* populations living in the soil [8, 9, 18].

Many methods have been used to identify *A. fumigatus* genotypes, including restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), multilocus enzyme electrophoresis (MLEE), microsatellite analysis, and multilocus-sequence typing (MLST) [1,19, 20]. Analyses using these methods have led to significantly improved understandings of the epidemiology of aspergillosis around the globe. These methods differ in their reproducibility and discriminatory power, as well as their ability to produce data for cross-laboratory comparisons [21, 22]. Among these methods, MLST has emerged as particularly useful in large-scale studies, especially in phylogenetic analyses and in tracing the origins of genotypes. Understanding the origin(s) and spread of drug

resistance will help develop better strategies to combat future development and spread of antifungal resistance. The objective of this study is to use MLST, in combination with other types of markers, to elucidate the origin(s) of MTR strains of *A. fumigatus* from India.

4.4 Materials and methods

4.4.1 Isolate collection

A total of 89 Indian *A. fumigatus* isolates were analyzed in this study, including 51 MTR and 38 triazole-susceptible isolates. These isolates were from a variety of clinical and environmental sources. The 65 environmental isolates were obtained from soil, air, bird droppings, and tea patties/plantations. The 24 clinical isolates were obtained from patients from three hospitals, namely, Vallabhbhai Patel Chest Institute (VPCI), Ram ManoharLohia Hospital (RML), and Rajiv Gandhi Cancer Institute & Research Center, Delhi, India (RGCIRC). Among the 51 MTR strains, 43 have been analyzed in a previous study [13] and eight were new isolates. Of the 38 susceptible isolates, 33 were from a previous study [13] and five were new. The susceptibilities of these 13 new isolates to azole drugs were tested following protocols described previously [13]. The genomic DNAs from all isolates were extracted following a protocol described previously [21] and diluted to around 50-100 ng/mL for DNA amplification using PCR. The mating type and multilocus sequence type were identified for all isolates, following protocols described below.

4.4.2 Mating type determination

Mating types for all 89 isolates were determined as previously described by Bain et al. [20]. Briefly, each PCR reaction had a total volume of 10 µl containing 5 µl 2x GoTaq Master Mix (Promega, Madison, WI, USA), 1µl of diluted template genomic DNA, 0.32 µl (0.32µM) each of AFM1 (5′-CGCTCCTCATCAGAACAACTCG-3′) and AFM2 5′-CCTTGACGCGATGGGGTGG-3′ forward primers, 0.64 µl of the shared reverse primer (0.64µM) AFM3 (5′-CGGAAATCTGATGTCGCCACG-3′), and 2.72µl of ddH_2O. The PCR protocol ran with an initial denaturation step of 95^oC for 5 min, followed by 35 cycles of 95^oC for 30 seconds, 60^oC for 30 seconds, and 72^oC for

1 min, and concluded with a final elongation step at 72^{o}C for 5 min. The PCR products were then loaded onto a 1% agarose gel and underwent gel electrophoresis at 150 V for 1 hour. The gel was then visualized under a UV transilluminator. The *MAT1-1* mating type was identified by its correspondant 834 bp band size, while *MAT1-2* by its 438 bp band size [20].

4.4.3 Multi-locus sequence typing

To allow effective comparison with sequence data from previous studies, seven gene fragments previously used to genotype strains of *A. fumigatus* were also used here [20]. The seven gene fragments were: *ANXC4, BGT1, CAT1, LIP, MAT1-2, SODB,* and *ZRF2*, which code for proteins annexin, beta-1,3-glucanosyl transferase, catalase, lipase, mating type protein, superoxide dismutase, and zinc transporter, respectively. PCR amplification was performed as previously described by Bain et al. [20]. Briefly, each PCR reaction contained 8 µl of 2x GoTaq Master Mix (Promega, Madison, WI, USA), 1 µl of forward primer (10 µM), and 1 µl of reverse primer (10 µM), 2 µl diluted template DNA, and 4 µl of ddH_2O. The PCR protocol ran with an initial denaturation step of 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 1min, 50^{o}C for 1min, and 70^{o}C for 1min, and a subsequent final elongation at 68^{o}C for 10 min. PCR products were then sent to the MoBix Laboratory (Hamilton, ON, Canada) for Sanger sequencing.

For each gene fragment, the obtained sequences were aligned with ClustalW and the strain relationships were analyzed using PAUP* 4.0b software [24]. Sequence information from all seven loci was then combined to identify the multilocus sequence types for our strains. To identify the relationships between our sequences and those from the *A. fumigatus* MLST database, we retrieved the sequences of all alleles from each of the seven loci from the MLST database and analyzed these sequences together with ours to reveal potential novel alleles and new multilocus genotypes.

4.4.4 *CYP51A* sequencing

The DNA sequence at the *CYP51A* gene was obtained for allelic identification and confirmation at this locus. To obtain the *CYP51A* sequences, we followed the PCR amplification protocol described in Mellado et al. [25]. Briefly, each 8 µl PCR reaction contained 4 µl of 2x GoTaq Master Mix (Promega, Madison, WI, USA), 0.5 µl of (10 µM stock solution) forward primer (5′-TCATATGTTGCTCAGCGG-3'), and 0.5 µl of (10 µM stock solution) reverse primer (5′-GGGGTCGTCAATGGACTA-3'), 1 µl of template DNA, and 2 µl ddH_2O. The PCR protocol ran with an initial denaturation step of 94^oC for 5 min, followed by 35 cycles of 94^oC for 1min, 50^oC for 1min, and 68^oC for 1min, and concluded with a final elongation at 68^oC for 10min. PCR products were then sent to the MoBix laboratory (Hamilton, ON, Canada) for Sanger sequencing.

4.4.5 PCR fingerprinting

To further investigate genetic variation among 89 Indian *A. fumigatus* strains, PCR fingerprinting was conducted. Here, PCR fingerprints were obtained using two separate primers, $(GACA)_4$ (5'-GACAGACAGACAGACA-3') and M13-core (5′-GTAAAACGACGGCCAGT-3′) in separate PCR reactions. For each PCR reaction, a total volume of 8 µl contained 4 µl of 2x GoTaq Master Mix (Promega, Madison, WI, USA), 2 µl of H_2O, 1 µl of primer, and 1 µl template genomic DNA. Both $(GACA)_4$ and M13 reactions underwent a modified PCR protocol derived from Meyer and Mitchell [26]. Briefly, the reaction was run at 93^oC for 5min, followed by 40 cycles of 93^oC for 20 sec, 50^oC for 60 sec, and 72^oC for 20 sec, concluding with a final elongation step at 72^oC for 6min. The M13 PCR products were separated through gel electrophoresis on 2% agarose gel for 24 hours at 30 V, and all bands were scored. The $(GACA)_4$ PCR products underwent gel electrophoresis on 1.25% gel for 1.5 hours at 150 V, and all bands were scored. The strain relationships based on PCR fingerprint profiles were inferred using the Neighbor-Joining method in the program MEGA6.

4.5 Results

We analyzed the patterns of genetic variation among 89 isolates from different geographic locations, hospitals, and ecological niches in India. Mating types and fingerprint patterns were determined for all isolates. All isolates, including 51 MTR and 38 triazole susceptible isolates, were sequenced at the seven MLST loci. The summary information for mating type and MLST genotype of each isolate is presented in Table 4.1. Below we describe these results.

4.5.1 Mating types

Mating type analysis shows that 66 of the 89 isolates are of the *MAT1-1* idiomorph, while 22 isolates *MAT1-2*. The mating type of one isolate is not known as PCR failed to amplify its mating type locus using existing primers (Table 4.1). The overall mating type ratio between *MAT1-1 and MAT1-2* is 3:1. Interestingly, the mating type ratios between resistant and susceptible populations of *A. fumigatus* within India are statistically significantly different [X^2 = 128.736 (df = 1), two tailed P-value < 0.0001]. Of the 51 MTR strains, 50 are *MAT1-1* and one (497/E1/12/3) is *MAT1-2*. In contrast, for the 38 triazole susceptible strains, 16 were *MAT1-1*, 21 were *MAT1-2*, and one remains unknown.

Table 4.1. Geographic, ecological, phenotypic, and genotypic information about all the isolates analyzed in this study

Strain[1]	Geographic Source	Ecological niche	*CYP51A* allele[2]	Mating type[3]	MLST Type[4]	Multi-Triazole Resistance[5]	Microsatellite Genotype[6] (STRAf)	PCR Genotype[7]	Combined Genotype[8]
211/Ei/11[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P11	C24
217/Ei/11/1[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P40	C16
218/Ei/11/1[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P37	C22
219/Ei/11/1[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P35	C21
221/Ei/11[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P32	C15
222/Ei/11/3[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P38	C17
225/Ei/11/2[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P32	C15
217/Ei/11/2[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P39	C17
218/Ei/11/2[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P32	C15
219/Ei/11/2[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P32	C15

222/Ei/11/1[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P32	C15
222/Ei/11/2[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P48	C35
225/Ei/11/1[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P31	C14
123/Ei/11/1[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P34	C18
123/Ei/11/3[1]	VPCI, Delhi	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P31	C14
162/Ei/11/1[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P31	C14
163/Ei/11/1[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P36	C20
165/Ei/11/2[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P16	C13
166/Ei/11/2[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P32	C15
162/Ei/11/2[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P31	C14
165/Ei/11/3[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P48	C35
165/Ei/11/4[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P31	C14

166/Ei/11/1[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P19	C13
166/Ei/11/3[1]	Bihar	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P27	C23
271/Ei/11/1[1]	Hoogli, Kolkata	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P32	C15
271/Ei/11/2[1]	Hoogli, Kolkata	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P25	C27
271/Ei/11/3[1]	Hoogli, Kolkata	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P25	C27
124/W/11[1]	VPCI, Delhi	Environmental/ Air	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P3	C19
373/Ei/12/3[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	I	Yes	14/20/9/31/9/10/8/10/28	P31	C34
379/Ei/12[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P42	C7
378/Ei/12/1[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P22	C28
382/Ei/12/2	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	N/A	P19	C60
383/Ei/12/1[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P15	C29
382/Ei/12/3	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	N/A	P19	C13
383/Ei/12/2[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P31	C14

383/Ei/12/3[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P28	C32
378/Ei/12/3[1]	Darjeeling	Environmental/ Soil	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P26	C30
257/Ei/11/1[1]	Bihar	Environmental	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P31	C14
257/Ei/11/2[1]	Bihar	Environmental	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P41	C17
257/Ei/11/3[1]	Bihar	Environmental	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P24	C26
437/Ei/12[1]	VPCI, Delhi	Environmental/ Air	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P32	C15
439/Ei/12[1]	VPCI, Delhi	Environmental/ Air	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P35	C21
497/Ei/12/1	Tamilnadu	Environmental/ Bird Droppings	TR34/L98H	1	11	Yes	N/A	P16	C62
497/Ei/12/3	Tamilnadu	Environmental/ Bird Droppings	Wild Type	2	5	Yes	20/29/15/31/42/18/10/9/5	P4	C41
287/Ei/11/1[1]	VPCI	Environmental/ Soil	Wild Type	2	14	No	26/10/11/28/13/8/8/10/15	P6	C9
290/Ei/11[1]	VPCI	Environmental/ Soil	Wild Type	1	14	No	17/21/15/9/12/7/13/15/8	P16	C50
308/Ei/12/1[1]	VPCI	Environmental/ Soil	Wild Type	1	A	No	23/19/8/23/13/12/13.3/7/5	P17	C10

314/Ei/12/1[1]	VPCI	Environmental/ Soil	Wild Type	1	H	No	23/19/8/23/12/ 12/13.3/7/5	P44	C11
315/Ei/12/1[1]	VPCI	Environmental/ Soil	Wild Type	1	14	No	17/21/15/9/12/ 7/13/15/8	P15	C51
378/Ei/12/2[1]	Siliguri	Environmental/ Soil	Wild Type	1	11	No	14/20/9/31/9/1 0/8/10/28	P15	C29
343/Ei/12/1[1]	Siliguri	Environmental/ Soil	Wild Type	Unknown	19	No	19/24/11/26/1 1/26/21.3/10/5	P23	C40
344/Ei/12/3[1]	Siliguri	Environmental/ Soil	Wild Type	1	11	No	11/22/13/16/1 0/7/12/11/5	P15	C55
350/Ei/12/3[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/18/18/13/1 0/10/9/9/5	P21	C54
351/Ei/12/1[1]	Siliguri	Environmental/ Soil	Wild Type	2	L	No	16/18/3/21/10/ 7/9/9/5	P19	C2
369/Ei/12/3[1]	Siliguri	Environmental/ Soil	Wild Type	1	K	No	11/21/14/21/9/ 7/11/10/5	P33	C56
371/Ei/12/2[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/21/8/24/10/ 10/12/4/5	P43	C3
374/Ei/12/2[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/21/8/24/10/ 10/12/4/5	P8	C5
375/Ei/12/3[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/21/8/24/10/ 10/12/4/5	P19	C4
379/Ei/12/2[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/21/8/24/10/ 10/12/4/5	P15	C53

379/Ei/12/3[1]	Siliguri	Environmental/ Soil	Wild Type	1	14	No	17/21/15/9/12/ 7/13/15/8	P15	C31
380/Ei/12/2[1]	Siliguri	Environmental/ Soil	Wild Type	2	B	No	24/10/8/61/12/ 7/9/11/10	P13	C59
381/Ei/12/1[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/21/8/24/10/ 10/12/4/5	P7	C6
382/Ei/12/1[1]	Siliguri	Environmental/ Soil	Wild Type	2	B	No	24/10/8/61/12/ 7/9/11/10	P6	C52
384/Ei/12/1[1]	Siliguri	Environmental/ Soil	Wild Type	2	J	No	11/21/8/24/10/ 10/12/4/5	P19	C4
388/Ei/12/1[1]	Siliguri	Environmental/ Soil	Wild Type	2	24	No	18/12/15/52/2 5/19/15/9/5	P7	C61
1042/11	VPCI	Clinical/Sputum	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P4	C12
942/10	VPCI	Clinical/Sputum	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P29	C32
343/P/11[1]	RGCIRC	Clinical/Sputum	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P19	C13
591/12[1]	VPCI	Clinical/Sputum	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P19	C13
598/12[1]	VPCI	Clinical/Sputum	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P30	C33
245/P/12	RML	Clinical/Nasal Polyp and Debris	TR34/L98H	1	11	Yes	14/20/9/31/9/1 0/8/10/28	P12	C25

1166/12	VPCI	Clinical/Endotracheal Aspirate	TR34/L98H	1	11	Yes	14/20/9/31/9/10/8/10/28	P19	C13
2581/11[1]	VPCI	Clinical/Sputum	Wild Type	2	G	No	19/12/8/13/9/22/18/11/15	P45	C49
487/09	VPCI	Clinical/Bronchial Wash	Wild Type	1	5	No	N/A	P1	C42
1286/09[1]	VPCI	Clinical/Sputum	Wild Type	1	34	No	20/12/15/17/11/33/7/11/8	P16	C37
1261/09[1]	VPCI	Clinical/Sputum	Wild Type	1	20	No	20/23/17/49/12/26/18.3/10/5	P2	C44
1272/09[1]	VPCI	Clinical/Sputum	Wild Type	2	5	No	25/23/11/0/9/7/7/10/8	P18	C57
894/09[1]	VPCI	Clinical/Endotracheal Aspirate	Wild Type	2	F	No	17/24/21/39/11/29/12.3/9/5	P13	C43
834/10[1]	VPCI	Clinical/Bronchial Wash	Wild Type	2	E	No	25/19/19/26/19/17/10/16/8	P8	C48
1072/10	VPCI	Clinical/Endotracheal Aspirate	Wild Type	1	25	No	N/A	P20	C36
1011/10[1]	VPCI	Clinical/Sputum	Wild Type	1	J	No	11/20/7/34/10/8/9/9/5	P47	C1
111/10[1]	VPCI	Clinical/Sputum	Wild Type	1	5	No	20/23/22/48/12/27/10/9/5	P19	C41
1608/09[1]	VPCI	Clinical/Sputum	Wild Type	2	34	No	20/12/16/34/22/23/22.3/9/5	P14	C45

1591/09	VPCI	Clinical/Sputum	Wild Type	2	D	No	N/A	P5	C8
462/09	VPCI	Clinical/Sputum	Wild Type	2	10	No	N/A	P15	C39
1573/09[1]	VPCI	Clinical/Sputum	Wild Type	1	C	No	15/21/18/24/12/16/16/5/0	P15	C46
1926/10[1]	VPCI	Clinical/Endotracheal Aspirate	Wild Type	1	5	No	25/23/31/49/8/7/18.3/10/8	P46	C58
1268/10[1]	VPCI	Clinical/Sputum	Wild Type	2	10	No	20/26/17/28/9/20/18.3/10/5	P9	C38
94/P/10	RML	Clinical/Sputum	Wild Type	2	14	No	25/20/20/23/22/33/10/11/10	P10	C47

[1]Isolates labeled "1" are those from the Chowdhary et al. 2012 study. Isolates not labeled are new isolates.
[2]*CYP51A* allele: "TR_{34}/L98H" represents a drug-resistant mutant allele while "Wild Type" represents an allele without these mutations.
[3]Mating type: "1" represents mating type idiomorph MAT1-1; "2" represents mating type MAT1-2. "Unknown": the mating type of this isolate could not be determined using the existing primers.
[4]MLST sequence types represent allelic combinations shown in Table 4.2.
[5]Isolates were tested for their susceptibilities to the following azole antifungal drugs: itraconazole, voriconazole, posaconazole, bromuconazole, cyproconazole, difenoconazole, epoxiconazole, penconazole, tebuconazole, triadimefon, metconazole, hexaconazole, and tricyclazole. Yes: resistant to these azoles; No: susceptible to these azoles.
[6]The microsatellite allele order corresponds to the following loci respectively: STRAf 2A/2B/2C/3A/3B/3C/4A/4B/4C.
[7]PCR genotype: identified using the concatenation of M13 and $GACA_4$ primers
[8]Combined genotype: identified using the combined genotype information based on PCR fingerprints, MLST data, STRAf and Mating Type

4.5.2 Multi-locus sequence typing

We successfully obtained DNA sequences from most of the loci for our *A. fumigatus* isolates (Table 4.2). These results were submitted to Genbank (KU575252-KU575867). Based on the obtained sequence information, the following number of alleles were identified among the 89 isolates at the seven loci: 3 at *ANXC4*, 3 at *BGT1*, 6 at *CAT1*, 2 at *LIP*, 2 at *MAT1*, 2 at *SODB* and 4 at *ZRF2* (Table 4.2). Among the MTR isolates, PCR failed to amplify the gene fragment at the *ANXC4* locus for four isolates (163/Ei/11/1, 222/Ei/11/3, 257/Ei/11/2, and 343/P/11). At the *SODB* locus, three isolates (211/Ei/11, 163/Ei/11/1, and 343/P/11) failed to amplify. Three additional attempts were made to produce these sequence data, all of which were unsuccessful. This result suggests potentially mismatched primers and novel alleles at these loci. However, in the absence of sequence information at the one or two loci for the above isolates and based on sequences at the other loci, we can conservatively identify isolates 163/Ei/11/1, 211/Ei/11, 222/Ei/11/3, 257/Ei/11/2, and 343/P/11 as sequence type 11. Of the 38 susceptible strains, only isolate 287/Ei/11/1 had missing information for two loci. We conservatively grouped it into the most closely related sequence type 14 (Table 4.2).

Table 4.2. Allelic information at seven MLST loci for the 89 Indian strains of *Aspergillus fumigatus* and for the 43 MLST genotypes in the database.

Name of sequenced Indian strains[1]	ANXC4	BGT1	CAT1	LIP	MAT1	SODB	ZRF2	Sequence type[2]
308/Ei/12/1(S)	1	1	1	1	2	1	1	A
380/Ei/12/2(S) 382/Ei/12/1(S)	1	1	2	1	1	1	1	B
1573/09(S)	1	1	3	1	1	2	1	C
1591/09(S)	1	1	3	1	2	2	1	D
834/10(S)	1	1	8 (n)[3]	1	2	1	1	E
894/09(S)	1	3	1	1	1	1	2	F
2581/11(S)	1	3	2	1	1	1	2	G
314/Ei/12/1(S)	1	3	1	1	2	1	2	H
373/Ei/12/3(R)	2	1	3	2	2	1	2	I
350/Ei/12/3(S) 371/Ei/12/2(S) 374/Ei/12/2(S) 375/Ei/12/3(S) 379/Ei/12/2(S)	2	3	2	1	1	1	1	J

381/Ei/12/1(S) 384/Ei/12/1(S) 1011/10(S) 369/Ei/12/3(S)	2	3	7(n)	1	1	1	1	K
351/Ei/12/1(S)	5	3	2	1	1	1	1	L
	1	1	1	1	1	1	1	1
	1	2	2	1	2	1	2	2
	1	3	2	1	2	1	1	3
	2	1	2	2	2	1	2	4
111/10(S) 487/09(S) 497/Ei/12/3(R) 1272/09(S) 1926/10(S)	1	3	2	1	2	1	2	5
	2	3	3	1	2	1	1	6
	1	1	2	2	2	1	1	7
	3	4	4	3	1	2	3	8
	2	1	3	1	1	1	1	9
462/09(S) 1268/10(S)	1	3	3	1	2	1	2	10
163/Ei/11/1(R) 343/P/11(R)	?[4]	1	2	1	1	?	1	11 (?)
222/Ei/11/3(R) 257/Ei/11/2(R)	?	1	2	1	1	1	1	11 (?)
211/Ei/11(R)	2	1	2	1	1	?	1	11 (?)
123/Ei/11/1(R) 123/Ei/11/3(R) 124/W/11(R) 162/Ei/11/1(R) 162/Ei/11/2(R) 165/Ei/11/2(R) 165/Ei/11/3(R) 165/Ei/11/4(R) 166/Ei/11/1(R) 166/Ei/11/2(R) 166/Ei/11/3(R) 217/Ei/11/1(R) 217/Ei/11/2 (R) 218/Ei/11/1 (R) 218/Ei/11/2(R) 219/Ei/11/1(R) 219/Ei/11/2(R) 221/Ei/11(R) 222/Ei/11/1(R) 222/Ei/11/2(R) 225/Ei/11/1(R) 225/Ei/11/2(R) 245/P/12(R) 257/Ei/11/1(R) 257/Ei/11/3(R) 271/Ei/11/1(R) 271/Ei/11/2(R) 271/Ei/11/3(R) 344/Ei/12/3(S)	2	1	2	1	1	1	1	11

373/Ei/12/3(R)								
378/Ei/12/1(R)								
378/Ei/12/2(S)								
378/Ei/12/3(R)								
379/Ei/12(R)								
382/Ei/12/2(R)								
382/Ei/12/3(R)								
383/Ei/12/1(R)								
383/Ei/12/2(R)								
383/Ei/12/3(R)								
437/Ei/12(R)								
439/Ei/12(R)								
591/12(R)								
598/12(R)								
942/10(R)								
1042/11(R)								
1166/12(R)								
	1	1	3	2	2	1	1	12
	1	1	2	1	2	1	2	13
94/P/10(S)								
287/Ei/11/1(S)								
290/Ei/11(S)	1	1	3	1	1	1	1	14
315/Ei/12/1(S)								
379/Ei/12/3(S)								
	2	1	2	1	2	1	1	15
	1	3	2	2	2	1	2	16
	3	5	2	1	1	2	4	17
	3	4	4	4	1	1	4	18
343/Ei/12/1(S)	1	3	3	1	2	1	1	19
1261/09(S)	1	1	2	1	2	1	1	20
	1	3	3	1	1	2	1	21
	3	4	5	4	1	2	3	22
	4	1	3	1	2	1	1	23
388/Ei/12/1(S)	2	1	2	2	2	1	1	24
1072/10(S)	2	3	2	1	2	1	1	25
	1	3	3	1	1	1	1	26
	1	1	3	1	1	3	1	27
	2	1	3	5	1	1	1	28
	1	3	2	1	1	1	1	29
	1	1	2	1	3	1	1	30
	3	4	4	4	1	2	3	31
	5	3	2	2	2	1	2	32
	2	1	4	2	4	1	1	33
1286/09(S)	2	3	2	1	2	1	2	34
1608/09 (S)								
	2	3	2	2	2	1	2	35
	2	1	6	2	2	1	1	36
	2	3	2	2	2	1	1	37

2	1	3	2	4	1	1	38
2	1	4	1	1	1	1	39
6	6	4	1	1	1	5	40
7	7	3	6	1	2	5	41
1	1	3	1	5	1	1	42
1	3	2	7	1	4	6	43

[1]Resistant strains identified by "R"; susceptible strains identified by "S".
[2]Numbered sequence types "1" to "43" are those identified by Bain et al. [19] and in the MLST database. Lettered sequence types "A" through "K" are reported for the first time using the Indian isolates of *A. fumigatus*.
[3]Novel alleles (i.e. not found in Bain et al., [19]) are identified by (n),
[4]locus with missing data marked by "?".

Multi-locus sequence types for all isolates are summarized in Table 4.1. The detailed sequence type profiles of the isolates as well as all the representative genotypes from the MLST database are presented in Table 4.2. A total of 21 MLST genotypes were found among the 89 isolates, including 3 for the 51 MTR isolates and 20 for the triazole-susceptible isolates. Our comparisons found that 67 of the 89 isolates shared nine sequence types with those previously reported in the *A. fumigatus* MLST database (http://pubmlst.org/afumigatus/). Following the notation by Bain et al. [19], strains in our study with these sequence types are labeled with numbers corresponding to those already in the database. The remaining isolates have novel sequence types that have not been reported in the literature or the database. To better distinguish these novel genotypes from the ones reported before, the new genotypes are labeled with letters (Tables 4.1 and 4.2).

The overwhelming majority of MTR strains (49 out of 51) are sequence type (ST) 11, a sequence type they share with two triazole-susceptible *A. fumigatus* isolates found within India, as well as several isolates from the United Kingdom, France, and Australia, as recorded in the literature [26]. The remaining two MTR strains were of different sequence types: (i) isolate 497/Ei/12/3 corresponded to ST5, a sequence type which has been previously described in the literature [19]; (ii) isolate 373/Ei/12/3, with a novel type ST I that did not appear among either the susceptible isolates or in the MLST database. Interestingly, isolate (373/Ei/12/3) did not contain any new alleles at any of the seven sequenced loci, but rather it was comprised of a unique combination of previously identified alleles. The MLST results alone suggest

that there are at least two independent origins of MTR among the 51 MTR isolates, one each for ST5 and ST11 and a likely sexual origin for the third MTR genotype (ST I). The results also indicate that long-distance dispersal is very common among the ST11 MTR isolates.

Among the 38 triazole-susceptible isolates, we found a total of 20 MLST genotypes. 11 of these 20 MLST genotypes are novel, not reported previously in the MLST database. Among these 11 novel genotypes, one was shared by two isolates, while another was shared by nine isolates. The 9 other genotypes were each represented by a single isolate. The cluster with two isolates had both strains from environmental soil samples in Siliguri. Similarly, in the second cluster, eight of nine isolates were also found in soil samples in Siliguri. However, one isolate was acquired from a clinical sputum sample at VPCI, roughly 1,500km from Siliguri. Interestingly, this isolate had a mating type different from the other eight isolates from Siliguri. These results suggest that most clonal distributions for azole susceptible isolates were local; however, long-distance dispersal followed by sexual recombination cannot be excluded. Interestingly, among isolates with identical MLST genotypes, not all shared the same mating type. Several examples of this phenomenon were seen in our data, including the following MLST genotypes: 5, 14, 34, and J. Sequence type J contained the nine isolates described above, with one VPCI clinical sputum isolate and eight soil isolates from Siliguri. Interestingly, the VPCI clinical sputum isolate, which was geographically distinct, also differed in mating type (*MAT1-1*). For ST5, three isolates (487/09, 111/10, 1926/10) are *MAT1-1*, while two (497/Ei/12/3 and 1272/09) are *MAT1-2*. Similarly, ST14 contained three isolates (290/Ei/11, 315/Ei/12/1, and 379/Ei/12/3) with the *MAT1-1* type, and two isolates (287/Ei/11/1, 94/P/10) with the *MAT1-2* type. Finally ST34 had one isolate (1286/09) with the *MAT1-1* type while the other (1608/09) had the *MAT1-2* type.

4.5.3 PCR fingerprinting

PCR fingerprinting using the $(GACA)_4$ and M13 primers identified a diversity of banding patterns among the 51 MTR *A. fumigatus* isolates from India. These 51 isolates shared a total of 26 fingerprint profiles (Fig 4.1). Eight fingerprint profiles were shared by two or more isolates each, with five profiles shared by two isolates

each, and three by eight isolates each. The remaining 18 fingerprints were each represented by one isolate. Overall, the PCR fingerprinting results suggest greater genetic heterogeneity among the Indian MTR isolates than results from MLST and mating type analyses.

As expected, the 38 triazole-susceptible strains of *A. fumigatus* showed a diverse range of fingerprint patterns (Fig 4.1). Surprisingly, some shared fingerprinting patterns with MTR isolates. For example, one PCR fingerprint profile contained two susceptible strains (380/Ei/12/2 and 894/09) and one MTR strain (245/P/12). Another contained seven susceptible strains and one MTR strain (383/Ei/12/1). Finally, the largest cluster contained six triazole-susceptible strains (1286/09, 290/Ei/11, 111/10, 384/Ei/12/1, 351/Ei/12/1, and 375/Ei/12/3) and eight MTR strains (497/Ei/12/1, 165/Ei/11/2, 1166/12, 382/Ei/12/3, 343/P/11, 166/Ei/11/1, 382/Ei/12/2, and 591/12). While some isolates with shared PCR fingerprints often had shared MLST and mating type as well, this was not always true.

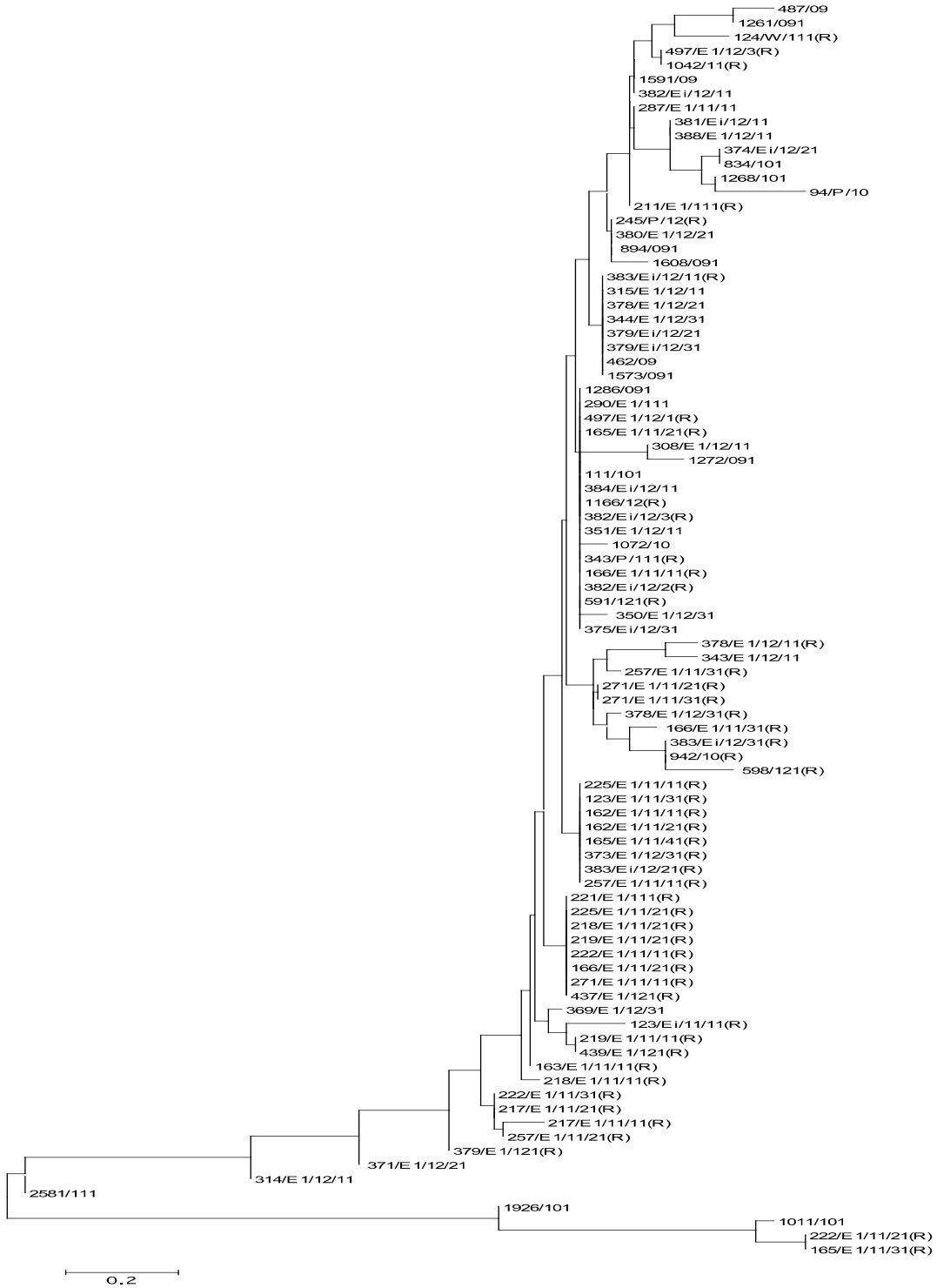

Figure 4.1 The relationships among 89 strains of Indian *A. fumigatus* based on PCR fingerprinting results. 'R' and 'S' in parentheses depict resistant and susceptible strains.

4.5.4 Combined genotype analyses

Based on the combined information of PCR fingerprinting, MLST data, STRAf, and mating type, we documented a total of 62 unique genotypes among the 89 isolates (Fig 4.2). Of these 62 genotypes, 28 were from the 51 MTR strains, while 37 were from the 38 triazole susceptible strains. Two concatenated genotypes (C29 and C41) contained both MTR (383/Ei/12/1 and 497/E1/12/3, respectively) and triazole susceptible strains (378/Ei/12/2 and111/10, respectively). The distribution patterns of these genotypes based on combined genetic information varied widely for the clinical strains. For example, one genotype (C13) was found in two different hospitals (VPCI and RGCIRC), consistent with a wide geographical distribution. However, some genotype clusters were only found from the same hospital (for example, C36, C37, C38, and C39 from VPCI).

The environmental strains also showed a distribution pattern similar to the clinical strains. For example, C14 was found across India (Bihar, Darjeeling, and Delhi, which are over 1,500 km apart). This is also true for C15 (VPCI and Kolkata) and C35 (Bihar and Delhi). On the other hand, similar clusters were also found together in the same geographical region. For example, C8-C11 were mostly found in Delhi, while C3-C6 were found solely in Siliguri.

The resistant population of *A. fumigatus* were also shown to have a reduced genetic diversity compared to susceptible populations based on the Wilcoxon paired rank test ($P<0.001$). The analysis was conducted using $GACA_4$ and M13 fingerprints, MLST alleles, mating type, and STRAf microsatellites.

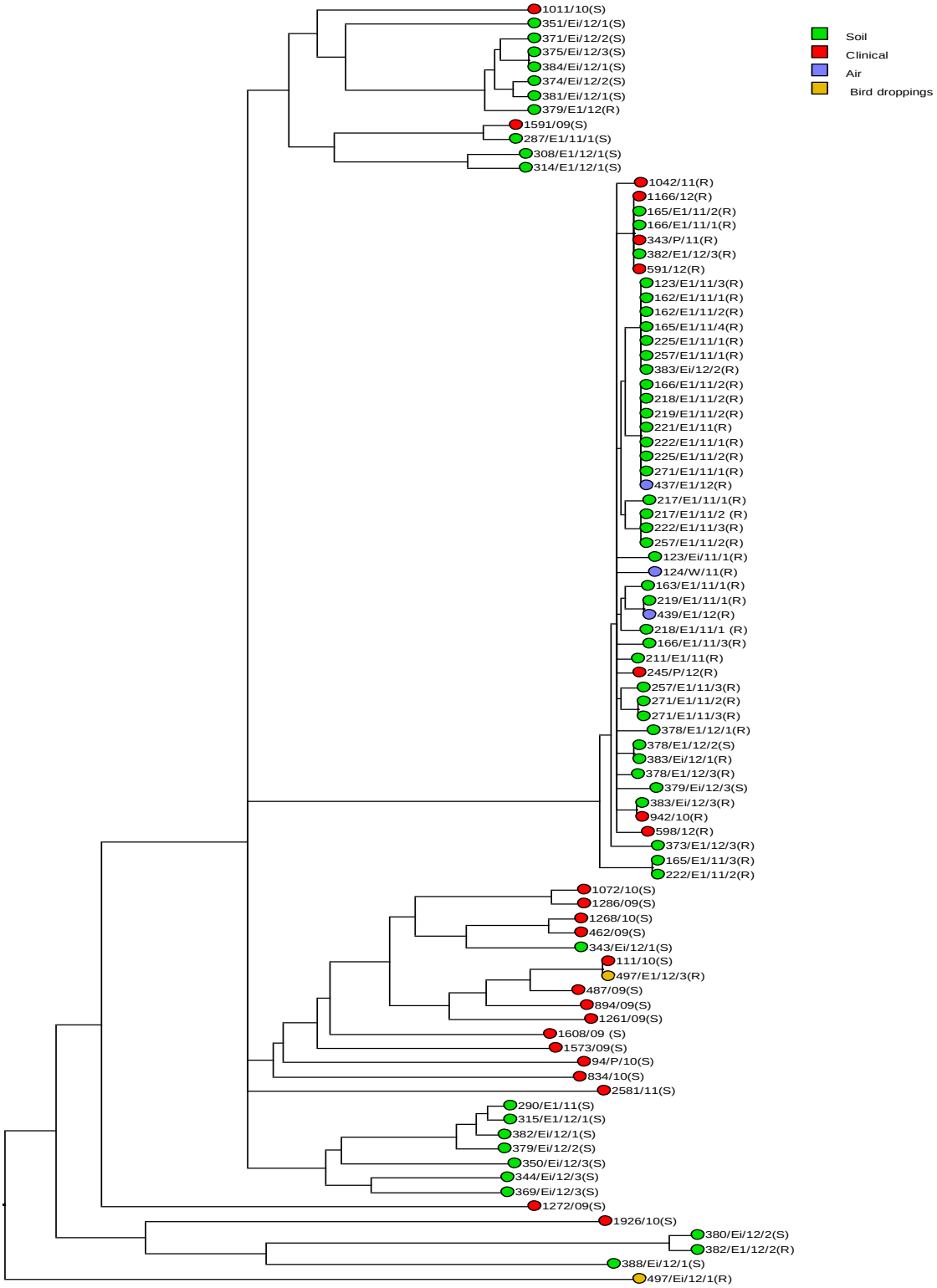

Figure 4.2 The relationships among 89 strains of Indian *A. fumigatus* based on combined genotype results. 'R' and 'S' in parentheses depict resistant and susceptible strains.

4.6 Discussion

A previous study indicated that all *A. fumigatus* strains from India with the TR_{34}/L98H mutation belonged to the same microsatellite genotype at 9 loci (STRAf 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C) [13]. In contrast, non-resistant strains of *A. fumigatus* showed a diversity of microsatellite genotypes. Here, similar to previous findings, the triazole-susceptible population sample showed significant genetic variations in both PCR fingerprinting and MLST genotypes. However, combined analyses using MLST, mating type, STRAf and PCR fingerprinting identified more genetic variation than previously found based on microsatellite markers alone for the Indian MTR *A. fumigatus* isolates. The new data suggested that there were multiple origins for MTR in India. However, evidence for clonal dispersal is still present. Below we discuss evidence for and implications of the multiple origins of MTR in *A. fumigatus* populations in India.

4.6.1 Multiple origins of the Indian *A. fumigatus* MTR strains

The MLST data indicated three MLST genotypes for the MTR isolates. One isolate (373/Ei/12/3) is ST I and has the expected TR_{34}/L98H mutation, while the other (497/Ei/12/3) is ST5, but does not have the TR_{34}/L98H mutation. Isolate 497/Ei/12/3 differs from the main resistant MLST genotype (ST11) at four of the seven loci (Tables 4.1 and 4.2). Additional susceptibility and genotyping tests were conducted to confirm the uniqueness of isolate 497/Ei/12/3. We confirmed that it was indeed resistant to all tested azoles and that it did not have either the TR_{34}/L98H mutation or the TR_{46}/Y121F/T289A mutation (another mutation associated with azole resistance) at the *CYP51A* locus. Furthermore, additional microsatellite genotyping identified that isolate 497/Ei/12/3 had a microsatellite allele profile different from the main genotype (Table 4.1). Thus, we believe that isolate 497/Ei/12/3 represents an independent origin of MTR, different from the origin of the major genotype with the TR_{34}/L98H mutation.

Recently, in the barley fungal pathogen *Rhynchosporium commune*, a likely novel mechanism for azole resistance was also suggested [28]. *R. commune* causes necrotic lesions on barley leaves, resulting in grain yield reductions of up to

30% if untreated [29]. In this case, chemical fungicides have been the main agents of control in agricultural fields and resistance not associated with mutation at the drug target was identified. The development of a novel mechanism in environmental isolate 497/Ei/12/3 was likely also driven by agricultural fungicides as it showed cross resistance to commonly used agricultural fungicides including bromuconazole, tebuconazole, hexaconazole, tricyclazole, cyproconazole, triadimefon, difenoconazole, epoxiconazole, metconazole and penconazole.

Similar to evidence from the MLST data, the mating type and PCR fingerprinting analyses also support the multiple origin hypothesis for Indian MTR *A. fumigatus* isolates. Indeed, though highly skewed, both *MAT1-1* and *MAT1*-2 idiomorphs were found among the resistant isolates. The most significant evidence for multiple origins of drug resistance was seen from PCR fingerprinting where 26 fingerprint profiles were found among the 51 MTR isolates analyzed here. In the strain relationship trees resolved by PCR fingerprinting, many of these MTR isolates were part of several clusters which also contained susceptible isolates. Indeed, in this respect, some MTR isolates were more closely related to azole susceptible strains than they were to each other.

The combined phylogenetic tree (Fig 4.2) using 37 different loci, including MLST, STRAf, fingerprinting, and mating type, similarly supports the multiple origins hypothesis. This analysis was able to demonstrate the presence of multiple clades due to the high genotyping resolution provided by combined genetic markers. On the whole, the combined analysis was effective in discriminating between clonal resistant populations and the diversity of susceptible isolates. At the same time, the analysis was also able to show that certain resistant strains may have different origins. Much like the fingerprinting analysis, the combined phylogenetic analysis reaffirmed that several MTR isolates were more closely related to susceptible isolates than other MTR isolates. In fact, as mentioned previously, two concatenated genotypes contained both resistant and susceptible isolates.

Interestingly, the concatenated genotype analysis placed the majority of triazole-susceptible clinical and environmental isolates in distinct genotype groups. This finding is consistent with niche differentiation in India. Furthermore, this suggests possible differences between clinical *A. fumigatus* strains prone to infecting

human hosts and potentially less pathogenic environmental strains. Further research would be useful in elucidating the relationship between the genetic and ecological profiles of Indian *A. fumigatus* and their associated pathogenicity and virulence.

The triazole-susceptible *A. fumigatus* strains within our data are associated with high genetic diversity and both mating types. Contrarily, resistant *A. fumigatus* strains are predominantly clonal and significantly associated with a single mating type. In conjunction with recent evidence showing the existence of another MTR resistance mechanism within India associated with the TR_{46}/Y121F/T289A mutation [11], we provide robust support for the "multiple origins with clonal spread" hypothesis of MTR isolates of *A. fumigatus* in India. Independent and spontaneous origins of azole resistance have been found in other fungal pathogens. For example, Xu et al. [21] found both clonal and spontaneous origins of fluconazole resistance in the yeast pathogen *Candida albicans.*

Our results show several new MLST genotypes that have not been reported previously. However, most Indian isolates share alleles and MLST sequence types with isolates from other countries, suggesting the existence of long-distance clonal dispersal between *A. fumigatus* populations from various countries. The suggested gene flow is consistent with our current understanding of the global distribution of *A. fumigatus*.

4.6.2 Comparison between our data and microsatellite Data

Chowdhary et al. [13] analyzed 44 MTR isolates of *A. fumigatus* from India by using 9 polymorphic microsatellite markers, 37 of which were further genotyped in the current study. Earlier analyses by Chowdhary et al. found all 44 strains to have an identical genotype at nine microsatellite loci. However, in our study, we found variation among the MTR strains using MLST markers. Specifically, isolate 373 Ei/12/3 was shown to have a sequence type different from the predominant clonal population, despite sharing the same microsatellite genotype. This result was unexpected given that microsatellite loci are known to have a higher discriminatory power (0.9994) [19] than MLST analysis (0.93) in *A. fumigatus* [20]. The housekeeping genes used for MLST are expected to be more conserved than

microsatellite loci; thus we expected a similar number or fewer genotypes among the resistant isolates using the MLST method.

At present, the reason for the unexpected result is not known. There are three possibilities. Firstly, there might be significant parallel mutations due to high strand slippage to result in identical microsatellite genotypes from different starting alleles and genotype backgrounds. Secondly, the chromosomal locations where the microsatellite markers are located might be very conserved in the drug-resistant strains, thus they might experience lower mutation rate than nucleotide substitutions at the MLST loci. Recently, whole genome analysis of azole susceptible and resistant *A. fumigatus* strains from clinical and environmental sources from India, the Netherlands, and the United Kingdom revealed that *A. fumigatus* population contained abundant signatures of recombination within indvidual countries and across Eurasia [30]. However, Indian MTR *A. fumigatus* isolates despite being isolated from both clinical and environmental sources across >1,000 km were highly related, suggesting a recent selective sweep of a highly fit genotype associated with the TR_{34} /L98H allele. All the eight Indian MTR *A. fumigatus* isolates analyzed by WGS also showed the same genotype (ST 11) in our study. The third possibility is related to mating and recombination in nature. Specifically, during the emergence of these azole-resistant genotypes, sexual mating and allelic re-assortment might have been prevalent and identical microsatellite genotypes could have been maintained or generated independently while MLST loci were re-assorted among the azole resistant progeny [13].

Along with a significant clonal component as originally revealed by Chowdhary et al. [13], our results strongly suggest that there are multiple origins for the MTR isolates of *A. fumigatus* in India. The likely paths to MTR include: (i) mutation from an existing susceptible genotype to a drug resistant one, (ii) recombinant origin, as originally proposed in Chowdhary et al. [13], and (iii) exotic introduction. Our combined genotype analyses based on all markers identified two likely recombinant origins. One recombination event is required to generate isolate 373/Ei/12/3. This is because isolate 373/Ei/12/3 has a unique allele "3" at the *lip* locus and this allele is not found in other resistant isolates (Table 4.2). The second (and potentially more likely) recombination event is required to generate the remaining

resistant isolates and to spread the TR_{34}/L98H mutation. The multiple origins hypothesis is further supported by another recent discovery of an MTR isolate with the TR_{46}/Y121F/T289A novel resistant mutation [11].

Based on the results of the Wilcoxon paired rank test (Table 4.3), we were able to elucidate the allelic diversity of the susceptible and resistant populations of *A. fumigatus*. The test shows statistically significant reduced allelic diversity among the resistant population compared to susceptible populations. Since mutations accumulate over time, this finding is consistent with the suggestion that MTR developed more recently via one of our proposed mechanisms.

The genetic variation observed among MTR *A. fumigatus* isolates have implications for the management of this pathogen within India, specifically as it relates to the overuse of triazoles in agriculture. The recent detection of similar Indian TR_{34}/L98H genotype in the environment of Tanzania also caused significant concerns [31]. In resource-limited countries, the medical implications pertaining to early diagnosis and effective treatment of azole-resistant aspergillosis pose a paramount challenge. Populations at risk in India and Africa primarily include patients with chronic aspergillosis and are specifically in the settings where a high burden of tuberculosis can lead to complications such as aspergilloma and chronic pulmonary aspergillosis in these patients. As azole antifungals are the recommended first-line drugs in the treatment and prophylaxis of aspergillosis, the high burden of environmental isolates harboring azole resistance poses a therapeutic challenge [14, 15, 32]. Furthermore, alternative therapeutic options such as Amphotericin B require appropriate medical infrastructure to allow intravenous administration. Finally, understanding the origins of triazole resistance provides a firm foundation for future efforts to track the MTR populations, especially with regards to its nosocomial spread.

Table 4.3. Comparison of genotypic diversities between triazole - resistant and triazole - susceptible samples of *Aspergillus fumigatus* from India

Population	Locus	Na[1]	h[2]	uh[3]	Population	Locus	Na[1]	h[2]	uh[3]
Triazole resistant	**ANXC4**	1	0.000	0.000	Triazole Susceptible	**ANXC4**	3	0.522	0.538
	BGT1	1	0.000	0.000		**BGT1**	2	0.478	0.492
	CAT1	2	0.040	0.041		**CAT1**	5	0.564	0.581
	LIP	2	0.040	0.041		**LIP**	2	0.059	0.061
	MAT1	2	0.040	0.041		**MAT1**	2	0.463	0.477
	SODB	1	0.000	0.000		**SODB**	2	0.059	0.061
	ZRF2	2	0.040	0.041		**ZRF2**	2	0.397	0.409
	2A	4	0.117	0.120		**2A**	12	0.858	0.884
	2B	4	0.117	0.120		**2B**	10	0.843	0.869
	2C	3	0.117	0.119		**2C**	16	0.874	0.902
	3A	3	0.079	0.081		**3A**	16	0.914	0.944
	3B	4	0.117	0.120		**3B**	9	0.815	0.841
	3C	3	0.079	0.081		**3C**	14	0.845	0.871
	4A	4	0.117	0.120		**4A**	12	0.878	0.905
	4B	4	0.117	0.120		**4B**	8	0.821	0.847
	4C	3	0.117	0.119		**4C**	5	0.588	0.607
	MT	2	0.115	0.117		**MT**	2	0.498	0.514
	PCR1	2	0.040	0.041		**PCR1**	2	0.213	0.220
	PCR2	1	0.000	0.000		**PCR2**	2	0.165	0.170
	PCR3	2	0.183	0.187		**PCR3**	2	0.165	0.170
	PCR4	2	0.495	0.505		**PCR4**	2	0.059	0.061
	PCR5	2	0.370	0.378		**PCR5**	2	0.059	0.061
	PCR6	2	0.078	0.080		**PCR6**	2	0.114	0.117
	PCR7	2	0.078	0.080		**PCR7**	2	0.114	0.117
	PCR8	2	0.390	0.398		**PCR8**	2	0.500	0.515

PCR9	2	0.348	0.355	**PCR9**	2	0.478	0.492
PCR10	2	0.078	0.080	**PCR10**	2	0.257	0.265
PCR11	2	0.495	0.505	**PCR11**	2	0.298	0.307
PCR12	2	0.040	0.041	**PCR12**	2	0.114	0.117
PCR13	2	0.245	0.250	**PCR13**	2	0.257	0.265
PCR14	1	0.000	0.000	**PCR14**	2	0.298	0.307
PCR15	1	0.000	0.000	**PCR15**	2	0.213	0.220
PCR16	2	0.150	0.153	**PCR16**	2	0.059	0.061
PCR17	2	0.183	0.187	**PCR17**	2	0.298	0.307
PCR18	2	0.078	0.080	**PCR18**	1	0.000	0.000
PCR19	1	0.000	0.000	**PCR19**	1	0.000	0.000
PCR20	1	0.000	0.000	**PCR20**	1	0.000	0.000

[1]Na refers to the number of alleles in the Indian samples at each of the locus
[2]Diversity index
[3]Unbaised diversity index

4.7 Acknowledgements

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4.9 Supporting information

Table S4.1. Accession numbers for MLST sequences submitted to Genbank.

Isolate ID	Accession Numbers
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211-Ei-11m	KU575256
211-Ei-11s	KU575257
211-Ei-11z	KU575258
217-Ei-11-1a	KU575259
217-Ei-11-1b	KU575260
217-Ei-11-1c	KU575261
217-Ei-11-1l	KU575262
217-Ei-11-1m	KU575263
217-Ei-11-1s	KU575264
217-Ei-11-1z	KU575265
218-Ei-11-1a	KU575266
218-Ei-11-1b	KU575267
218-Ei-11-1c	KU575268

		219-Ei-11-2a	KU575315
		219-Ei-11-2b	KU575316
218-Ei-11-1l	KU575269	219-Ei-11-2c	KU575317
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221-Ei-11b	KU575281	222-Ei-11-2a	KU575329
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378-Ei-12-3c	KU575503	439-Ei-12b	KU575551
378-Ei-12-3l	KU575504	439-Ei-12c	KU575552
378-Ei-12-3m	KU575505	439-Ei-12l	KU575553
378-Ei-12-3s	KU575506	439-Ei-12m	KU575554

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314-Ei-12-1c	KU575579	369-Ei-12-3b	KU575627
314-Ei-12-1l	KU575580	369-Ei-12-3c	KU575628
314-Ei-12-1m	KU575581	369-Ei-12-3l	KU575629
314-Ei-12-1s	KU575582	369-Ei-12-3m	KU575630
314-Ei-12-1z	KU575583	369-Ei-12-3s	KU575631
315-Ei-12-1a	KU575584	369-Ei-12-3z	KU575632
315-Ei-12-1b	KU575585	371-Ei-12-2a	KU575633
315-Ei-12-1c	KU575586	371-Ei-12-2b	KU575634
315-Ei-12-1l	KU575587	371-Ei-12-2c	KU575635
315-Ei-12-1m	KU575588	371-Ei-12-2l	KU575636
315-Ei-12-1s	KU575589	371-Ei-12-2m	KU575637
315-Ei-12-1z	KU575590	371-Ei-12-2s	KU575638
378-Ei-12-2a	KU575591	371-Ei-12-2z	KU575639
378-Ei-12-2b	KU575592	374-Ei-12-2a	KU575640
378-Ei-12-2c	KU575593	374-Ei-12-2b	KU575641
378-Ei-12-2l	KU575594	374-Ei-12-2c	KU575642
378-Ei-12-2m	KU575595	374-Ei-12-2l	KU575643
378-Ei-12-2s	KU575596	374-Ei-12-2m	KU575644
378-Ei-12-2z	KU575597	374-Ei-12-2z	KU575645
343-Ei-12-1a	KU575598	375-Ei-12-3a	KU575646
343-Ei-12-1b	KU575599	375-Ei-12-3b	KU575647
343-Ei-12-1c	KU575600	375-Ei-12-3c	KU575648
343-Ei-12-1l	KU575601	375-Ei-12-3l	KU575649
343-Ei-12-1m	KU575602	375-Ei-12-3m	KU575650

375-Ei-12-3s	KU575651	388-Ei-12-1s	KU575699
375-Ei-12-3z	KU575652	388-Ei-12-1z	KU575700
379-Ei-12-2a	KU575653	1042-11a	KU575701
379-Ei-12-2b	KU575654	1042-11b	KU575702
379-Ei-12-2c	KU575655	1042-11c	KU575703
379-Ei-12-2l	KU575656	1042-11l	KU575704
379-Ei-12-2m	KU575657	1042-11m	KU575705
379-Ei-12-2s	KU575658	1042-11s	KU575706
379-Ei-12-2z	KU575659	1042-11z	KU575707
379-Ei-12-3a	KU575660	942-10a	KU575708
379-Ei-12-3b	KU575661	942-10b	KU575709
379-Ei-12-3c	KU575662	942-10c	KU575710
379-Ei-12-3l	KU575663	942-10l	KU575711
379-Ei-12-3m	KU575664	942-10m	KU575712
379-Ei-12-3s	KU575665	942-10s	KU575713
379-Ei-12-3z	KU575666	942-10z	KU575714
380-Ei-12-2a	KU575667	343-P-11b	KU575715
380-Ei-12-2b	KU575668	343-P-11c	KU575716
380-Ei-12-2c	KU575669	343-P-11l	KU575717
380-Ei-12-2l	KU575670	343-P-11m	KU575718
380-Ei-12-2m	KU575671	343-P-11s	KU575719
380-Ei-12-2s	KU575672	343-P-11z	KU575720
380-Ei-12-2z	KU575673	591-12a	KU575721
381-Ei-12-1a	KU575674	591-12b	KU575722
381-Ei-12-1b	KU575675	591-12c	KU575723
381-Ei-12-1c	KU575676	591-12l	KU575724
381-Ei-12-1l	KU575677	591-12m	KU575725
381-Ei-12-1s	KU575678	591-12s	KU575726
381-Ei-12-1z	KU575679	591-12z	KU575727
382-Ei-12-1a	KU575680	598-12a	KU575728
382-Ei-12-1b	KU575681	598-12b	KU575729
382-Ei-12-1c	KU575682	598-12c	KU575730
382-Ei-12-1l	KU575683	598-12l	KU575731
382-Ei-12-1m	KU575684	598-12m	KU575732
382-Ei-12-1s	KU575685	598-12s	KU575733
382-Ei-12-1z	KU575686	598-12z	KU575734
384-Ei-12-1a	KU575687	245-P-12a	KU575735
384-Ei-12-1b	KU575688	245-P-12b	KU575736
384-Ei-12-1c	KU575689	245-P-12c	KU575737
384-Ei-12-1l	KU575690	245-P-12l	KU575738
384-Ei-12-1m	KU575691	245-P-12m	KU575739
384-Ei-12-1s	KU575692	245-P-12s	KU575740
384-Ei-12-1z	KU575693	245-P-12z	KU575741
388-Ei-12-1a	KU575694	1166-12a	KU575742
388-Ei-12-1b	KU575695	1166-12b	KU575743
388-Ei-12-1c	KU575696	1166-12c	KU575744
388-Ei-12-1l	KU575697	1166-12l	KU575745
388-Ei-12-1m	KU575698	1166-12m	KU575746

1166-12s	KU575747	834-10m	KU575795
1166-12z	KU575748	834-10s	KU575796
2581-11a	KU575749	834-10z	KU575797
2581-11b	KU575750	1072-10a	KU575798
2581-11c	KU575751	1072-10b	KU575799
2581-11l	KU575752	1072-10c	KU575800
2581-11m	KU575753	1072-10l	KU575801
2581-11s	KU575754	1072-10m	KU575802
2581-11z	KU575755	1072-10s	KU575803
487-09a	KU575756	1072-10z	KU575804
487-09b	KU575757	1011-10a	KU575805
487-09c	KU575758	1011-10b	KU575806
487-09l	KU575759	1011-10c	KU575807
487-09m	KU575760	1011-10l	KU575808
487-09s	KU575761	1011-10m	KU575809
487-09z	KU575762	1011-10s	KU575810
1286-09a	KU575763	1011-10z	KU575811
1286-09b	KU575764	111-10a	KU575812
1286-09c	KU575765	111-10b	KU575813
1286-09l	KU575766	111-10c	KU575814
1286-09m	KU575767	111-10l	KU575815
1286-09s	KU575768	111-10m	KU575816
1286-09z	KU575769	111-10s	KU575817
1261-09a	KU575770	111-10z	KU575818
1261-09b	KU575771	1608-09a	KU575819
1261-09c	KU575772	1608-09b	KU575820
1261-09l	KU575773	1608-09c	KU575821
1261-09m	KU575774	1608-09l	KU575822
1261-09s	KU575775	1608-09m	KU575823
1261-09z	KU575776	1608-09s	KU575824
1272-09a	KU575777	1608-09z	KU575825
1272-09b	KU575778	1591-09a	KU575826
1272-09c	KU575779	1591-09b	KU575827
1272-09l	KU575780	1591-09c	KU575828
1272-09m	KU575781	1591-09l	KU575829
1272-09s	KU575782	1591-09m	KU575830
1272-09z	KU575783	1591-09s	KU575831
894-09a	KU575784	1591-09z	KU575832
894-09b	KU575785	462-09a	KU575833
894-09c	KU575786	462-09b	KU575834
894-09l	KU575787	462-09c	KU575835
894-09m	KU575788	462-09l	KU575836
894-09s	KU575789	462-09m	KU575837
894-09z	KU575790	462-09s	KU575838
834-10a	KU575791	462-09z	KU575839
834-10b	KU575792	1573-09a	KU575840
834-10c	KU575793	1573-09b	KU575841
834-10l	KU575794	1573-09c	KU575842

1573-09l	KU575843	1268-10c	KU575856
1573-09m	KU575844	1268-10l	KU575857
1573-09s	KU575845	1268-10m	KU575858
1573-09z	KU575846	1268-10s	KU575859
1926-10a	KU575847	1268-10z	KU575860
1926-10b	KU575848	94-P-10a	KU575861
1926-10c	KU575849	94-P-10b	KU575862
1926-10l	KU575850	94-P-10c	KU575863
1926-10m	KU575851	94-P-10l	KU575864
1926-10s	KU575852	94-P-10m	KU575865
1926-10z	KU575853	94-P-10s	KU575866
1268-10a	KU575854	94-P-10z	KU575867
1268-10b	KU575855		

Chapter 5: Limited evidence of fungicide-driven triazole resistant *Aspergillus fumigatus* in the Hamilton Region.

5.1 Preface

It is well known that the use of triazole based fungicides can lead to clinically important resistant *A. fumigatus* strains. Although there is a notable influx of high risk patients into Hamilton, it is still unclear if triazole fungicide use on farms around Hamilton poses any serious threat to these patients. In this study we investigate if triazole fungicide use can lead to treatment failure in aspergillosis patients in Hamilton. Our results suggest that triazole fungicide use plays a limited role in the emergence of clinically important resistant strains in Hamilton. Authors of this article are Eta Ashu, Ga Young Kim, Patrick Roy-Gayos, Kelly Dong, Adrian Forsythe, Victoria Giglio, Gregory Korfanty, Deborah Yamamura, and Jianping Xu. I am the primary contributor of this work. This study was conceived by JX and EA. Strains for this study were obtained by EA, GYK and DY. Genotyping was performed by EA, GYK and PRG. Susceptibility testing was done by EA, KD, AF,VG and GK. This study is in press and will be published by the Canadian Journal of Microbiology. This chapter is formatted as per the journal's requirements.

5.2 Abstract

Aspergillus fumigatus is a ubiquitous opportunistic fungal pathogen that can cause aspergillosis in humans. Over the last decade there have been increasing global reports of treatment failure due to triazole resistance. An emerging hypothesis for the origin of clinical triazole-resistance is that the use of triazole fungicides in agriculture can propagate triazole-resistant strains of clinical importance. Here we test this hypothesis in Hamilton, Ontario, Canada by examining a total of 195 agricultural, urban and clinical isolates using 9 highly polymorphic microsatellite markers. For each isolate, the *in vitro* susceptibility to itraconazole and voriconazole, two triazole drugs commonly used in the management of patients, was also determined. Our analyses suggested frequent gene flows among the agricultural, urban, and clinical samples of *A. fumigatus* and found evidence for widespread sexual recombination within and among the different populations. Interestingly, all 195 isolates analyzed in this study were susceptible to both triazoles tested. However, compared to the urban population, agricultural and clinical populations showed significantly reduced susceptibility to

itraconazole and voriconazole. Our results suggest evidence for individual selective pressures on *A. fumigatus* niche populations in Hamilton. Frequent gene flow and genetic recombination among these populations suggest greater attention should be paid to monitor *A. fumigatus* populations in Hamilton and other similar jurisdictions.

5.3 Introduction

Aspergillus fumigatus is an opportunistic fungal pathogen that can thrive in a broad range of ecological niches. There are two distinct features of this saprophyte that enhances its pathogenicity: small size of airborne spores and thermo-tolerance (Dagenais & Keller 2009). Each day, hundreds of *Aspergillus fumigatus* spores are inhaled by most individuals but without causing any obvious infections. However, in immunocompromised individuals, *A.fumigatus* can cause a group of infections collectively known as aspergillosis, a disease with high morbidity and mortality (Dagenais & Keller 2009).

Triazoles class antifungal drugs, such as itraconazole and voriconazole, have emerged as the first-line drug in the treatment and prevention of aspergillosis. Similarly, triazole fungicides such as propiconazole, prochloraz, pebuconazole and priadimenol are used to manage plant fungal infections in agriculture (Bowyer & Denning 2014). Triazoles impede proliferation in *A. fumigatus* by inhibiting lanosterol-14-alpha-demethylase (CYP51A), a protein that is essential for the synthesis of ergosterol in fungal cell membranes. Over the last decade, there have been increasing reports of triazole resistance in clinical and environmental isolates in countries across Eurasia, North and South America, including United Kingdom, Netherlands, Germany, India, United States and Colombia (Verweij *et al.* 2016). Two rationales have been proposed to explain recent increases in triazole resistance: use of triazoles for prophylaxis in highly immunocompromised patients and extensive use of triazole fungicides in agriculture (Enserink 2009). In favor of the latter hypothesis, there have been several reported cases of triazole-resistant aspergillosis in triazole-naïve patients in the Netherlands, India and the United Kingdom (Chowdhary *et al.* 2012a; Enserink 2009; Howard *et al.* 2009; van der Linden *et al.* 2013). Furthermore, it has been shown that clinically resistant strains of *A. fumigatus* exhibit cross-resistance to agriculture triazole fungicides. This is largely due to the fact that both

agricultural and clinical triazoles have similar molecular structures and adopt similar conformations while docking to their target protein, CYP51A (Chowdhary *et al.* 2012b; Snelders *et al.* 2012).

Understanding the frequency of triazole resistance in clinical settings and its relation to agricultural fungicide use is of significant importance to agronomists, mycologists, physicians, public health practitioners and policymakers worldwide. Thus far, three major field studies have tested if agricultural fungicide use can lead to triazole-resistant *A. fumigatus*. One of these field studies was done in the United Kingdom and showed evidence supporting the agricultural fungicide-driven resistance hypothesis (Bromley *et al.* 2014). However, the other two studies done in Japan showed little evidence to support the said hypothesis (Kano *et al.* 2014; Toyotome *et al.* 2016). These studies suggest that there might be some geographic specificity in the relationship between agricultural triazole fungicide use and resistance. At present, the agricultural fungicide-driven resistance hypothesis has yet to be investigated using a molecular epidemiological approach which tracks resistant strains from farms, through urban areas to carriage in patients within a community.

Located at the west end of Lake Ontario, Hamilton is home to ~500,000 people. Hamilton hosts 12 specialized healthcare facilities and hospitals. Some of these hospital and healthcare facilities serve as referral centers for patients in Southern Ontario, thus there is a frequent influx of patients, including those that are immunocompromised, into Hamilton. Although there is frequent influx of high-risk patients and use of triazole fungicides on farms around Hamilton, very little is known about the prevalence of triazole-resistant *A. fumigatus* or the link between agricultural triazole fungicide use and the acquisition of clinically important resistant strains.

In order to understand the prevalence of triazole-resistant *A. fumigatus* and the link between triazole fungicide use in agriculture and the acquisition of clinically important resistant strains, we examined agricultural, urban and clinical *A. fumigatus* populations in and around Hamilton. We hypothesized that there would be triazole resistant strains in agricultural fields with long-term triazole fungicide use history. In addition, given the prevalence of asexual reproduction in this species and the high dispersal ability of its asexual conidia spores, we postulated that there would be shared

resistant genotypes between the environmental and clinical populations. Our postulate was based on a continent-island gene flow model wherein the environmental samples acts as the continent source population and the clinical samples the island population. Our proposed continental population in Hamilton consists of two main demes (urban and agricultural), which we assumed to have frequent gene flow (Fig 5.1). Supposing the agricultural population was under a strong triazole selective pressure (Fig 5.1A), our proposed model would suggest an eventual convergence of resistant phenotypes and genotypes between agricultural and urban populations due to frequent gene flow between the two populations (Fig 5.1B). In this model, change in allelic frequency (Δq) due to gene flow from the continent to the island population can be calculated using the formula $\Delta q = -m(qo - Q)$ (Macdonald 2004). Where, m is the proportion migrants in the island population, Q the frequency of resistant strains in the continental population, and qo the frequency of resistant strains in the island population. At equilibrium ($qo = Q$), after several cycles of gene flow, resistant allele frequency in continental and island populations are expected to converge as well. Prior to equilibrium, qo is expected to be less than Q. Being a potential source population for triazole resistance, the environmental population was expected to show a higher diversity of resistant strains. In addition, environmental and clinical populations were expected to share similar triazole resistance mechanism.

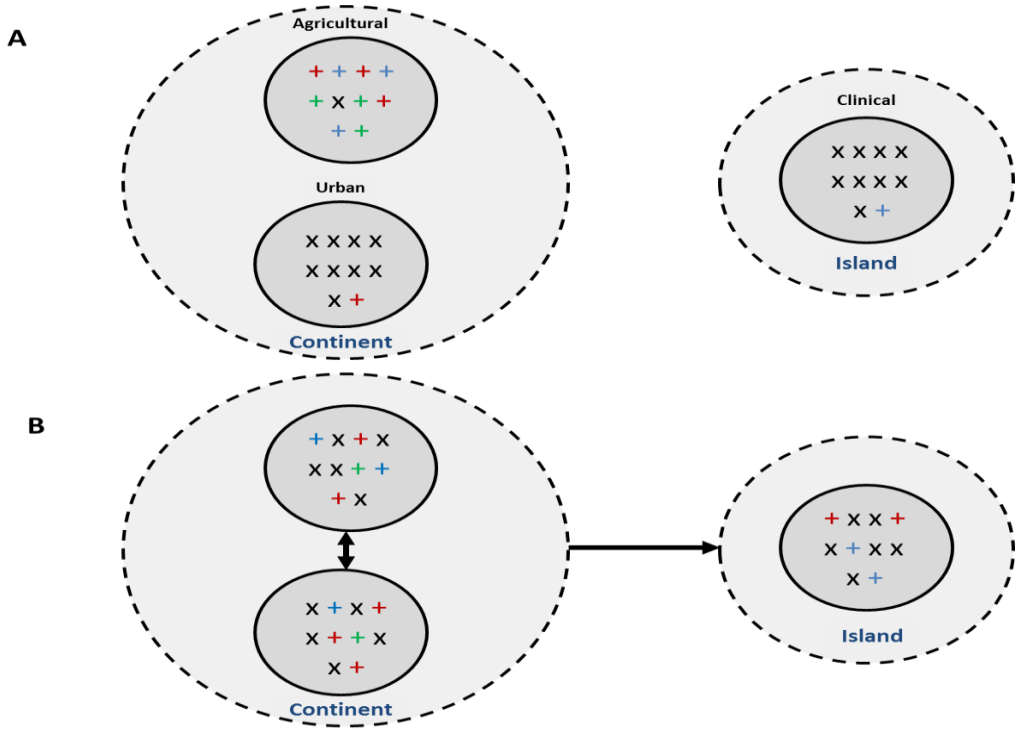

Figure 5.1. Continent-island schematic. Plus signs are indicative of resistant strains while crosses represent susceptible strains. Colored plus signs depict diversity of resistance. Double arrow thick line is indicative of equal gene flow in either direction while the thick single arrow line represents a predominantly one-way gene flow. A) A model where a triazole selective pressure is solely exerted on the agricultural population. B) Prior model after many cycles of gene flow.

5.4 Materials and methods

5.4.1 Environmental and clinical isolates

A total of 781 soil isolates were obtained from 3 agricultural and 6 urban sites between September 2014 and January 2015 (Fig 5.2). Agricultural samples were obtained from 3 farms around St. George, a township which is ~35km West of Hamilton, Ontario. Wheat, soybeans or barley were grown on these farms. Triazole fungicides were used on these farms every other year for at least the previous 10 years. Triazole fungicides used were Stratego (propiconazole and tebuconazole), Prosaro (prothioconazole and tebuconazole), and Headline (pyraclostrobin and tebuconazole). Urban sampling was done throughout the city of Hamilton: at McMaster University, Aviary Park, Gore Park, Concession Park, Gage Park, and Pier Park. These urban sites have no known fungicide usage. For each collection site, soil

samples were obtained ~ 5 meters apart from each other in all four major cardinal directions. Environmental sampling was done by adding about 2g of soil to microcentrifuge tubes each containing 1mL of Sabouraud Dextrose Broth (SDB) supplemented with chloramphenicol (50mg/L) (Sigma-Aldrich: Mississauga).

Clinical *A. fumigatus* isolates were obtained from the Microbiology Laboratory at Hamilton Health Sciences, Hamilton General Hospital site. This laboratory serves as the regional mycology laboratory for Hamilton Health Sciences and St. Joseph's Healthcare. Between January and October 2015, clinically significant *A. fumigatus* isolates from respiratory, wound, tissue and sterile fluid were evaluated. A retrospective chart review [approved by the Hamilton Integrated Research Ethics Board (project number: 3328-C)] to obtain patient demographics and prior antifungal use was performed.

Figure 5.2. Map of Hamilton showing sampling sites. The agricultural site (black) consisted of three different farms (not shown in this figure). Map was retrieved and modified from Google maps.

5.4.2 *A. fumigatus* identification

In order to differentiate *A. fumigatus* from *A. lentulus, A. udagawae, A. fumigatiaffinis, A. novofumigatus, A. fumisynnematus, A. viridinutans, A. fischeri* and *A. thermomutatus* (section Fumigati), both environmental and clinical isolates were incubated on Sabourauds Dextrose Agar (SDA) supplemented with chloramphenicol at 50^{o}C for 48 hours (Samson *et al.* 2007; Lamoth 2016). *A. fumigatus* was then identified by macroscopic and microscopic features such as colony color, texture, conidial heads and seriation. *A fumigatus* was further confirmed by using a set of 9 species-specific microsatellite markers(Valk *et al.* 2005).

5.4.3 Genotyping

The aforementioned set of highly polymorphic microsatellite markers was also used to genotype our set of *A. fumigatus* isolates. Genotyping was done as previously described by Valk and colleagues (Valk *et al.* 2005). Briefly, each reaction contained a total volume of 13.5 uL, which was made up of 0.25 ul of each primer (0.16 uM), 7 uL of 2x GoTaq Master Mix (Promega: Markham, Canada), and 5 uL of nuclease free water. Amplification conditions involved using an initial denaturation step of 95^{o}C for 10 min, followed by 30 cycles of 95^{o}C for 30 seconds, 60^{o}C for 30 seconds, 72^{o}C for 1 min, and by a final elongation step at 72^{o}C for 5 min. Capillary electrophoresis was carried out at the Mobix Laboratory (Hamilton, ON, Canada). Peak Scanner software v1.0 (ThermoFisher Scientific) was used for fragment analysis. In order to further categorize the genetic makeup of our set of isolates, a mating type PCR was carried out as previously described (Chang *et al.* 2016). The *MAT1-1* and *MAT1-2* mating types were identified by their corresponding band sizes of 834 and 438 bp, respectively

5.4.4 Antifungal susceptibility testing

The *in vitro* susceptibility of our set of isolates to itraconazole and voriconazole (Selleckchem, San Francisco) was tested following the M38-A2 guidelines of the Clinical and Laboratory Standard Institute (Clinical and Laboratory Standards Institute 2008). Based on previously described epidemiological cutoff values, isolates

with itraconazole and voriconazole MICs of ≥ 2mg/L were considered resistant (Pfaller *et al.* 2011; Pfaller et al. 2009; Rodriguez-Tudela et al. 2008). To prepare the isolates for testing, all isolates were plated on SDA at 37°C for 48 hours. Spores were harvested by washing culture plates with 1mL of 0.085% of sterile saline and adjusted to a range of approximately $4x10^5$ to $5x10^6$ CFU/mL, these suspension were then diluted 50 times in standard medium. In-house strains with known MICs were used as controls (accession numbers, KU575343 and KU575805). Strains KU575343 and KU575805 are two *A. fumigatus* strains that have been previously confirmed by means of β-tubulin gene sequencing, microscopic and macroscopic morphology. These strains were used because their MICs fall on the 20th and 80th centile of the range of itraconazole and voriconazole concentrations tested. Additionally, 5 isolates obtained in this study were further tested at the Public Health of Ontario laboratory in Toronto. This representative set of our collection was further tested to corroborate our susceptibility results to that done in a routine clinical laboratory that uses CLSI guidelines. Drug response in our set *A. fumigatus* isolates was further assessed by obtaining optical density readings at 590nm.

5.4.5 Population and statistical analysis

Simpson's unbiased diversity index(uh) and analysis of molecular variance (AMOVA) as implemented by GENALEX (V 6.5) were used to determine levels of ecological niche differentiation and genetic diversity (Peakall & Smouse 2012).In order to score levels of linkage disequilibrium, MULTILOCUS (V 1.3b) was used to determine the index of association (Smith *et al.* 1993). Significance was determined by 1000 permutations. Analysis of variance was used to elucidate differences in triazole susceptibility between ecological niche groups at different drug concentrations. The Wilcoxon rank sum test was used to elucidate differences in triazole susceptibility between ecological niche populations.

5.5 Results

5.5.1 Environmental and clinical *A. fumigatus* isolates in and around Hamilton

A. fumigatus isolates were distinguished from other thermophilic fungi by their distinct greyish-green suede-like colonies. Furthermore, microscopic observation confirmed the presence uniseriate and club-shaped conidial heads characteristic of *A. fumigatus.* Isolates not amplified by the aforementioned set of highly discriminatory microsatellite markers were not included in our analysis. A total of 124 environmental and 71 clinical *A. fumigatus* isolates were identified. Clinical isolates were obtained from a total of 56 patients, 30 female and 26 male. Of all 56 patients; 27 lived in Hamilton; 4 in St. Catharines; 3 each in Grimsby, Burlington, and Nigara Falls; while 2 each lived in Haldimand, Simcoe, Brantford and Welland. One patient each lived in Oakville, Guleph, Waterloo, Milton and Glencairn. Three patients' postal codes were unknown. Patients' ages ranged from 8 to 97 years, with their median and modal ages being 64 and 65 years, respectively. More than one *A. fumigatus* isolate was obtained from a total of 11 patients, 2 isolates each were obtained from 9 patients, while 3 and 5 isolates were each obtained from 2 patients. When two or more *A. fumigatus* isolates were obtained from a single patient, they often did not share the same microsatellite genotype, suggesting the existence of multiple carriages among patients studied (Table 5.1). Ten of the fifty-six patients surveyed in this study had unknown triazole histories while 4 patients had previously used either fluconazole or voriconazole within three months prior to their sampling dates. A total of 6 *A. fumigatus* isolates were obtained from all 4 patients who had previously used triazoles. Sixty-two *A. fumigatus* isolates were each obtained from urban and agricultural niches. The difference in isolation rates between West, Centre, and East Hamilton was statistically insignificant. There was also no statistical significant difference in isolation rates between urban and agricultural ecological niches. The distribution of *A. fumigatus* isolated by location is shown in Table 5.2.

Table 5.1. Patients from who two or more isolates were obtained. Identical superscript letters denote isolates from the same patient. Isolates obtained from a single patient that were identical in all 9 microsatellite alleles are higlighted.

Patient number	Isolate source	2A	2B	2C	3A	3B	3C	4A	4B	4C
15-4[a]	BAL	11	30	13	3	7	18	13	6	7
15-5[a]	BAL	11	29	13	3	10	18	14	4	7
15 -6[b]	BAL RLL	13	31	15	27	10	33	8	9	10
15-7[b]	BAL RLL	12	31	14	4	11	19	12	8	9
15-19[c]	Sputum	11	30	13	10	9	25	12	7	8
15-58[c]	Sputum	11	30	13	10	9	25	12	7	8
15-21[d]	ETT	10	29	12	20	12	32	11	8	9
15-22[d]	ETT	16	34	18	24	30	16	7	7	8
15-32[e]	BAL LUL	28	46	30	36	9	5	16	7	8
15-36[e]	BAL RUL	4	23	6	12	9	25	8	6	6
15-42[f]	BR wash	23	41	25	6	2	25	14	6	10
15-52[f]	BR wash	22	40	25	28	9	13	8	6	7
15-43[g]	BAL	21	39	23	11	3	27	8	8	6
15-57[g]	Sputum	21	39	23	11	3	27	8	6	9
15-46[h]	Sputum	17	35	19	9	9	24	8	9	10
15-48[h]	Sputum	14	32	16	2	9	15	15	8	15
15-49[i]	BAL RLL	8	27	10	30	10	12	14	6	7
15 -50[i]	BAL RLL	11	30	13	34	15	12	15	8	7
15 -62[i]	BAL LLL	10	29	12	9	8	31	13	6	7
15 -65[i]	BAL LLL	14	33	16	28	9	13	12	3	8
15 -68[i]	BAL RLL	14	33	16	28	9	13	12	7	8
15 -66[j]	Sputum	9	28	11	11	17	11	7	4	5
15 -67[j]	Sputum	9	28	11	11	17	11	7	4	5
15 -61[k]	BR wash	6	25	8	41	16	12	7	10	11
15 -63[k]	Tissue	9	28	11	2	31	17	7	7	8
15 -69[k]	Tissue	4	23	6	11	8	24	8	6	7

BR wash-bronchial wash
BAL-bronchoalveolar lavage
RLL-right lower lung
LLL-left lower lung
RUL-right upper lung
LUL-left upper lung
ETT-endotracheal tube

Table 5.2. Proportions of *A.fumigatus* isolates obtained from 6 urban sites and 3 agricultural fields.

Sample Region	Sample Size	Number (%) of Thermophilic fungal isolates	Number (%) of *A. fumigatus* isolates
Urban			
West			
McMaster University	38	26 (68)	10 (26)
Aviary Park	94	62 (66)	17 (18)
Centre			
Gore Park	36	25 (69)	4 (11)
Concession Park	36	24 (67)	6 (17)
East			
Gage Park	36	22 (61)	0 (0)
Pier Park	91	43 (47)	26 (29)
Agricultural			
Field A	34	21 (62)	0 (0)
Field B	33	11 (33)	0 (0)
Field C	383	205 (54)	65 (17)
Total	781	439 (56)	128 (16)

5.5.2 Evidence of gene flow

AMOVA showed that only 0.4 % of total genetic variation in the whole Hamiltonian *A. fumigatus* population was contributed by grouping our set of isolates by ecological niche, i.e. urban, agricultural and clinical. Although very small, differences between the clinical population and the other 2 populations were statistically significant, consistent with limited differentiations [clinical and urban PhiPT=0.006 (P=0.03), clinical and agricultural PhiPT=0.006 (P=0.05); PhiPT refers to pairwise population heterogeneity index of the proportion of total genetic variance]. However, there was no significant differentiation between the urban and agricultural populations (PhiPT=0.001, P=0.33).

5.5.3 Susceptibility patterns

All analyzed 195 isolates were susceptible to itraconazole and voriconazole. Our results were concordant with those from Public Health of Ontario laboratory. Compared to the urban population, both agricultural and clinical populations showed a significant decrease in their susceptibility to itraconazole (in both cases, P value <

0.001) (Table 5.3). Similarly, spectrophotometry revealed that agricultural and clinical populations grew significantly better than the urban population at itraconazole concentrations higher than 0.25mg/L (Fig 5.3). Comparably, the clinical population also grew significantly better than the urban and agricultural populations at voriconazole concentrations higher than 0.125mg/L (Fig 5.3). These results suggest that even though there is currently no evidence for triazole resistance in *A. fumigatus* from Hamilton, there is significant evidence of decreased triazole susceptibility in both clinical and agricultural populations.

Table 5.3. *In-vitro* antifungal susceptibility profile of medical triazoles against environmental *A. fumigatus* isolates from Hamilton.

Triazole drug	Niche	Drug concentration (mg/L)						No. Tested	Modal MIC
		0.03	0.06	0.125	0.25	0.5	1		
Itraconazole	Urban	0	0	6	56	0	0	62	0.25
	Agricultural	0	0	0	17	45	0	62	0.5
	Clinical	1	0	0	13	57	0	71	0.5
	Total	1	0	6	86	102	0	195	0.5
Voriconazole	Urban	1	0	4	14	43	0	62	0.5
	Agricultural	0	0	0	25	37	0	62	0.5
	Clinical	1	0	1	56	13	0	71	0.25
	Total	2	0	5	95	93	0	195	0.25

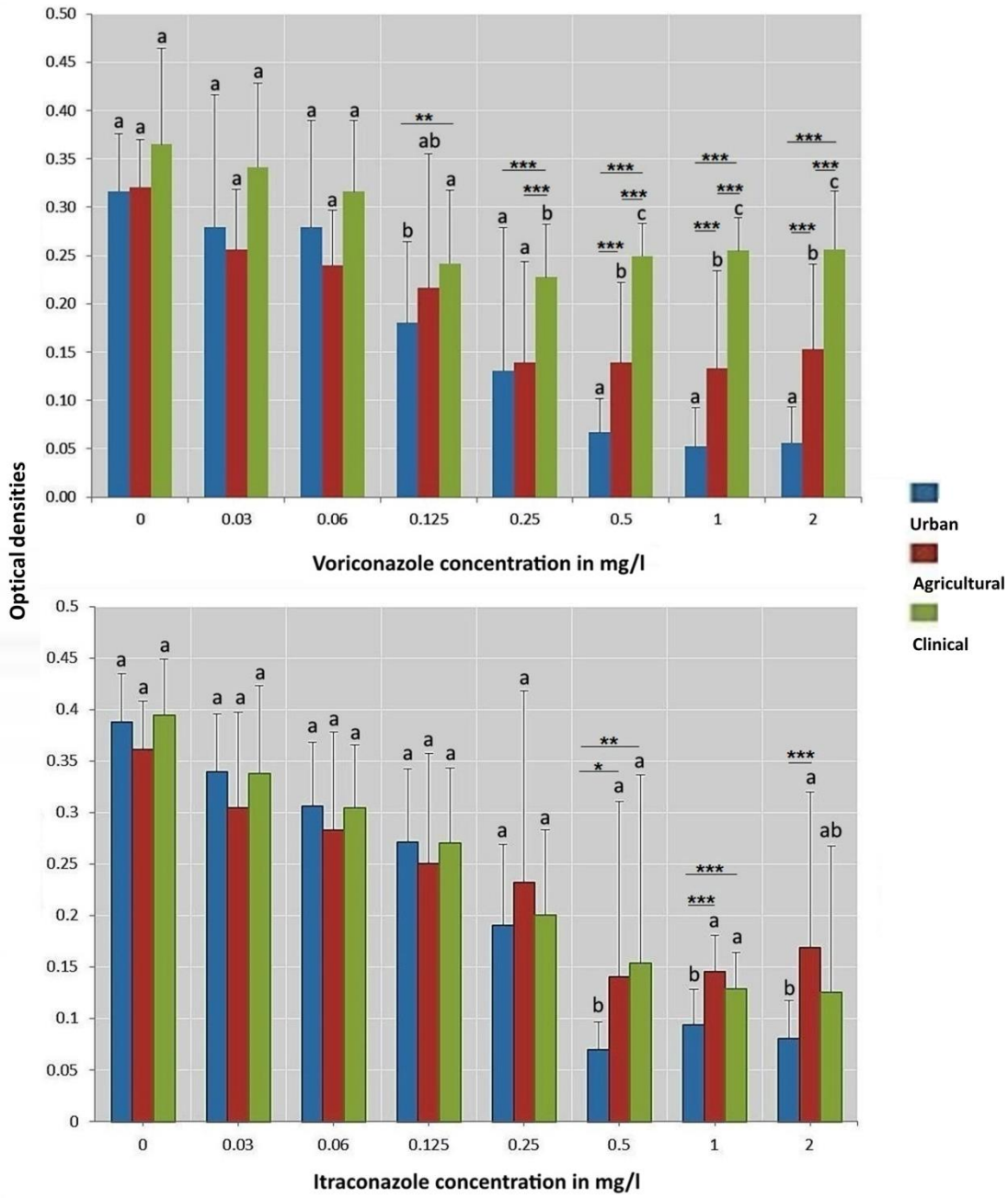

Figure 5.3. Plots showing growth response of urban, agricultural and clinical populations to itraconazole and voriconazole. Differing letters at a given concentration indicate a significant difference in the mean optical density. The number of stars depicts the level of significance. Adjustments were made to P-values using the Bonferroni method.

5.5.4 Patterns of diversity and evidence of recombination

We found high levels of allelic and genotypic diversities within ecological niche populations of *A. fumigatus* from Hamilton. Out of 62 urban isolates, we identified 60 genotypes. Similar to the urban population, 2 genotypes were shared by 4 clinical isolates; the remaining isolates belonged to 67 different genotypes. Within the agricultural population, 1 genotype was shared by 3 isolates; the remaining isolates belonged to 59 different genotypes. Simpson's unbiased allelic diversity indices for urban, agricultural and clinical populations were 0.893, 0.891 and 0.890 respectively. Locus 3C showed the most allelic polymorphism in urban (uh=0.951) and clinical (uh=0.946) populations, while locus 3A had the highest diversity in the agricultural population (uh=0.943). Locus 4C showed the least allelic polymorphism in agricultural (uh=0.788) and clinical (uh=0.805) populations. Locus 4A in the urban population had an unbiased diversity index of 0.772. Consistent with high levels allelic polymorphism, no genotype was shared between the ecological populations.

All three ecological niche populations showed significant evidence of phylogenetically incompatible pairs of loci. Specifically, phylogenetic incompatibility indices for urban, agricultural and clinical populations were 0.97, 0.94 and 0.92, respectively. These results are consistent with widespread recombination in natural populations of this organism in Hamilton. However, there is also evidence for some clonality within each population. Though the index of association (I_A) values were low (0.81 for the urban, 0.88 for the clinical, and 1.14 for the agricultural populations), all 3 populations strongly rejected the null hypothesis of random recombination ($P<0.01$).

Although slightly skewed in urban and agricultural populations, mating type ratios were also consistent with sexual recombination. Thirty-eight of all urban isolates belonged to MAT1-1 mating type, while 24 were categorized as MAT1-2. Similarly, 40 and 22 agricultural isolates were categorized as MAT1-1 and MAT1-2 mating types. The clinical population's mating type ratio was however a lot closer to 1:1. The mating type loci for 4 clinical isolates could not be amplified using previously described AFM1, AFM2 and AFM3 primers (Paoletti *et al.* 2005).

5.6 Discussion

In several geographic regions, the effectiveness of triazoles in the management of patients with aspergillosis has been compromised due to increasing resistance to the azole class of antifungals. It has been found that the use of triazole fungicides in agriculture can cause resistance in environmental *A. fumigatus* strains of clinical importance. However, there has been no report of such a link in Canada. Here, we investigated the potential link between triazole fungicide use and the acquisition of clinically important resistant strains in Hamilton, Ontario. While all 195 isolates examined in this study were susceptible to both itraconazole and voriconazole, there was evidence of increased MICs in both clinical and agricultural *A. fumigatus* isolates over the urban isolates. Our results suggest that continued triazole selection pressure could drive some of these isolates to become resistant. Furthermore, we found evidence of widespread recombination and gene flow among the local ecological niche samples. Such gene flows and sexual recombination could have a significant implication in the initiation and spread of resistant genes throughout the Hamiltonian population. Below we discuss the implications for our results to public health practitioners and farmers in Canada.

5.6.1 Link between triazole fungicide use and the acquisition of clinically important resistant strains

Using cutoffs of 2 mg/L, all isolates examined in this study were susceptible to both itraconazole and voriconazole. This suggests that widely reported multi-azole resistance such as that caused by TR34/L98H mutations might not yet be a problem in the Hamilton Region. Our results showed limited evidence of a link between agricultural triazole fungicide use and the acquisition of clinically important resistant strains in Hamilton. However, the agricultural and clinical *A. fumigatus* populations were significantly less susceptible to itraconazole and voriconazole than the urban population (Fig 5.3). Likewise, the clinical population showed an overall reduced susceptibility to voriconazole than both the agricultural population and even more so the urban population (Fig 5.3). We hypothesized that in the advent of a significant link between triazole fungicide use and the acquisition of resistance in strains of clinical

importance, agricultural and urban populations would share similar triazole susceptibility profiles and be more resistant than the clinical population (Fig 5.1). We however found that the urban population was significantly more susceptible to both itraconazole and voriconazole than the other 2 populations. This was surprising given that we found significant evidence of gene flow, more so between agricultural and urban populations. Taken together, these results are consistent with independent selective pressures acting on ecological niche populations of *A. fumigatus* in the Hamilton Region.

Although the MIC values for all isolates were below the epidemiological cutoffs, differences in triazole susceptibility profiles among the three ecological niches suggest local adaptations to distinct selective pressures. Two agricultural fungicides used on the sampled farms in this study, propiconazole and tebuconazole, are structurally similar to medical triazoles and they dock similarly to the CYP51A protein (Snelders *et al.* 2012). The continuous use of these fungicides could have contributed to the decreased itraconazole and voriconazole susceptibility in the agricultural population of *A. fumigatus* around Hamilton. Acquired triazole resistance in *A. fumigatus* due to repeated exposure to triazoles has been previously demonstrated both *in vivo* and *in vitro* (Dannaoui *et al.* 2001; Escribano *et al.* 2012).

Similar to what has been reported elsewhere (Toyotome *et al* 2016*;* Meletiadis *et al.* 2012; Pfaller *et al.* 2011; Pfaller *et al.* 2009), our voriconazole modal MIC in environmental and clinical populations were 0.5 mg/L and 0.25 mg/L, respectively. However, a more in-depth analysis of voriconazole susceptibility using spectrophotometry revealed that, clinical isolates grew significantly better at higher concentrations than agricultural and urban populations (Fig 5.3). Escribano and colleagues previously reported that after progressive and direct exposure to itraconazole, there was a statistically significant increase in the geometric mean MICs of two other triazoles (Escribano *et al.* 2012). It is worth noting that although prior itraconazole exposure led to a significant increase in the geometric mean MICs of voriconazole and posaconazole, itraconazole MICs were the most affected among the three triazoles. In this study, although the clinical population showed reduced susceptibility to both itraconazole and voriconazole when compared to the urban population, voriconazole MICs were more affected than itraconazole MICs (Fig 5.3).

However, given that only 2 patients surveyed in this study had been previously exposed to voriconazole, selection by voriconazole was unlikely the reason for the overall reduced voriconazole susceptibility in the clinical sample. Instead, adaptation to host microenvironments by the secretion of SrbA (sterol regulatory element-binding protein) or similar proteins might have been responsible. A previous study showed that adaptation to hypoxic environments due to altered expression of SrbA could lead to resistance to voriconazole (Willger *et al*. 2008).

At present, the dominant mutations associated with triazoles resistance in *A. fumigatus* are found in the CYP51A gene. However, Escribano and colleagues also noted that increases in geometric mean MICs of *A.fumigatus* strains can be independent of the presence of *CYP51A* mutations (Escribano *et al.* 2012). Indeed, sequencing 1kb of the *CYP51A* gene of 95 randomly chosen isolates from our sample set revealed no mutations known to be associated with decreased triazole susceptibility (data not shown). Although mechanisms leading to decreased triazole susceptibility in clinical and agricultural populations are not fully understood, several mechanisms could have contributed, including target overexpression, up-regulation of multidrug transporter, secretion of sterol regulatory element-binding proteins, and/or other cellular stress responses.

Abundant evidence of gene flow between ecological niche populations was found in Hamilton (PhiPT=0.006). Surprisingly, on a population scale, these exchanges do not seem to cause a convergence of triazole susceptibility phenotypes between these populations. The results are consistent with our inference of independent selective triazole pressures on ecological niche populations of *A. fumigatus* in Hamilton. A possible explanation for the observed difference in triazole susceptibility despite abundant evidence of gene flow is that, decreased itraconazole and voriconazole susceptibility in agricultural and clinical populations is likely caused by non-*CYP51A* mechanisms which may have a fitness cost on these populations. A previous study showed that a *CYP51A* wild type isolate with an acquired non-*CYP51A* itraconazole resistance mechanism (>16mg/L) experienced a significant increase in susceptibility (1mg/L) after five weeks of proliferation in triazole-free medium (Escribano *et al.* 2012). It is plausible that a majority of strains migrating from the

farm to the urban environment adapt to their new environment by losing triazole tolerance, which may help restore their fitness in the urban, triazole-free environment.

Contrary to our results, Bromley et al showed evidence of fungicide-driven resistance in Manchester, UK (Bromley *et al.* 2014). Their study however did not include a clinical population which could be useful in tracking resistant strains from farms through urban settings to carriage in patients. Nonetheless, they sampled for a period of over 3 years which was longer than what was done in this study. Given that we sampled within a period of only 4 months, we could not completely exclude the possibility of a link between triazole fungicide use and the acquisition of clinically important resistant strains. However, in the event of a link, our results suggest that triazole fungicide use in Hamilton is not a dominant mechanism for the spread of triazole resistance.

5.6.2 The importance of crop rotation

Although triazole fungicide usage patterns may not necessarily follow that of combined fungicide usage, it is worth nothing that the combined increased usage of fungicides in Ontario is among the highest in Canada (Table 5.4). Generally speaking, a tendency towards specialized and intensive crop production is thought to have played a major role in increasing fungicide use. Specialized and intensive farming is also thought to have resulted in little to no crop rotation in provinces like Ontario (Gossen *et al.* 2014). Crop rotation has been shown to reduce infection severity of blackleg on Western Canadian canola plants (Kutcher *et al.* 2013). Similarly, Bailey and colleagues showed that populations of fungal pathogens *S. tritici*, *B. sorokiniana* and *S. nodorum* in wheat decreased after increasing rotating crop diversity (Bailey *et al.* 2001). Sufficient crop rotation oftentimes imply less fungal plant disease and consequently less fungicide use. Although increased usage of fungicides in Ontario is amongst the highest in Canada, the farms from which we sampled rotated wheat, soybeans and barley. Furthermore, triazole fungicides were only used every other year. We hypothesized that crop rotation and farmer's fungicide use patterns could explain the observed overall triazole susceptibility pattern (MICs < 2mg/L) in the agricultural *A. fumigatus* population.

Table 5.4. Percentage of farmland with crops to which fungicides (including triazoles) were applied in Canada from 1996-2006. Source: Statistics Canada.

Provinces	1996	2001	2006	% change from 1996 to 2006
Manitoba	8.3	15.7	16.5	98.70
Saskatchewan	3.7	5.9	6.6	77.57
Ontario	5.2	5.3	7.2	40.09
Alberta	5.7	5.6	6.8	19.26
New Brunswick	17.6	17.8	19.9	13.16
Newfoundland and Labrador	2.2	3.7	2.5	10.74
Quebec	3.8	4.0	4.0	5.21
British Columbia	4.4	4.0	4.3	-3.56
Nova Scotia	9.7	10.1	9.2	-5.17
Prince Edward Island	26.0	25.4	24.4	-6.22

5.6.3 The need for continuous surveillance in Hamilton and other similar jurisdictions

Given that wild type CYP51A isolates seldom have itraconazole MICs of $\geq$ 1mg/L (Ingen *et al.* 2015; Meletiadis *et al.* 2012), we compared our results to wild type isolates obtained from around the world. Compared to modal itraconazole MICs in wild type isolates reported elsewhere (Toyotome *et al.* 2016; Meletiadis *et al.* 2012; Pfaller *et al.* 2011; Pfaller *et al.* 2009; Rodriguez-Tudela *et al.* 2008), the modal MIC reported in this study is slightly higher. For example, Pfaller and colleagues previously determined the modal itraconazole MIC for a set of 1221 wild type isolates obtained from over 60 medical centers worldwide to be 0.25 mg/L (Pfaller *et al.* 2011). Interestingly our modal itraconazole MIC comprises up to 76.7% of all agricultural and clinical isolates; which is noticeably higher than was reported by

Rodriguez-Tudela et al. 2008 (54.8%), Pfaller et al 2009 (41.3%) and Toyotome et al. 2016 (65%). In light of the overall observed reduced itraconazole susceptibility, there is a need for close monitoring in hospitals and on farms that use azole fungicides in Hamilton as a strong selection pressure could drive some of these isolates to become resistant to clinical triazoles drugs. Despite following CLSI guidelines and using multiple controls, authors acknowledge that lab-to-lab variability in susceptibility testing is a potential limitation to the above inference.

Different from most previous studies where abundant evidence for clonality was often found, the Hamiltonian samples showed notable evidence of recombination. For instance, significant numbers of both mating types were found in all 3 ecological populations. While recombination is known to allow for faster adaptation to stressful environments, significant amounts of gene flow can easily disseminate resistance mechanism in the advent of an onset of triazole-resistance on farms. We thus call on necessary public health stakeholders such as Public Health Ontario to monitor *A. fumigatus* populations in Hamilton and other similar jurisdictions.

5.6.4 Epidemiology

According to statistic Canada, only 23.7% of Hamilton's population is 60 years and over. However, this age group was disproportionately represented among patients surveyed in this study (59%), suggesting age as a possible risk factor for the acquisition of aspergillosis in Hamilton, Ontario. However, given the objectives of this study, researchers did not include a control group and hence could not further test this hypothesis. Further epidemiological surveys would be necessary to ascertain whether age among other factors should be considered when developing prevention measures against aspergillosis in Hamilton. Evidence of multiple carriages was found in 82% (9/11) of patients from who two or more isolates were obtained. This result is not unique to this study, 75% (9/12) of studied patients from three European hospital were also shown to carry at least 2 genotype (Bertout *et al.* 2001). Multiple carriages are of public health and clinical significance for two main reasons. First, multiple carriages can complicate antifungal therapy leading to treatment failure or disease persistence, as both triazole susceptible and resistant *A.fumigtaus* genotypes can be isolated from the same patient (Mortensen *et al.* 2011). The cohabitation of both

triazole susceptible and resistant genotypes can lead to transfer and spread of resistance alleles from resistant to susceptible strains within and even between host microenvironments. Second, in the event of aspergillosis outbreaks, tracking infections to infection sources can be troublesome as *A.fumigatus* genotypes can vary greatly within body sites.

5.7 Conclusion

This study, which aimed to establish the link between agricultural triazole fungicide use and the acquisition of clinically important resistant strains, is the first to be conducted in Canada. Although our results suggest that agricultural azole fungicide use has not caused triazole resistance among clinical samples of *A. fumigatus* in Hamilton, we cannot exclude the possibility that other regions could have such a link. For example, increased usage of fungicides in Canada is highest in Manitoba and Saskatchewan (Table 5.4). Such a greater selective pressure could have facilitated the emergence and spread of triazole-resistant strains. Investigating the fungicide driven hypothesis in those provinces, with a focus on the local areas with the highest triazole fungicide use, could prove vital to public health scientists and policy makers in Canada.

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Chapter 6: Evidence of unique genetic diversity in *Aspergillus fumigatus* isolates from Cameroon

6.1 Preface

Despite current knowledge and advances in characterizing global populations of *A. fumigatus,* the fine-scale population genetics of *A. fumigatus* in Africa is still unknown. Here, we obtained and analyzed *A. fumigatus* from Cameroon, West Africa. Our results suggest that the Cameroonian population is different from populations around the world. Our findings could benefit the development of geography-specific diagnostic markers. Authors of this article are Eta Ashu, Gregory Korfanty, and Jianping Xu. This study was conceived by EA and JX. Genotyping and susceptibility testing was performed by EA and GK. This study is in press, and will be be published by Mycoses. References in this chapter appear as they are in the original manuscript.

6.2 Abstract

Aspergillus fumigatus is a saprophytic fungus that can cause lethal invasive aspergillosis in immunocompromised patients. Recent studies have shown that Eurasian and North American populations of *A. fumigatus* often consist of genetically diverse strains. However, very little is known about African populations of *A. fumigatus.* The objective of this study was therefore to characterize the diversity, reproductive structure and triazole susceptibility patterns of *A. fumigatus* in Cameroon, West Africa. A total of 495 soil samples were obtained from 9 different collection sites in three regions of Cameroon. Nine highly polymorphic microsatellite markers were used to genotype all 51 identified *A. fumigatus* isolates. *In vitro* susceptibility to itraconazole and voriconazole was tested by means of micro broth dilution.The nine microsatellite loci resolved all 51 Cameroonian *A. fumigatus* isolates into 45 genotypes. Consistent with recombination, 32 of all 36 possible pairwise loci combinations in Cameroon are phylogenetically incompatible. Interestingly, evidence for geographic sub-structuring was found within Cameroon and the sub-population with the most evidence of recombination was also the least susceptible sub-population to the triazole antifungals tested. Furthermore, we found that the Cameroonian collection was significantly differentiated from those in Eurasia and North America. Overall, our results indicate the genetic uniqueness of Cameroonian *A. fumigatus*

populations and that additional novel genetic diversity likely exist in other parts of Africa.

6.3 Introduction

Aspergillus fumigatus is a ubiquitous saprophytic fungus capable of infecting humans and livestock. Largely due to its highly mobile and resilient asexual spores, *A. fumigatus* can travel long distances and propagate in a wide array of environmental conditions. Hundreds of these spores are inhaled daily by humans and can cause lethal aspergillosis in the absence of adequate immune responses.[1] Being one of the most prominent human fungal pathogens, *A. fumigatus* infections can cause mortalities as high as 90% in high-risk groups. High-risk groups include those who suffer from leukemia, have undergone organ transplants and are on immunosuppressants, as well as patients infected with the human immunodeficiency virus.[2] Aside from asexual reproduction and propagation by conidia, *A. fumigatus* can also reproduce sexually. In *A. fumigatus*, mating between isolates of *MAT1-1* and *MAT1-2* mating types is followed by meiosis and ascospore formation.[3] The mating type locus in *A. fumigatus* has shown to contribute to pathogenesis, with *MAT1-1* strains being more invasive and virulent than *MAT1-2* strains.[4,5]. In addition to virulence, understanding the pervasiveness of sexual reproduction in fungal populations is of notable importance to public health scientists, as the persistence of fungal pathogens in human populations, including those resistant to antifungal drugs, can be facilitated by increased recombination and genetic diversity.[6]

Previous studies have shown that geographic populations of *A. fumigatus* often consist of genetically diverse strains.[7,8] For instance, Klaassen and colleagues found up to 5 genetically differentiated *A. fumigatus* clusters in the Netherlands.[7] A recent study by Ashu and colleagues extended the aforementioned observation by showing that *A. fumigatus* populations in Belgium, France, Germany, India, Norway, Spain, Switzerland and United States also contained genetically divergent strains and populations.[9] However, at present, very little is known about the genetic diversity of African populations of *A. fumigatus*. Elucidating the genetic diversity in African populations of *A. fumigatus* might be of critical importance given the high number of AIDS-related immunocompromised patients, and that several human pathogens have

been previously suggested to have evolved out of Africa.[10-12] Interestingly, West African isolates of *Aspergillus flavus*, a species closely related to *A. fumigatus*, are known to be geographically and physiologically divergent from those in North America.[13] Whether a similar difference exists between the West African *A. fumigatus* population and those from outside of Africa is unknown. Characterizing the genetic diversity of *A. fumigatus* in Africa could further our understanding of the global evolution of *A. fumigatus* and benefit the development of geography-specific diagnostic markers.

Aside from genetic diversity, understanding the origins and dissemination of triazole resistant *A. fumigatus* has also become a major research and public health interest. Targeting the enzyme lanosterol 14 α-demethylase (CYP51A), triazoles are routinely used in the treatment of aspergillosis. However, increased frequency in triazole resistance is a growing concern as rapid emergences of triazole resistant *A. fumigatus* have been reported across many parts of the world.[14] In addition, it has been previously hypothesized that recent increases in triazole resistance in immunocompromised patients have environmental origins.[15] In April of 2014, the first evidence of environmental triazole resistance in Africa was reported in Tanzania, where up to 20% of soil samples from the surroundings of Kilimanjaro Christian Medical Centre (KCMC) harbored resistant *A. fumigatus*. These resistant isolates had the same resistance mechanism as the dominant resistance mechanism previously found in Dutch and Indian isolates.[16] In September 2016, the same mechanism of resistance was reported in isolates obtained from otitis media patients in Bugando Medical Center, Tanzania. [17] These findings underline the need for continuous surveillance as resistant *A. fumigatus* can be disseminated through long-distance dispersal events.

In this study, we isolated *A. fumigatus* isolates from soil samples in Cameroon and obtained their genotypes and triazole susceptibility profiles. Our objectives were to characterize for the first time the diversity, mode of reproduction, and triazole susceptibility patterns of *A. fumigatus* in the Cameroonian environment. Given the mobile nature of asexual *A. fumigatus* spores and the finding of triazole resistant strains elsewhere in Africa and outside of Africa, we expected that triazole resistance might be present in the Cameroonian environment. In addition, since *A. fumigatus*

samples from most other geographic areas showed evidence for abundant gene flow at local, regional and continental scales [7-9], we also hypothesized that evidence for gene flow would be detected among local samples within Cameroon and between the Cameroonian samples and other geographic populations of *A. fumigatus*. Within Cameroon, the sampled regions were located relatively close to each other, closer than most of the reported regional samples from elsewhere that showed no genetic differentiation.

6.4 Materials and methods

6.4.1 Sampling and isolation of *A. fumigatus*

In total, 495 soil samples were obtained from 9 different collection sites in 3 Cameroonian regions (Figure 6.1). For each site, soil samples (~1 gram each) were obtained ~ 5 meters apart from each other in all four major cardinal directions. In the Northwest Region, 49 soil samples were obtained from Babanki, 50 from Bambui and, 51 each from Mbingo and Njinikejem. Forty-seven soil samples were obtained from Makepe in the Littoral Region. In the Centre Region, 99 soil samples were obtained from Eloundem, 51 from Mbalgong, 52 from Simbock, and 45 from Mbandoumou. All soil samples were obtained from farmlands with unknown triazole fungicide application histories.

In order to isolate *A. fumigatus* from the soil samples, 0.5g of soil was incubated at 50°C in 1mL of Sabouraud Dextrose Broth supplemented with chloramphenicol (50mg/L) for 2 days. Mycelium from tubes showing characteristic mycelial growth was sub-cultured on to Sabouraud Dextrose agar (SDA) and grown for two days at 50°C. *A. fumigatus* was then identified macroscopically by its characteristic suede-like greenish hydrophobic colonies and microscopically by its columnar vesicle and uniseriate conidial head. Molecular identification was done by means of 9 highly polymorphic microsatellite markers which can differentiate *A. fumigatus* from 11 other *Aspergillus* species.[18] The same set of markers was used for strain genotyping and the differentiation of strains.

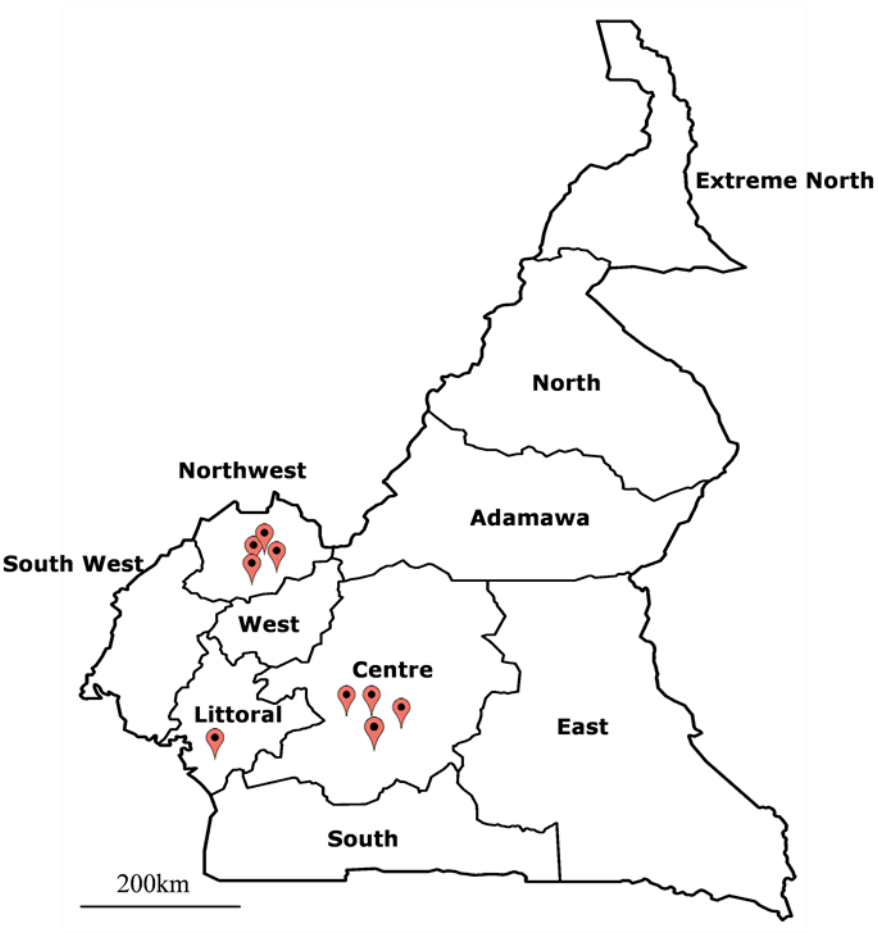

Figure 6.1. Regions and locations from which *A. fumigatus* isolates were obtained in Cameroon.

6.4.2 Genotypic and population genetic analyses

Genotyping was carried out using 9 highly polymorphic microsatellite markers as previously described.[9,18] In order to determine the levels of genetic diversity and geographic differentiation, Simpson's unbiased allelic (or genotypic) diversity Index (uh) and analysis of molecular variance (AMOVA) were estimated using GENALEX (version 6.5). Uh is a measure of allelic (or genotypic) diversity quantified on a scale of 0-1. An uh value of 0 means no variation where all strains have the same allele at the specific locus or all strains have the same multilocus genotype. In contrast, an uh value of 1 means high genetic variation where every strain has a different allele at a specific locus or each strain has a different multilocus genotype. The index of association (I_A) as implemented by MULTILOCUS (version 1.3b) was used to assess

linkage disequilibrium.[19] The I_A quantifies the extent of linkage disequilibrium within a population by comparing the observed association between alleles at different loci with that under the assumption of random mating and recombination. Here, statistical significance of the test was determined by 1000 permutations. STRUCTURE software (Stanford, California, USA) version 2.3 was used for model-based Bayesian clustering of the Cameroonian sample and those previously obtained from Eurasia, Australia, North and South America.[9] Admixture and the correlated allele options were chosen for ancestry and allele frequency models. Simulations were run for 1 to 14 populations (K). The MCMC sampling scheme was run for only 6,000 iterations with a burn-in period of 1,000 due to our huge data set (1275 genotypes). Nonetheless, our run length was validated by the fact that summary statistics values (Alpha, Fst, and likelihood) seemed to have converged. The average log probability [LnP (D)] of each K value and the rate of change in the log probability of data between successive runs of K were used to identify the optimal number of clusters.[20,21] Discriminant Analysis of Principal Components (DAPC) as implemented by ADEGENET package in R (Vienna, Austria) version 3.0 was used to further examine the existence of distinct genetic sub-clusters in Cameroon. The Bayesian Information Criterion (BIC) was used to determine the optimal number of genetic clusters as previously described. [22]

6.4.3 Mating type Identification

In order to determine the mating type of each isolate and help characterize the mode of reproduction in the Cameroonian population of *A. fumigatus*, we used a mating type-specific PCR. Each PCR reaction had a total volume of 11 µl containing 5 µl of 2x GoTaq Master Mix (Promega:Markham, Canada), 1µl of diluted template genomic DNA, 0.32 µl (0.29 µM) each of AFM1 (5′-CCTTGACGCGATGGGGTGG-3′) and AFM2 (5′-CGCTCCTCATCAGAACAACTCG-3′) forward primers, 0.64 µl (0.58 µM) of the shared reverse primer AFM3 (5′-CGGAAATCTGATGTCGCCACG-3′), and 3.72µl of autoclaved ddH_2O. The PCR protocol ran with an initial denaturation step of 95^{o}C for 5 min, followed by 35 cycles of 95^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 1 min, and concluded with a final elongation step at 72^{o}C for 5 min. PCR products were run on 1% agarose gel in Tris - Acetic Acid – EDTA buffer

at 100V for 1.5 hours. The gel was then stained and visualized as previously described.[8]

6.4.4 Azole susceptibility testing

The *in vitro* susceptibility of all 51 Cameroonian *A. fumigatus* isolates to itraconazole and voriconazole (Selleckchem, San Francisco) was determined following the Clinical and Laboratory Standards Institute's (CLSI) M38-A reference method.[23] Briefly, spore solutions were adjusted to an optical density between 0.09 and 0.13 at 530nm. For both itraconazole and voriconazole, susceptibility of all isolates were tested at concentrations of 0.03 to 16 mg/L. In-house strains (accession numbers, KU575343 and KU575805) with known MICs to these drugs were used as controls. Microdilution trays were then incubated at 35^{o}C without agitation. End points were visually determined after 48 hours.

6.4.5 Statistical analysis

The Fisher's exact test (two-sided) was used to determine whether geographic samples of *A. fumigatus* from Cameroon were different in their mating type distributions. Similarly, the Wilcoxon rank sum test was used to elucidate differences in triazole susceptibility between geographic samples.

6.5 Results

6.5.1 Isolation rates of *A. fumigatus*

Of all 495 soil samples, 1(1/47), 7(7/201) and 43(43/247) *A. fumigatus* isolates were obtained from the Littoral, North West and Central Regions, respectively. The only isolate obtained in the Littoral Region was from Makepe. Within the North West Region; 1, 2 and 4 isolates were obtained from Babanki, Mbingo, and Bambui, respectively. In the Centre Region; 3, 11, 14 and 15 isolates were obtained from Mbandoumou, Simbock, Eloundem and Mbalgong, respectively (Table 6.1). The rate of *A. fumigatus* isolation from soil samples from the Center Region was statistically higher than that from the North West Region (P value= < 0.001). The Littoral Region

was excluded from the test of significance due the fact that only one *A. fumigatus* isolate was obtained from that region.

Table 6.1. Detailed microsatellite genotype information of the 51 Cameroonian isolates of *A. fumigatus* obtained and analyzed in this study. This table details the source of the isolates collected as well as respective alleles obtained at loci 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B and 4C

ID	Location	Province	2A	2B	2C	3A	3B	3C	4A	4B	4C
15	**Mbingo**	**North West**	5	24	7	25	7	31	3	3	4
44	**Mbingo**	**North West**	3	22	3	3	11	34	7	7	8
65	**Bambui**	**North West**	9	28	11	20	13	38	8	9	10
67	**Bambui**	**North West**	34	53	37	26	19	42	3	9	4
79	**Bambui**	**North West**	11	30	3	1	9	32	8	8	8
82	**Bambui**	**North West**	13	32	3	22	20	43	7	8	7
158	**Makepe**	**Litoral**	14	33	8	6	12	36	6	6	10
205	**Babanki**	**North West**	5	24	7	25	9	32	2	2	4
298	**Eloundem**	**Centre**	5	24	7	25	18	32	2	2	4
304	**Eloundem**	**Centre**	8	27	10	35	7	30	3	3	4
307	**Eloundem**	**Centre**	7	26	9	32	7	30	7	7	11
308	**Eloundem**	**Centre**	8	27	10	14	11	34	4	3	4
320	**Eloundem**	**Centre**	5	24	7	18	8	30	3	3	4
321	**Eloundem**	**Centre**	5	24	7	26	8	30	2	2	4
322	**Eloundem**	**Centre**	5	24	7	15	8	31	3	3	4
326	**Eloundem**	**Centre**	10	29	11	16	8	44	3	3	4
327	**Eloundem**	**Centre**	7	28	9	23	21	43	3	3	4
333	**Eloundem**	**Centre**	8	27	10	11	3	36	3	3	4
338	**Eloundem**	**Centre**	5	24	7	26	12	36	3	3	4
340	**Eloundem**	**Centre**	6	25	8	11	3	31	3	3	4
341	**Eloundem**	**Centre**	8	27	10	11	7	31	3	3	4
343	**Eloundem**	**Centre**	5	11	7	25	7	30	3	3	3
350	**Mbalgong**	**Centre**	9	28	10	4	11	34	6	6	10
351	**Mbalgong**	**Centre**	9	28	11	6	11	34	6	6	10

352	**Mbalgong**	**Centre**	9	27	11	6	11	34	6	6	10
353	**Mbalgong**	**Centre**	8	27	11	6	11	34	6	6	10
355	**Mbalgong**	**Centre**	6	25	8	16	7	30	3	3	4
359	**Mbalgong**	**Centre**	9	28	11	6	11	34	6	6	10
362	**Mbalgong**	**Centre**	9	27	11	6	11	34	6	9	10
363	**Mbalgong**	**Centre**	9	28	11	6	11	34	6	6	10
370	**Mbalgong**	**Centre**	9	27	11	6	11	34	6	6	10
371	**Mbalgong**	**Centre**	8	27	2	6	11	34	6	6	10
372	**Mbalgong**	**Centre**	9	28	2	6	11	34	6	6	10
386	**Mbalgong**	**Centre**	9	28	3	19	11	34	6	6	10
390	**Mbalgong**	**Centre**	8	27	11	7	11	34	6	6	10
391	**Mbalgong**	**Centre**	9	28	10	6	10	34	6	6	10
396	**Mbalgong**	**Centre**	9	28	10	6	11	34	6	6	10
417	**Simbock**	**Centre**	9	28	2	6	11	34	6	6	10
419	**Simbock**	**Centre**	9	27	11	6	7	31	3	3	4
421	**Simbock**	**Centre**	5	24	6	24	21	44	4	3	4
422	**Simbock**	**Centre**	9	28	11	24	29	44	3	3	4
428	**Simbock**	**Centre**	5	24	7	14	6	50	3	3	4
429	**Simbock**	**Centre**	9	27	2	6	11	34	6	6	10
431	**Simbock**	**Centre**	9	28	12	6	11	34	6	6	10
435	**Simbock**	**Centre**	9	28	10	6	11	33	6	6	10
443	**Simbock**	**Centre**	9	28	11	6	11	33	6	6	10
446	**Simbock**	**Centre**	9	28	10	6	11	34	6	6	10
447	**Simbock**	**Centre**	9	28	11	6	11	34	6	6	10
471	**Mbandoumou**	**Centre**	5	24	7	25	8	31	2	2	4
473	**Mbandoumou**	**Centre**	5	24	7	16	21	44	6	3	4
480	**Mbandoumou**	**Centre**	9	28	11	20	15	38	8	9	10

6.5.2 Patterns of genetic diversity

We found notable allelic and genotypic diversities in the Cameroonian sample of *A. fumigatus*. The nine microsatellite loci resolved the 51 *A. fumigatus* isolates into 45 genotypes (uh=0.74). A total of 96 alleles were found at the nine loci, with an average of over 10 alleles per locus. Among the nine loci, the most polymorphic was 3A (15 alleles, uh= 0.834), and the lowest being 4C (6 alleles, uh= 0.602). Saturation analyses revealed all markers used in this study were informative of *A. fumigatus* diversity in Cameroon, as a minimum of 9 loci were required to resolve all 51 isolates into 45 genotypes. Of the 96 alleles, 55 were shared by at least 2 isolates from Cameroon while the remaining 41 alleles were found in only one strain each. Overall, linkage disequilibrium analysis revealed evidence of clonal reproduction (I_A=3.25) and the null hypothesis of random recombination was rejected (P value < 0.001). Nonetheless, consistent with some recombination, we found evidence of phylogenetically incompatible pairs of loci (compatibility index=0.11).

6.5.3 Differences between Cameroonian samples and those in Eurasia and North America

The Cameroonian samples were compared with those reported recently from other geographic regions and genetic populations.[9] Our comparisons suggested that the Cameroonian samples of *A. fumigatus* are genetically different from other regions. Firstly, about 21% (20/96) of all alleles at the nine microsatellite loci in Cameroon are so far only found in Cameroon (these are called private alleles for the Cameroonian sample). Among these 20, 5 (25%) occurred with a frequency of greater than 10% in Cameroon (Table 6.2). In contrast, aside from the Netherlands that had a private allele frequency of ~16%, the frequencies of private alleles in all other geographic populations were less than 3% [9]. Secondly, our pairwise population genetic comparisons suggested significant differentiations between the Cameroon samples and those from other previously sampled geographic populations (Table 6.3), with the most prominent differentiation identified between Cameroon and India (PhiPT =0.375, P=0.001; PhiPT refers to pairwise population heterogeneity index of the proportion of total genetic variance). Such large differentiations were still found even

after clone correction of both the Cameroonian sample and those recently analyzed from outside of Cameroon (Table 6.4). Here, in the clone-corrected analyses, each individual multilocus genotype was represented by only one isolate in each geographic population. Lastly, except for four isolates that were assigned to two existing genetic populations dominated by strains from other geographic regions, STRUCTURE clustered the remaining 47 Cameroonian isolates into a single genetic population that consisted of isolates exclusively from Cameroon. The optimal predicted number of populations (K) for all 1,275 isolates was nine, one more than those found recently for the analyzed global samples [9].

Table 6.2. List of private alleles from Cameroon. Alleles from Cameroon when compared with 9 other geographic *A. fumigatus* populations. Alleles that occur at frequencies larger than 10% are starred.

Locus	N° of alleles in all 10 populations	N° in alleles in Cameroon	Unique alleles in Cameroon	Frequency of unique alleles in Cameroon
2A	26	11	3, 5*, 6, 7 and 8*	0.020, 0.235, 0.039, 0.039 and 0.137
2B	26	12	32, 33 and 53	0.020, 0.020 and 0.020
2C	36	10	2 and 6	0.078 and 0.020
3A	84	19	1, 3, 4 ,6* and 7	0.020, 0.020, 0.020, 0.392 and 0.020
3B	41	15	None	
3C	49	11	None	
4A	27	6	3* and 4	0.314 and 0.039
4B	28	6	3	0.078
4C	35	6	3 and 4*	0.020 and 0.431

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Table 6.3. Pairwise differentiations between the Cameroonian isolates and 9 other geographic populations of *A. fumigatus.* PhiPT values are in the lower diagonal of the pairwise comparison table. Probabilities based on 999 permutations are shown above the diagonal.

Belgium	France	Germany	India	Netherlands	Norway	Spain	Switzerland	USA	Cameroon	
	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	**Belgium**
0.035		0.001	0.001	0.001	0.001	0.001	0.002	0.001	0.001	**France**
0.058	0.049		0.001	0.001	0.001	0.001	0.001	0.001	0.001	**Germany**
0.225	0.183	0.273		0.001	0.001	0.001	0.001	0.001	0.001	**India**
0.021	0.015	0.034	0.180		0.001	0.001	0.023	0.001	0.001	**Netherlands**
0.051	0.063	0.083	0.230	0.032		0.001	0.001	0.001	0.001	**Norway**
0.034	0.031	0.041	0.208	0.023	0.050		0.001	0.001	0.001	**Spain**
0.021	0.017	0.036	0.218	0.005	0.033	0.012		0.001	0.001	**Switzerland**
0.060	0.053	0.054	0.257	0.044	0.074	0.037	0.035		0.001	**USA**
0.170	0.169	0.178	0.357	0.152	0.202	0.161	0.155	0.172		**Cameroon**

Table 6.4. Pairwise differentiations between clonally corrected geographic populations of *A. fumigatus.* PhiPT values are in the lower diagonal of the pairwise comparison table. Probabilities based on 999 permutations are shown above the diagonal.

Belgium	France	Germany	India	Netherlands	Norway	Spain	Switzerland	USA	Cameroon	
	0.161	0.001	0.001	0.171	0.001	0.009	0.396	0.001	0.001	**Belgium**
0.003		0.001	0.002	0.143	0.001	0.001	0.013	0.001	0.001	**France**
0.032	0.027		0.001	0.001	0.001	0.001	0.001	0.001	0.001	**Germany**
0.022	0.033	0.025		0.002	0.002	0.004	0.005	0.013	0.001	**India**
0.002	0.003	0.021	0.019		0.001	0.001	0.073	0.001	0.001	**Netherlands**
0.025	0.041	0.055	0.028	0.026		0.001	0.001	0.001	0.001	**Norway**
0.007	0.024	0.025	0.017	0.014	0.026		0.262	0.134	0.001	**Spain**
0.000	0.010	0.023	0.017	0.003	0.021	0.001		0.060	0.001	**Switzerland**
0.015	0.027	0.020	0.015	0.016	0.036	0.003	0.005		0.001	**USA**
0.136	0.151	0.152	0.146	0.132	0.174	0.136	0.137	0.134		**Cameroon**

6.5.4 Evidence of sub-structured populations

Within Cameroon, evidence for genetic differentiation among local populations was found. The largest differentiations between pairs of local populations were identified between Eloundem and two other locations. Specifically, 45% and 26% of total genetic variation between Eloundem and Mbalgong (P=0.001), and between Eloundem and Simbock (P=0.001) were respectively contributed by geographic separations. Due to their small sample sizes, local populations from Bambui, Babanki, Mbingo, Mbandoumou and Makepe were excluded from the AMOVA of sub-populations.

The genetic differentiations revealed by microsatellite markers were also supported by data on mating type alleles. Specifically, the mating type ratio between *MAT1-1 and MAT1-2* in Eloundem was approximately 1:1. In contrast, all 15 isolates from Mbalgong were *MAT1-1*, while all but one isolate from Simbock was *MAT1-1*. The mating type ratio in Eloundem was significantly different from those in both Mbalgong and Simbock (P values = 0.0007 and 0.033, respectively). We further tested whether the approximately equal mating type ratio in the Eloundem population had greater evidence for recombination than the Mbalgong and Simbock populations. Indeed, we found that the Eloundem sample had a lower I_A value than those from Mbalgong and Simbock (I_As = 1.2, 2.8 & 3.7, respectively). Of all 36 possible pairwise loci combinations, 6, 2 and 5 of those loci pairs in Eloundem, Mbalgong and Simbock are phylogenetically incompatible.

The existence of sub-structuring within the Cameroonian population of *A. fumigatus* was also supported by results from STRUCTURE and DAPC analyses. In general, the two analyses showed an overall consistent pattern (Fig 6.2). Interestingly, the large STRUCTURE cluster containing 47 Cameroonian isolates were further split into 3 tightly clustered DAPC sub-populations, while the remaining 4 variant strains belonging to two different STRUCTURE clusters were assigned to a single DAPC population (Fig 6.2B).

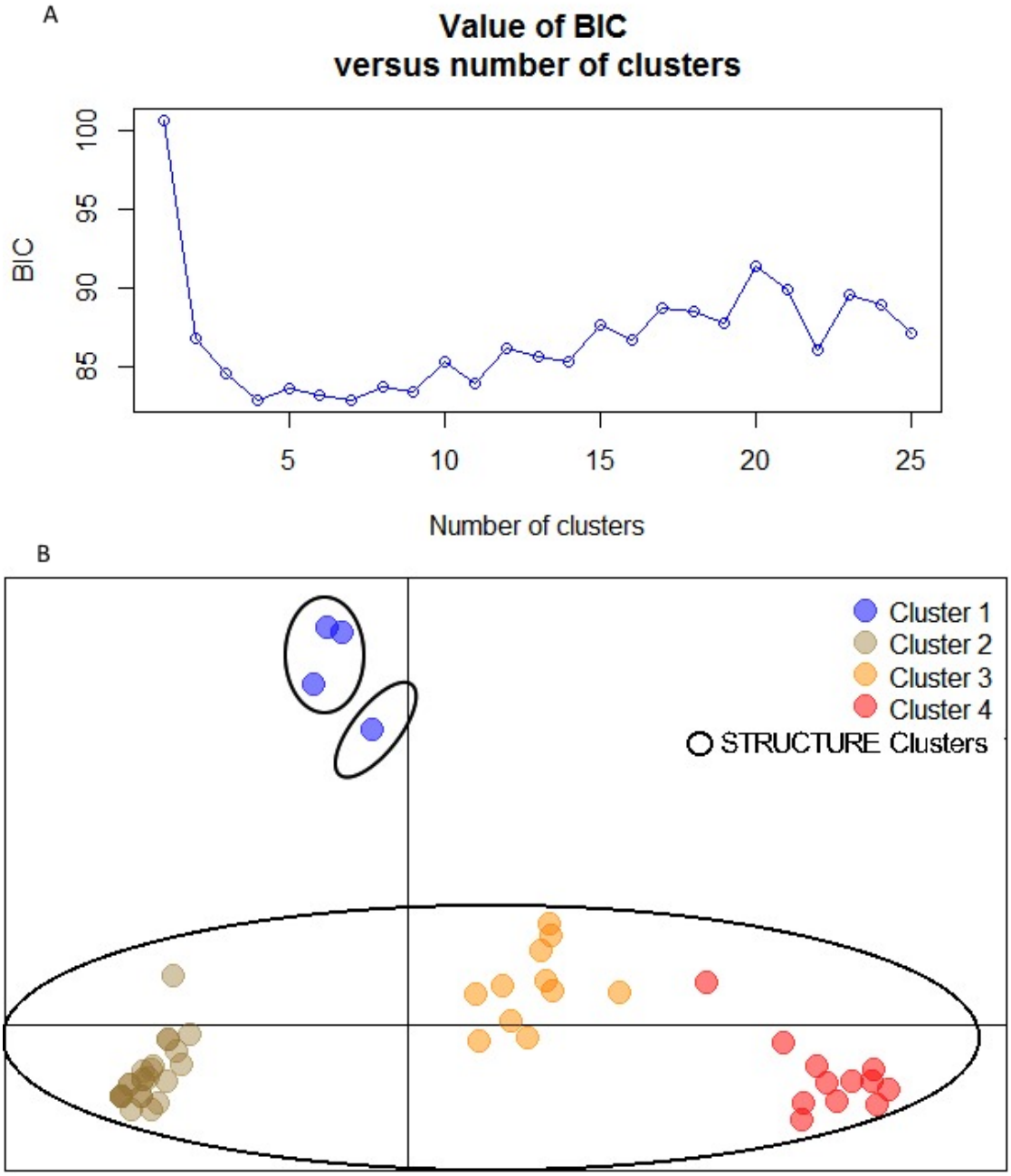

Figure 6.2. Estimated number of genetic clusters in the Cameroonian sample based on DAPC analysis. (A) Plot of the optimal number of clusters (K) versus the Bayesian information criterion (BIC). The optimal number of clusters predicted was four. (B) DAPC scatterplot. Each isolate is represented as a dot; thick black circles depict STRUCTURE inferred populations.

6.5.5 Azole susceptibility patterns

All 51 *A. fumigatus* isolates were susceptible to both itraconazole and voriconazole (Table 6.5). However, though no triazole - resistance was detected among the 51 strains from Cameroon, variations in MICs among isolates and local populations for both triazole drugs were found. The overall modal MIC for itraconazole and voriconazole were 1mg/l and 0.25mg/l, respectively. Interestingly, compared to the sample from Simbock, the Eloundem sample had a significantly higher MIC to itraconazole (P value = 0.019). Similarly, the Eloundem sample was significantly more tolerant to voriconazole than that from Mbalgong (P value = 0.033).

Table 6.5. In-vitro antifungal susceptibility profile of medical triazoles against environmental *A. fumigatus* isolates from Cameroon

Antifungal	Location	Drug concentration (mg/l)			
		0.125	0.25	0.5	1
Itraconazole	Mbingo	0	1	0	1
	Bambui	3	1	0	0
	Makepe	0	0	0	1
	Babanki	0	0	0	1
	Eloundem	0	5	0	9
	Mbalgong	3	2	2	8
	Simbock	7	1	0	3
	Mbandoumou	0	2	0	1
Total		13	12	2	24
Voriconazole	Mbingo	0	1	1	0
	Bambui	0	4	0	0
	Makepe	0	1	0	0
	Babanki	0	0	1	0
	Eloundem	0	8	6	0
	Mbalgong	1	14	0	0
	Simbock	0	11	0	0
	Mbandoumou	0	3	0	0
Total		1	42	8	0

6.6 Discussion

This study provided the first glimpse of a West African population sample of *A. fumigatus*. Our analyses identified significant novel allelic and genotypic diversities in that region. Interestingly, unlike the low genetic differentiation among geographic populations of *A. fumigatus* from other geographic regions from Eurasia and North America [7-9], the Cameroonian sample of *A. fumigatus* was significantly differentiated from regional populations analyzed so far from other parts of the world. Whilst our analyses revealed abundant evidence of clonal reproduction, we identified a notably diverse sub-population in Eloundem that was differentiated from other local populations within Cameroon and this sub-population had signatures of recombination. Below we discuss the relevance of our results and its implications to the global epidemiology of *A. fumigatus*.

One of the most notable results obtained here was that the Cameroonian population of *A. fumigatus* was significantly differentiated from previously reported populations in Eurasia and North America. While evidence of geographic differentiation has been previously demonstrated in *Aspergillus* and in other fungal species[9][24,25], the extent of divergence of the Cameroonian population from other populations of *A. fumigatus*, by comparison, was unexpected in this species. Interestingly, similar differences have been shown between West African and North American communities of *Aspergillus flavus.*[13] A potential future research avenue will be to investigate whether the Cameroonian sample of *A. fumigatus* is physiologically divergent from those in Eurasia and North America. Generally speaking, a genetically differentiated population can be due to multiple factors including natural selection, genetic drift and mating patterns. For instance, the Indian population of *A. fumigatus* was significantly differentiated from other geographic populations largely due to the selective sweep of a multiple triazole-resistant microsatellite genotype across India.[8][26,27] While assumed to be neutral[7], the microsatellite loci used in this study, which are identical to those used in the Indian study, might be linked to genes under selective constraints. As a result, through hitchhiking, selection could indirectly cause differential survival of different microsatellite genotypes and lead to population subdivision. However, what the

selected gene(s) might be and how tightly they might be linked to the analyzed microsatellite loci remain to be determined.

Interestingly, at seven of the nine assayed microsatellite loci (the exceptions were loci 2B and 3C), the alleles from Cameroon were generally shorter (i.e. having fewer tandem repeats) than those previously reported in other world regions. For example, ~ 88% (45/51) of all alleles at locus 4A in Cameroon have ≤ 6 repeats, much shorter than those reported elsewhere. Even at a highly polymorphic locus such as 3A, where up to 113 repeats have been previously reported in other geographic populations [9], about half of the alleles at this locus have ≤ 7 repeats in the Cameroonian sample. Similar patterns were also observed at the other five loci analyzed (Table 6.1). Overall, 85% (17/20) of all private alleles (i.e. alleles uniquely found in a given population) in Cameroon have fewer tandem repeats than those reported previously (Table 6.2). Assuming a stepwise mutational model, the large difference in microsatellite allele sizes suggest that the Cameroonian *A. fumigatus* samples may have diverged from other geographic populations for a long time. The long-time separation coupled with genetic drift would contribute to the observed differentiations. However, whether the stepwise mutational model could be applied here requires further investigation.

Mating patterns can also influence the genetic relationships among populations. Although sexual recombination can be beneficial in that it increases genetic variation, clonal reproduction is often observed in natural populations of *A. fumigatus*. Interestingly, in this study, clonally correcting geographic populations of *A. fumigatus* led to only slight decreases in pairwise differentiations between the Cameroonian population and those from elsewhere, suggesting that clonality accounts for relatively little of the observed differentiation between the Cameroonian *A. fumigatus* population and those from other countries. Since none of the analyzed geographic populations were similar to the Cameroonian sample, biased gene flow was unlikely the reason why the Cameroonian sample in this study was so distinct (Table 4). Lastly, the observed differentiation between the Cameroon and non-Cameroon *A. fumigatus* populations could be simply due to sampling effects. For example, the sample sizes of most geographic populations analyzed so far are relatively small compared to their true population sizes in nature.

STRUCTURE analyses assigned all 1275 global *A. fumigatus* multilocus genotypes into nine genetic populations. Forty-one of all 45 genotypes in Cameroon were assigned to the same genetic population, which so far included isolates only from Cameroon. Although adding the Cameroonian sample to previously described global samples resulted in the reassignment of some isolates to different genetic populations [9], the number of predicted genetic clusters outside of Cameroon did not change. Similarly, results reported here and those previously described suggest that diversity levels and proportions of genetic populations could differ among geographic regions.[9] Broadly speaking, the geographic center of origin of any genetic cluster should show the highest diversity levels. However, due to no and under-sampling in many countries and the limits in currently used clustering algorithms (multivariate and Bayesian model-based), predicting the exact number of *A. fumigatus* genetic clusters globally and the geographic origins of these clusters remains a challenge. Nonetheless, the existence of a unique genetic population in Cameroon underlines the importance of characterizing *A. fumigatus* populations in un-sampled and under-sampled geographic regions such as Africa and the Middle East.

A major epidemiological relevance of the unique diversity observed for the Cameroonian sample is that it can be used as a geographic identifier. Generally speaking, for an allele to be used as an identifier for a geographic population, it needs to be unique, stable and very frequent in that population. Here, albeit having similar diversity levels as those from around the world, the Cameroonian population of *A. fumigatus* had the highest proportion of private alleles. With the exception of Netherlands (49/296), the proportion of private alleles (20/96) in Cameroon was at least 8 times more than any other geographic population we analyzed. A quarter of all Cameroonian private alleles were frequently found in the Cameroonian population of *A. fumigatus*. Except for one private allele (62 repeats at locus 3A) that was relatively frequent (18.5%) in the Belgian population, private alleles in other geographic populations all occurred at frequencies of less than 3%. Private alleles 6, 3 and 4 at loci 3A, 4A and 4C occurred at frequencies of up to ~43% in Cameroon (Table 6.1), suggesting these alleles could be used in identifying the geographic origin of *A. fumigatus* isolates to Cameroon. However, before such a test could be applied,

samples from other parts of Africa and the Middle East should be analyzed to determine the uniqueness of alleles found so far only in Cameroon. Indeed, the observed high proportion of private alleles in Cameroon could be due to the lack of sampling from Africa and the Middle East.

Contrary to what has been reported within India and Netherlands [7][26], our analyses showed evidence of geographic sub-structuring within Cameroon. At present, the reasons for this observation in Cameroon are not known. However, factors such as the mode of pathogen reproduction, physical barriers, climatic differences, and human activities among the local environments could all have contributed to the geographic sub-structuring. Interestingly, we found different regions showing different degrees of clonality and recombination. For example, overall, there were approximately ~3 times more *MAT1-1* than *MAT1-2* mating types. However, about half of all *MAT1-2* mating type strains were found at a single location — Eloundem. Eloundem had a balanced distribution of *MAT1-1* and *MAT1-2* mating types and was significantly differentiated from other sampled sites. Secondly, compared to more industrialized nations where environmental triazole resistance has been reported [28-30], Cameroon has a relatively low utilization of fungicides (Fig 6.3). However, a noteworthy increase in fungicide use since 2001 might have contributed to the overall reduced susceptibility (1mg/l) to itraconazole seen in some regions such as Eloundem in Cameroon. Indeed, it has been previously demonstrated that increased genetic variation by means of sexual recombination can lead to adaptation of hostile environments in fungi.[31,32] Thus, it's possible that the population in Eloundem might be under certain selective pressure (including triazole drugs), leading to more frequent sexual reproduction, the generation of diverse genotypes, and the spread of triazole-tolerant alleles and genotypes, making this population different from those in other regions in Cameroon. Although the mechanism of reduced itraconazole susceptibility in Cameroon is currently unknown, target overexpression, cellular stress response and multidrug transporter up-regulation are possibilities to be considered for future research.

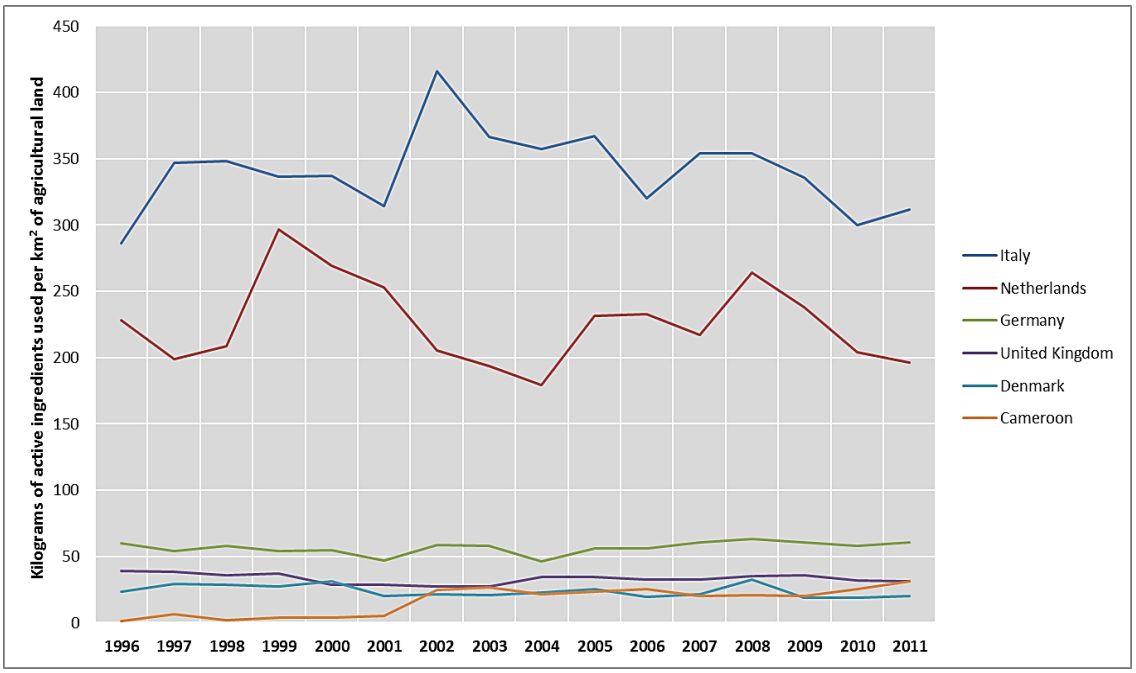

Figure 6.3. Plot showing trends in fungicides and bactericides use in Cameroon, Italy, Germany, Denmark, the Netherlands and the United Kingdom between 1996 and 2011. Data for the years 2012-2016 for all six countries is currently unavailable. Sources: Food and Agriculture Organization of the United Nations and World Bank.

Investigation of climatic classifications and *A. fumigatus* isolation rates suggested that *A. fumigatus* has increased isolation rates from tropical savanna climatic regions (Central Region) than from Monsoon climatic regions (Littoral and Northwest Regions). Climatic data suggest a moderate variation in temperature and precipitation between the two biomes. However, due to its non-fastidious growth requirements, *A. fumigatus* is able to grow in a broad range of ecological niches.[33] Difference in climatic regions is therefore unlikely the cause of the lower isolation rates in the Littoral and Northwest Regions. However, it is plausible that *A. fumigatus* abundance might have been indirectly influenced by the climate. For instance, allium plants have been shown to possess fungicidal activities against *A. niger*, *A. flavus* and *A. fumigatus.* The inhibitory effect of these plants against these *Aspergillus* species was negatively correlated with temperature.[34] This effect could indirectly contribute to increased abundance of *A. fumigatus* in the Central Region where the temperature is higher. Another possibility is the potential differences in soil microbiota among various geographic regions. For example, antagonistic relationships between *A. fumigatus* and certain soil microorganisms might have been more prevalent in the

Littoral and Northwest Regions than in the Central Region. We are currently investigating the differences in overall fungal diversity in soil samples from the three regions in Cameroon to test this possibility.

In conclusion, our analyses identified that soil samples from different regions differed in their *A. fumigatus* abundance. Contrary to previous findings in other geographic regions, we report evidence of geographic sub-structuring of *A. fumigatus* samples in Cameroon. We identified a sub-population with abundant evidence for sexual reproduction and decreased susceptibility to the antifungals tested. Overall, the Cameroonian population was differentiated from those in Eurasia and North America. The identification of a novel genetic cluster unique to Cameroon underlines the need to characterize *A. fumigatus* populations in un-sampled and under-sampled geographic regions. Taken together, our results suggest that there is likely vast and unknown diversity in Africa and that local biotic and abiotic factors can play an important role in shaping the evolution *A. fumigatus*.

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Chapter 7: Conclusion

Overall, my thesis expands current understanding on the population structure and molecular epidemiology of *A. fumigatus* at diverse scales. Below, I summarize some of my major findings and how they have advanced our current understanding of this constantly evolving human pathogenic fungus, and also make suggestions for future research.

7.1 Main findings

In chapter 2 we showed evidence of trade-offs between sexual and asexual reproduction during the initiation and expansion of most fungal outbreaks. In *A. fumigatus,* although sexual reproduction can lead to the initiation of aspergillosis outbreaks, most outbreaks are initiated due to significant changes to host immuno-competency and/or changes to the environment in which the host and the pathogen interact. Expanding on work previously done by Engering, Hogerwerf, & Slingenbergh (2013), we propose a framework that relates modes of reproduction to major events that can lead to the initiation and propagation of fungal disease outbreaks, including aspergillosis (Fig 2.1).

Next, we used over 2000 geographically and ecologically diverse *A. fumigatus* isolates to address various fundamental epidemiological and evolutionary questions. We showed that contemporary gene flow is likely the reason for limited but statistically significant genetic differentiation among geographic and ecological populations of *A. fumigatus*. Furthermore, our findings suggested that differences in triazole usage among countries have likely contributed to the differentiation among the triazole-resistant *A. fumigatus* geographic populations at the global scale. We extended the findings of Pringle et al. (2005) by identifying 8 genetically differentiated clusters, 7 of which are globally distributed. Similarly we found evidence of recombination in all analyzed genetic clusters, most especially in Pop #8, the dominant genetic cluster containing most of the triazole-resistant strains where no evidence of recombination was previously found (Klaassen, Gibbons, Fedorova, Meis, & Rokas, 2012).

Subsequently, we investigated whether MTR in strains carrying the TR34/L98H mutation in India originated only once. Our results suggested that MTR isolates with the TR34/L98H mutation originated multiple times in India. MTR

(TR34/L98H) in India likely evolved from single mutations in native susceptible genotypes, recombinant events, and/or exotic introductions. Our findings extended that of Chowdhary et al.(2012), who suggested that MTR resistance originated once from a cross between an exotically introduced azole-resistant strain and a native azole-susceptible strain, followed by a mutation.

The main finding from the study in chapter 5 of my thesis was that there is limited evidence showing that azole fungicides caused azole resistance in the clinical *A. fumigatus* population in Hamilton. However, we found significant evidence for decreased susceptibility to triazole drugs in both clinical and agricultural *A. fumigatus* population in Hamilton. This study is the first to be conducted in Canada and highlights the need to monitor *A. fumigatus* populations in other regions of Canada.

Lastly, we reported that soil samples from different Cameroonian regions differed in their *A. fumigatus* abundance. Contrary to previous studies (Klaassen et al., 2012; Chowdhary et al., 2012), we report evidence of geographic sub-structuring of the Cameroonian *A. fumigatus* population. This study is the first snapshot of an African *A. fumigatus* population. We found that the Cameroonian population is significantly differentiated from those in Eurasia and North America. Our results suggested that additional novel diversity likely exist in other unsampled Cameroonian regions and in Africa as a whole.

7.2 Suggestions for future research

Taken together our findings provide insights into the origin and spread of anti-fungal resistance, contributing toward developing effective long-term management strategies against aspergillosis outbreaks in Hamilton and the world at large. Furthermore, we provide important background data for future assessment of global migration patterns of *A. fumigatus.* However, despite the contributions made by my PhD research, many pertinent questions still remain unanswered.

In chapter 2, one important issue raised was that of cryptic sexual cycles in apparently asexual fungi. A potential future research avenue will be investigating whether these are indeed sexual cycles and what environmental and nutritional factors influence these putative cryptic sexual cycles. Comprehensive surveys of sex related

genes in these fungi will be useful in characterizing these cryptic sexual cycles. The discovery of sexual cycles in fungi thought to be strictly clonal can impact our understanding of the population biology of such species, enabling the development and implementation of better control measures.

In light of the development of better control measures, chapter 3 provides important background that can be used for the assessment of *A. fumigatus* gene flow patterns. We hypothesize a full island model. However, before such analyses can be carried out *A. fumigatus* populations in unsampled and under-sampled world regions such as Africa, Australia and South America need to be characterized.

Bearing in mind that MTR likely originated multiple times in India, future research should be aimed at drug discovery. Also, the use of triazole alternatives in both agriculture and clinical practice could reduce the selective pressure of triazole drugs on the Indian *A. fumigatus* population, eventually improving the potency of clinical triazoles.

Although our results suggest that agricultural triazole fungicides have not caused triazole resistance in the clinical *A. fumigatus* population in Hamilton, increased usage of fungicides in Canada is highest in Manitoba and Saskatchewan (Table 5.4). Investigating the fungicide-driven resistance hypothesis in those two provinces may prove vital to public health scientists and policymakers in Canada. Furthermore, identifying which regions within these provinces have the highest fungicide use could prove to be quite useful as the fungicide-driven resistance hypothesis is geography-specific.

Lastly, investigating the reason for the regional differences in *A. fumigatus* isolation in Cameroon could lead to the potential discovery of soil microorganisms with antagonistic properties against *A. fumigatus.* Also, another important research avenue will be to investigate whether the Cameroon population is physiologically different from populations around the world, most especially as pertains to virulence, pathogenicity, transmissibility etc.

7.3 References

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