Genetic and environmental influences on the germination of basidiospores in the *Cryptococcus neoformans* species complex

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Lay Abstract

In basidiomycetous fungi, the viability of gametes is an important component of sexual fitness and can have implications for speciation events. The *Cryptococcus neoformans* species complex are a group of opportunistic pathogens, for which hybridization can be facilitated readily under laboratory conditions, creating offspring that are generally completely inviable or have low germination potential. Antagonistic genetic interactions are mostly responsible for offspring inviability, yet the impacts of environmental factors is not known. Multiple genetic crosses between *Cryptococcus* strains were used as a model to investigate species relationships by examining the impacts of genetic and environmental factors on offspring germination potential.

Abstract

In basidiomycetous fungi, the viability of gametes is an important component of sexual fitness and can have implications for speciation events. Prior estimates of basidiospore germination are highly variable and the occurrence of reproduction between these lineages suggests that reproductive isolation is incomplete. Genetic incompatibilities during meiosis have been attributed to much of the offspring inviability. However, the influence of environmental factors on basidiospore germination in *Cryptococcus* are not well known. In this study, we used human opportunistic yeast pathogens, *Cryptococcus neoformans* and *Cryptococcus deneoformans*, as models to investigate the potential effects of selected genetic and environmental factors on basidiospore germination. We evaluated basidiospore germination of six genetic crosses by pairing a total of five strains, three intraspecific crosses and three interspecific crosses, between *C. neoformans* and *C. deneoformans*. The offspring of genetic crosses were incubated under multiple media and temperature treatments and scored for their germination ability. In general, spores from intraspecific crosses had greater germination potential than those from interspecific crosses. Growth under high temperatures was the most significant influence on basidiospore germination on these crosses. Furthermore, there were notable interaction effects between environmental factors and parental strains or strain pairs on basidiospore germination. The interaction between the sex-specific genes and environmental pressures impact reproductive barriers and blur species distinctions within the *Cryptococcus neoformans* species complex. And so, reduced viability of hybrid offspring can have implications for *Cryptococcus* speciation, ecology, and pathogenesis as hybridization events are an effective method of increasing pathogenicity, expanding species distributions and increasing tolerance to novel environments or hosts.

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Acronyms

BDM Bateson-Dobzhansky-Muller

CNSC *Cryptococcus neoformans* Species Complex

MAT Mating Type Locus

MM Minimal Medium

MYA Million Years Ago

UV Ultra-Violet

YEPD Yeast Extract Peptone Dextrose

Chapter 1

General Introduction

1.1 Introduction

The genus *Cryptococcus* was first conceptualized by Kützing in 1883 to incorporate multiple yeast species that were incapable of producing endospores (asexual spores inside of hyphae) (Casadevall and Perfect, 1998). The most widely studied of these species, *Cryptococcus neoformans*, was described by Busse in 1894, who isolated a culture from a patient. Although *Cryptococcus* is generally believed to be an opportunistic pathogen, affecting immunocompromised patients who suffer from disorders such as AIDS/HIV (Park et al., 2009), recent infections of seemingly healthy individuals have resulted in the expansion towards primary pathogen status (Hoang et al., 2004). The development of genetic tools and the ease with which cells are manipulated in the laboratory has lead to the widespread use of this species in experimental studies on the ecology and epidemiology of this pathogen (Lin and Heitman, 2006; Hull and Heitman, 2002).

Many species of *Cryptococcus* are facultative pathogens, causing diseases in both animals and humans while being prolific in a variety of habitats outside of their respective hosts. Early surveys established a preference for soils (Emmons, 1951) and pigeon excreta (Emmons, 1960) the prevalence of *C. neoformans* and *C. deneoformans* in samples of

decaying wood likely identifies a mutual primary niche for *Cryptococcus* (Lazéra et al., 2000). Most infections are thought to have originated from environmental reservoirs and spontaneous infections can occur in a variety of vertebrate hosts, including cats, dogs, dolphins, sheep, and bird species (Brewer and Wood, 1908; Casadevall and Perfect, 1998; Frothingham, 1902; Weidman and Ratliff, 1934). Further studies using both clinical samples and animals, as models for infection, established the pathogenic capabilities of *C. neoformans*. The isolation of environmental populations and application of these cultures *in vivo* demonstrated that *Cryptococcus* could cause infections in guinea pigs (Brewer and Wood, 1908). Many early studies of cryptococcal infections have increased awareness of *Cryptococcus* as a human pathogen (See Casadevall and Perfect (1998)).

Prior to 1981, cryptococcosis was a rare infection, with only ~100 cases being reported per year on average in the United States (Kaufman and Blumer, 1977). However, the medical importance of *C. neoformans* increased during the AIDS epidemic (Casadevall and Perfect, 1998). For many AIDS patients infected with cryptococcosis, the affliction is considered to be incurable and must be treated with constant application of anti-fungals (Mitchell and Perfect, 1995). These infections remain an extremely high risk for immunocompromised individuals, with case-fatality reaching 70% for cryptococcal meningitis in sub-Saharan African populations (Park et al., 2009), while recent outbreaks of cryptococcosis remain an epidemiological concern (Hoang et al., 2004).

1.1.1 *Cryptococcus neoformans* Species Complex

Many changes have been made to *Cryptococcus* nomenclature in a series of attempts to resolve this species complex. Preliminary morphological characterization incorrectly classified this species under a collection of different names, including *Saccharomyces neoformans*, *Cryptococcus hominis*, and European blastomycosis among others (Littman and Zimmerman, 1956. The confusion continued even with the implementation of new

techniques for differentiating *Cryptococcus* species. Specific antigens on the surface of the polysaccharide capsule have been used as one of the main characteristics to identify distinct species of *Cryptococcus*. Evans (1949) established this criteria by passing a solution containing cell wall components through rabbits. The antigenic characterization of *Cryptococcus* was further described by Benham (1956), leading to the establishment of the serotype groups A, B, and C. The discovery of an additional serotype, D, as well as individuals that had both A and D serotypes (AD), are the result of a study of environmental isolates by Wilson et al. (1968). Of the serotypes described above, all were placed under *C. neoformans*, with separate varietal status for serotypes A, D, and AD as *C. neoformans var. neoformans* and serotypes B, and C as *C. neoformans var. gattii* (Kwon-Chung et al., 1982). Yet, with the use of this method, species designations underwent several iterations during the rest of the 20th century.

The application of molecular marker information in the taxonomy of this species complex justified the separation of serotype A from serotype D into a distinct variety, *C. neoformans var. grubii* (Franzot et al., 1999). Distinct molecular groupings were then designated to all *C. neoformans* lineages through the application of genotyping: *C. neoformans var. neoformans* (VNIV), *C. neoformans* var. grubii (VNI/VNII), serotype AD (VNIII), *C. neoformans var. gattii* (VGI/VGII/VGIII/VGIV) (Ellis et al., 2000; Meyer et al., 1999. Based on these classifications, *C. neoformans var. gattii* split into its own species (Kwon-Chung et al., 2002. This was supported by additional investigations by Bovers et al. (2008), suggesting that serotype and molecular classifications were inconsistent. A recent reclassification has reverted *C. var. neoformans* and *C. var. grubii* back to their species status and assigned new species names (*C. deneoformans* and *C. neoformans*, respectively); in addition to these changes, three new species were established for the *gattii* lineage, culminating in seven separate species (Table 1.1) (Hagen et al., 2015).

TABLE 1.1: Strains of the *Cryptococcus neoformans* and *Cryptococcus gattii* species complex.

Variety/Strain	**Serotype**	**Molecular Type**	**MATa Strain**	**MATα Strain**	**References**
C. neoformans	A	VNI VNII	KN99a	KN99α CDC15α	Belay et al., 1996 Boekhout et al., 2001 Meyer et al., 2009 Meyer et al., 2011
C. deneoformans	D	VNIV	JEC20a NIH433	JEC21α	Boekhout et al., 2001 Bovers et al., 2008
C. gattii	B	VGI	NIH3233	WM163	Boekhout et al., 2001 Bovers et al., 2008 Hagen et al., 2010 Hagen et al., 2012 Meyer et al., 2003
C. bacilisporus	C/B	VGIII	Serotype C NIH191	Serotype B NIH444	Boekhout et al., 1997 Boekhout et al., 2001
C. deuterogattii	B	VGII	LA 55	CBS H-21968	Bovers et al., 2008 Hagen et al., 2010 Hagen et al., 2012 Kidd et al., 2004 Kidd et al., 2005
C. tetragattii	C	VGIV	-	CBS H-21969	Bovers et al., 2008 Hagen et al., 2010 Hagen et al., 2012
C. decagattii	B	VGIIIc/VGIV	CBS H-21970	CBS-6993	Hagen et al., 2010 Hagen et al., 2012

With their contemporary classification, serotypes A and D are representative of the species *C. neoformans* and *C. deneoformans*, respectively, these species form the *Cryptococcus neoformans* species complex (CNSC). Both species have been found to be capable of causing infection in humans (Dromer et al., 1996; Favalessa et al., 2014) and animal models (Cafarchia et al., 2006; Lazéra et al., 2011), and can be isolated from a wide range of natural habitats (Boekhout et al., 2001; Meyer et al., 2011). Both have widespread global distributions, with *C. deneoformans* being more prevalent in northern regions and in environmental samples than in clinical samples, commonly isolated from soil, bird guano, and on tree species (Lazéra et al., 1996; Levitz, 1991). *C. neoformans* isolates are responsible for a greater proportion of clinical infections and are generally associated with better growth *in vivo* (Lin et al., 2008). The collection of *gattii* species (Hagen et al., 2015) is mostly limited to tropical and subtropical environments (Kwon-Chung and Bennett, 1984).

The divergence between serotype A and D lineages occurred at least 1̃8MYA (Xu et al., 2002). However, hybridization between these species is still possible (Litvintseva et al., 2007), as demonstrated by the manipulation of a cross between two parents of A and D serotype, creating serotype AD offspring (Lengeler et al., 2001). However, hybrid basidiospores were frequently found to be aneuploid or diploid, had low germination (5-20%), or displayed abortive phenotypes (Lengeler et al., 2001; Vogan et al., 2013).

1.1.2 Sexual Life Cycle

Successive generations in *Cryptococcus* are created through asexual reproduction, in which haploid yeast cells give rise to progeny cells under normal, nutrient-rich conditions. However, under a set of specific environmental conditions, low moisture and limited nitrogen, sexual reproduction can be stimulated in nature or facilitated in the laboratory (Kwon-Chung, 1976). *Cryptococcus* has a bipolar mating system, in which haploid cells

contain alternative alleles at the sex-determining regions (mating type locus, MAT) of the genome with two different mating types: MAT**a** / MATα. Laboratory strains can be manipulated to switch mating types through outcrossing to a strain of opposite mating type and then backcrossing a single progeny population to the original parent strain 10 times (Heitman et al., 1999).

Mating events most commonly occur with the proper pairing of cells of opposite mating types. In some instances, instances of same-sex mating (monokaryotic fruiting) in diploid cells provide more flexibility among mating strategies in *Cryptococcus* populations, and have been observed to have a benefit to fitness for populations occupying novel niches (Fraser et al., 2005). After mating and cell fusion, the mated zygote transitions into the teleomorphic state (also called the perfect state), as characterized by filamentous growth and by the formation of basidia and basidiospores. The mating process is first initiated by the reception of pheromones from both parent cells. When in the presence of a candidate mate of opposite mating type, designated pheromones for MATα and MAT**a** (MFα1 and MFa1, respectively) are synthesized and released en masse, to be received by receptors on the reciprocal cell (McClelland et al., 2002; Moore and Edman, 1993). These small, hydrophobic peptides can be synthesized and used for studies *in vitro* (Chung et al., 2002; Davidson et al., 2000). The reception of these pheromones triggers a cascade of cellular changes. Primarily, it stimulates the directional growth of conjugation tubes from each parent. These specialized hyphal strands make contact and fuse together, forming a dikaryotic basidia containing unfused parental haploid nuclei. The separate parental nuclei fuse only right before undergoing meiosis to generate haploid recombinant nuclei. Among basidiomycete species, four daughter nuclei typically enter four basidiospores. However, the sexual cycle unique to *Cryptococcus* incorporates multiple rounds of mitosis to produce four long chains of basidiospores, each containing tens of spores in length (Kwon-Chung, 1980). From these chains, basidiospores are dispersed and germinate to produce new colonies under permissive conditions.

For many fungal species, spores are specialized cells that are easily transmitted across long ranges to initiate growth in new environments. This strategy is particularly useful for environmental microbes that have become established in a niche with specific growing conditions. When conditions change, spores enable an escape from environmental stressors (Brown and Hovmøller, 2002). For example, only sexual spores are produced in *Saccharomyces cerevisiae*, their growth is stimulated by nitrogen-limitation and poor carbon sources (Freese et al., 1982). While promoting long-range dispersal, spores also increases the resilience of many species by withstanding stressful conditions, such as: desiccation, high temperatures, oxidative stress (Botts et al., 2009) and UV radiation (Litvintseva et al., 2007). To cope, spores are often protected by thick cell walls, protective solutes, or exhibit plasticity in transcription for protection (Casadevall et al., 2003). For example, heat shock proteins extend the viability of fungal spores by prolonging dormancy (Wyatt et al., 2013). Fungal spores have a crucial role in the life cycles of numerous species. The dispersion and resilience of spores has ecological significance, by allowing species to reach novel environments and survive hostile conditions, as well as pathogenic significance as they also allow spread and resilience to host conditions.

1.1.3 Hybridization and Speciation

Estimates of fungal species richness have been increasing in recent years as a result of widely accessible genetic tools (O'Brien et al., 2005). Large-scale sampling of highly conserved regions among fungal genomes have extrapolated from the current fungal species richness to estimate that approximately 5 million species may exist in the fungal kingdom (Costello et al., 2012). In addition, studies of the mechanisms of speciation in fungi are underrepresented compared to other taxa (Coyne and Orr, 2004; Giraud et al., 2008). This becomes problematic for determining the taxonomic classifications

between a group of closely related species, as in a species complexes, which may contain previously undescribed or cryptic species.

Hybridization events between groups of closely related species, as in those that occupy a species complex, have been demonstrated to produce offspring with reduced viability (Greig, 2009; Hittinger, 2013). As sequence divergence increases, hybrid spore viability declines rapidly (Hittinger, 2013). Even among strains of *Cryptococcus* that mate efficiently, the germination potential of sexual progeny will not be 100% (Lengeler et al., 2001). As sexually incompatible species produce few or no viable offspring, general inferences can be made in connection to the degree of divergence between species. Measurements of viability among hybrid offspring serves as an indirect means of determining the strength of reproductive barriers and their influence on speciation events.

Divergence between populations arises with prolonged isolation, during which, various mechanisms introduce genetic diversity thereby preventing the exchange of genetic material between individuals prior to reproduction or result in genetic incompatibilities after reproduction has occurred (Mayr, 1964). Genetic isolation through pre-zygotic barriers is impacted by patterns of species distribution, behaviour, or mechanisms of reproduction prior to the fusion of cells. Many of these effects are readily studied in multicellular organisms (Coyne et al., 1994; Grant, 1994; Mendelson, 2003), but inherently difficult to study in unicellular organisms. As a result more interest has been directed to measuring the mechanisms of post-zygotic reproductive isolation in fungi (Dettman et al., 2007; Giraud et al., 2008). These include impacts on offspring viability that occur after cell fusion, including the mortality/non-viability of the zygote and hybrid sterility. The area where diverging populations overlap act as an intermediate step during speciation. Hybrids created from populations experiencing reproductive isolation are often less fit than offspring created from a single species. In contrast, with continued interaction between diverging populations, hybridization can blur species distinctions

and even lead to the breakdown of speciation barriers (Barton, 2001). The creation of viable hybrid offspring allows for mixing of genes in the successive generation and decreases the genetic distance between populations (Wu, 2001). It is also possible for hybrids that maintain their fertility to establish new populations, giving rise to novel species (Abbott et al., 2013).

A general understanding of how genetic incompatibility evolves in affecting the fitness of hybrids is still lacking. Among the causes of genetic isolation, large-scale genomic changes, such as extra-chromosomal (cytoplasmic symbionts, transposable elements) or chromosomal (polyploidization, rearrangements) are a common occurrence among hybrids of plant and animal species (Coyne and Orr, 2004). The impacts of large scale rearrangements on speciation have been explored in *Anopheles gambiae* (Coluzzi et al., 2002), multiple lineages of bacteria (Hughes, 2000), and among the *Saccharomyces cerevisiae* species complex (Fischer et al., 2006). Large-scale changes to parent genomes, such as the improper chromosome pairing and segregation play a major role in speciation (Rieseberg, 2001). *Cryptococcus* lineages have experienced notable cases of chromosomal rearrangements in the genomes of *C. neoformans* and *C. deneoformans* (Sun and Xu, 2009).

There is increasing evidence of specific allelic interactions that contribute to reproductive incompatibilities (Wu and Ting, 2004). Genic changes (incompatibilities between the genes of the diverging species) contributing to hybrid inviability or sterility in *Drosophila* were recently described (Ortiz-Barrientos et al., 2007). The divergence of alleles at two loci and the subsequent interaction between isolated populations of the same species is known as the Bateson–Dobzhansky–Muller (BDM) model of incompatibility. The BDM model explains post-zygotic reproductive isolation through the interaction of complementary alleles at two loci that have experienced sequence divergence, becoming incompatible, and subsequently lead to dampened or total loss of function (Dobzhansky,

1936; Muller, 1942). This model was first conceptualized by Bateson in a series of essays from the early 1900's which laid the groundwork experimental studies by Dobzhansky and Muller (Orr, 1996). Further study of the genetic basis for speciation used a model genetic system of multiple closely related species of *Drosophila* (Muller with the genetic model species *D. melanogaster* and *D. simulans*, and Dobzhansky with the North American species *D. pseudoobscura* and its relatives) (Orr, 1996). Their experiments demonstrated the genetic basis for hybrid incompatibility and led to the conception of the Bateson-Dobzhansky-Muller Model. This model suggests that hybrid incompatibility is due to the genetic interaction of more than one allele (Coyne and Orr, 2004). Genetic changes within the heterozygous hybrids of an ancestral population have low fitness. These mutations become fixed and are then incompatible with the ancestral type allele. Caused by complementary changes for the same allele in separate populations or individuals that are descended from the same ancestral populations.

Basidiospores from hybrid crosses of the CNSC have been shown to have low viability when germination is facilitated under standard laboratory conditions (Lengeler et al., 2001; Vogan et al., 2013. These results suggest that there is significant post-zygotic reproductive isolation between *C. neoformans* and *C. deneoformans*. The main goal of this thesis is to further the understanding of germination within hybrid offspring created from crossing various strains from the CNSC. Using genomic structural information from *C. neoformans* and *C. deneoformans* strains, we are interested in determining the roles of genome structure and environmental factors that impact the viability of hybrid offspring.

Spore viability is major trait that directly affects the occurrence of speciation, but also the tolerance of hybrids to different niches and hostile environments such as host defences. In addition, sexual spore viability can be highly dependent on environmental conditions (Rousseau et al., 1972; Salvadó et al., 2011). However, the potential effects

of environmental factors on basidiospore germination in CNSC have yet to be examined. The results of my study should have implications for speciation and pathogenesis research in this important species complex as well as in other fungi in general.

Chapter 2

Genetic and environmental influences on the germination of basidiospores in the *Cryptococcus neoformans* species complex

2.1 Preface

It is clear that incompatibilities exist between the genomes of *C. neoformans* and *C. deneoformans*. Incompatibilities and large-scale genomic rearrangements are a major source of post-zygotic genetic isolation in other species. The extent to which this is represented by the *Cryptococcus neoformans* Species Complex *in vivo* is not clear. In addition, environmental factors can have a great impact on sporogenesis and germination of basidiospores produced through sexual reproduction in *Cryptococcus*.

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I am the primary contributor of this work. Jianping Xu conceived the experiments, with the majority of the experiments conducted by me, with additional data from microdissection of JEC20a X KN99α and KN99a X JEC21α crosses conducted by Aaron Vogan. Analyses and writing of the manuscript were also predominantly completed by me, with much help and input from Jianping Xu and Aaron Vogan.

Abstract

In basidiomycetous fungi, the viability of basidiospores is an important component of sexual fitness. However, relatively little is known about the genetic and environmental factors influencing basidiospore germination. In this study, we used human opportunistic yeast pathogens, *Cryptococcus neoformans* and *Cryptococcus deneoformans*, as models to investigate the potential effects of selected genetic and environmental factors on basidiospore germination. A total of five strains with known genome structure were used to construct six crosses, three of which were between strains within the same species, while the remaining three were hybrid crosses between *C. neoformans* and *C. deneoformans*. Offspring from these crosses were incubated on two media (a nutrient-limiting and a nutrient-rich) and at three temperatures (23°C, 30°C, and 37°C). In general, spores from intra-specific crosses had greater germination potential than those from inter-specific crosses. Of the two environmental factors, temperature showed a greater influence than nutrient medium, with the 37°C environment yielding lower germination than 23°C and 30°C environments in most crosses. Furthermore, there were notable interaction effects between environmental factors and parental strains or strain pairs on basidiospore germination. We discuss the implications of these results on pathogenesis and speciation in this human fungal pathogen and for other fungi in general.

2.2 Introduction

In sexual eukaryotes, the viability of gametes is a key component of their reproductive fitness and low gamete viability is frequently used as an indicator of post-zygotic reproductive isolation between parental populations. In contrast to pre-zygotic reproductive isolations that are commonly mediated by physical, temporal, and/or mechanical barriers, post-zygotic reproductive isolations are caused by genetic factors (Coyne and Orr, 2004). At present, most studies on the mechanisms of post-zygotic reproductive isolation have involved model systems in *Drosophila* and *Saccharomyces*, while few species in other eukaryotic groups have been investigated (Giraud et al., 2008). Unlike in plants or animals, the products of sexual reproduction in many fungi can be directly cultured and examined for their viability, making fungi ideal organisms to study post-zygotic reproductive isolation. In addition, laboratory studies with fungal spores can permit the inclusion of large sample sizes and multiple experimental repeats, making inferences about the impacts of various environmental factors on spore germination feasible. However, very little is known about the factors that influence sexual spore viability and post-zygotic reproductive isolation in fungi (Vogan and Xu, 2014).

The *Cryptococcus neoformans* species complex (CNSC) has become a model for understanding fungal pathogenesis and fungal genetics. Members of this species complex are the major pathogens responsible for fungal meningitis, especially among immunocompromised individuals. The CNSC consists of two divergent species, *C. neoformans* and *C. deneoformans*, and their associative hybrids. Of the two species, *C. neoformans* dominates clinical populations, and has a global distribution – but is particularly prevalent in sub-Saharan Africa and Asia (Jarvis et al., 2010; Park et al., 2009; Xu et al., 2011). In contrast, *C. deneoformans* is more commonly isolated in Europe and is generally less virulent in animal models than *C. neoformans* (Dromer et al., 1996; Kwon-Chung et al., 1992; Mylonakis et al., 2004). It is estimated that *C. neoformans*

and *C. deneoformans* have diverged from each other for over 18 million years (Xu et al., 2000b).

Despite the long-term divergence, there is little evidence of pre-zygotic reproductive isolation between *C. neoformans* and *C. deneoformans*. Many strains of these two species can mate relatively easily under laboratory conditions and hybrids are commonly found in both natural environments and in clinical samples (Brandt et al., 1996; Litvintseva et al., 2007; Yan et al., 2002). Interestingly, most hybrids from natural populations or laboratory crosses are aneuploid or diploid, and are heterozygous at multiple loci, while the parental strains are haploid (Lengeler et al., 2001; Sun and Xu, 2007; Xu et al., 2002), which is consistent with chromosome nondisjunction during meiosis (Sun and Xu, 2009; Vogan et al., 2013). The fact that hybrid offspring often display traits that differ significantly from both parent populations is of practical concerns for human pathogens. In the case of hybrid vigor, select individuals with these traits can be advantageous within certain ecological niches (Shahid et al., 2008). For example, certain hybrids have been found to be tolerant/resistant to high levels of anti-fungal drugs (Xu et al., 2001; Shahid et al., 2008), UV radiation (Litvintseva et al., 2007), and high temperatures (Lin et al., 2007). Such fitness advantages have likely contributed to the broad distribution of CNSC hybrids in both their geographical ranges and ecological niches. Recent surveys have reported 30% of all clinical isolates in Europe are AD hybrids (Dromer et al., 2007), whereas 7.1% of environmental isolates from North America were representative of AD hybrids (Litvintseva et al., 2005).

Basidiospores from hybrid crosses have been shown to have low viability, compared to genetic cross between congenic species (90% germination [Velagapudi et al., 2009]). With only 5 - 20% of basidiospores germinating into mature colonies under standard laboratory conditions (Lengeler et al., 2001; Vogan et al., 2013), these results suggest that there is significant post-zygotic reproductive isolation between *C. neoformans* and

C. deneoformans. However, at present, only a small proportion of non-viable basidiospores from between *C. neoformans* and *C. deneoformans* crosses could be explained by the classical Bateson-Dobzhansky-Muller (BDM) interactions (Vogan and Xu, 2014). The currently described incompatible loci within hybrid offspring can be contributed to 20% spore inviability, given that reciprocal allelic combinations are present in equal numbers among progeny (Vogan et al., 2014). Thus, other genetic factors or environmental factors must play a role in the low basidiospore germination in hybrid crosses. Gock et al. (2003) showed that the germination of various Ascomycete spores (*e.g. Aspergillus penicillioides*, *Penicillium roqueforti*) were influenced by environmental factors such as temperature and water activity. Similarly, extracts from the substrate of the button mushroom, *Agaricus bisporus*, have been found to facilitate basidiospore germination (Feofilova et al., 2012). The potential effects of environmental factors on a diversity of physiological and life history traits in fungi, including CNSC (Sia et al., 2000), likely also impact basidiospore germination which has yet to be examined. Given incompatible loci only account for a small proportion of offspring inviability, we hypothesize that large-scale genetic interactions between parental genomes in the CNSC impact basidiospore germination. Furthermore, that environmental factors may influence intra-specific crosses differently than inter-specific crosses.

The objectives of this study were to examine the extent of basidiospore germination differences within and between the two species of the CNSC. Specifically, we selected five strains with known genome structure differences to construct six different crosses, including three intra-specific crosses and three inter-specific crosses. Basidiospores from these crosses were plated onto two different media and incubated at three temperatures to examine the potential influences of temperature and medium on basidiospore germination potential.

2.3 Methods

2.3.1 Strains

Four laboratory strains and one clinical isolate were used in this study. The four laboratory strains correspond to two pairs of isogenic isolates, with one pair, JEC20a and JEC21α, belonging to *C. deneoformans* (serotype D); and another pair, KN99a and KN99α, belonging to *C. neoformans* (serotype A). The isogenic strain pairs differ only at the mating type locus (Kwon-Chung and Bennett, 1978; Nielsen et al., 2003). Strains JEC20a and KN99a belong to mating type **a** while strains JEC21α and KN99α have the α mating type. The clinical isolate used in this study was CDC15α, of *C. neoformans* (serotype A) (Sun and Xu, 2009). CDC15α was obtained in a national survey by the US Center for Disease Control and Prevention (Brandt et al., 1995) and is known to differ from KN99a and KN99α strains by large scale genomic rearrangements involving Chromosomes 3 and 11 (Sun and Xu, 2009).

2.3.2 Crosses

The two MAT**a** and three MATα strains were used to create six crosses. Three of the crosses were between strains within the same species: one intra-specific cross was within *C. deneoformans* (JEC20a X JEC21α) and two were within *C. neoformans* (KN99a X KN99α, KN99a X CDC15α). The remaining three crosses (JEC20a X CDC15α, KN99a X JEC21α, JEC20a X KN99α) were inter-species, between strains of *C. deneoformans* and *C. neoformans*.

In preparation for mating, cells stored at -80°C were first cultured on yeast extract peptone dextrose (YEPD) agar medium and incubated at 30°C for five days. Actively growing cultures were then re-suspended in sterile distilled water and adjusted to a

concentration of 105 cells/µl. For each cross, 50µl of the adjusted cell suspension from each of the two parents was thoroughly mixed together. The mixed cell solutions were spotted onto separate plates containing V8-juice agar, a specific medium to induce sexual mating in CNSC (Kwon-Chung, 1976). Each plate contained three spots of the mixed parental cells and one spot for each of pure parental cells as negative controls. Each spot contained 10µl of the cell suspension, equivalent to about 106 cells. In total, 30 mating plates per cross were prepared and incubated at 23°C for four weeks to allow for mating and sexual spore formation. For species within the CNSC, a successful mating is indicated by the formation of hyphae along the periphery of the parental yeast colony. The hyphae typically extend away from the original parental yeast cell spot, with the ends of these hyphae differentiating into basidia, the sexual structures of the CNSC, which subsequently produce chains of basidiospores (Kwon-Chung, 1976).

2.3.3 Germination of Basidiospores

To evaluate basidiospore germination, two approaches were taken. The first approach examined basidiospores individually isolated using a micromanipulator (Singer Instruments, England). In this approach, the hyphae containing basidiospores were first identified using a microscope. The agar medium containing the hyphae was then cut using a sterile scalpel and transferred to a complementary space in a new plate containing YEPD medium. Multiple basidia from the section of transplanted agar, with the chains of basidiospores attached, were then picked and placed onto fresh areas of the plate. Basidiospores were then individually picked and transferred to pre-determined spots on the same agar plate. Plates containing dissected basidiospores were incubated at 23°C for one week to ensure that any slow-germinating or slow-growing basidiospores could establish a visible colony. Because of the high workload involved with micromanipulation, only two crosses (crosses JEC20a X KN99α and KN99a X JEC21α) were examined

by this method and the dissected basidiospores from this cross were only incubated on the rich medium YEPD at 23°C. The proportion of basidiospores germinated were calculated as the number of visible colonies (*i.e.* germinated spores) formed divided by the total number of dissected basidiospores for each cross.

A second approach was used to examine the basidiospore germination rates of all six crosses at all six incubation conditions. In this approach, after four weeks of mating on V8-juice agar medium at 23°C, sections of agar containing only hyphae and basidiospores (*i.e.* no parental yeast cells) were cut and transferred to a new blank plate and the hyphae and basidiospores were washed following the method outlined by Choi et al. (1999). Specifically, using a pipette, $50 - 100\mu$m of a sterile 0.5% Tween 20 solution (Sigma Aldrich: Mississauga) was applied to the mycelial surface of each agar block and spores were gently taken up along with the solution by the pipette and transferred to a sterile 1.5ml micro-centrifuge tube. The spore solutions were examined using a light microscope to determine the density of basidiospores as well as to ensure the absence of hyphae in each solution; any spore suspensions containing hyphae were discarded. Basidiospore suspensions were then concentrated/diluted with additional 0.5% Tween-20 solution to a final density of approximately 2 - 3 times 10^3spores/ml. Diluted spore suspensions were spread-plated on either the YEPD agar medium or the Minimal Medium (MM, yeast nitrogen base with ammonium sulfate but without amino acids). On each plate, 100µl of basidiospore suspension was spread evenly over the agar surface using 1mm diameter sterile glass beads.

Using the second approach, basidiospores from each of the six crosses were plated on a total of 72 plates with 36 containing the YEPD medium and 36 containing the MM media. Of the 36 plates, 12 were incubated at each of three temperatures (23, 30, or 37°C). A total of 432 plates were used for the six crosses. The number of visible colonies formed by germinated spores was counted on each plate at two and seven days after

incubation. All visible colonies were counted. The germination of basidiospores was determined as a ratio of the number of colonies observed to the estimated total number of basidiospores plated.

2.3.4 Genome Structural Differences

Data on chromosomal structural differences among strains JEC21α, H99α, and CDC15α were obtained from Sun and Xu (2009). Since JEC20a is isogenic with JEC21α and both KN99a and KN99α are isogenic with H99α (Heitman et al., 1999; Nielsen et al., 2003), we assume that JEC20a and JEC21α have the same genome structure (except at the mating type locus) (Marra et al., 2004) and that KN99a, KN99α, and H99α would have the same genome structure (Nielsen et al., 2003). Based on the chromosomal structural differences, we estimated the total percentage of non-syntenic blocks (including all known simple inversions, complex rearrangements, and translocations) over the whole genome between each of the six pairs of strains (Sun and Xu, 2009).

2.3.5 Data Analyses

Statistically significant differences in the proportion of basidiospores germinated between crosses and the effects of genetic and environmental factors contributing to the differences were analyzed using multifactorial ANOVA (Germination ~Temperature*Media*Cross). Additional post-hoc Tukey Honest Significant Difference (HSD) tests, Pearson's correlation, and η^2 were completed using R (V3.1.3; packages: stats, lsr, agricolae, ggplot2, cowplot) (R Core Team, 2015).

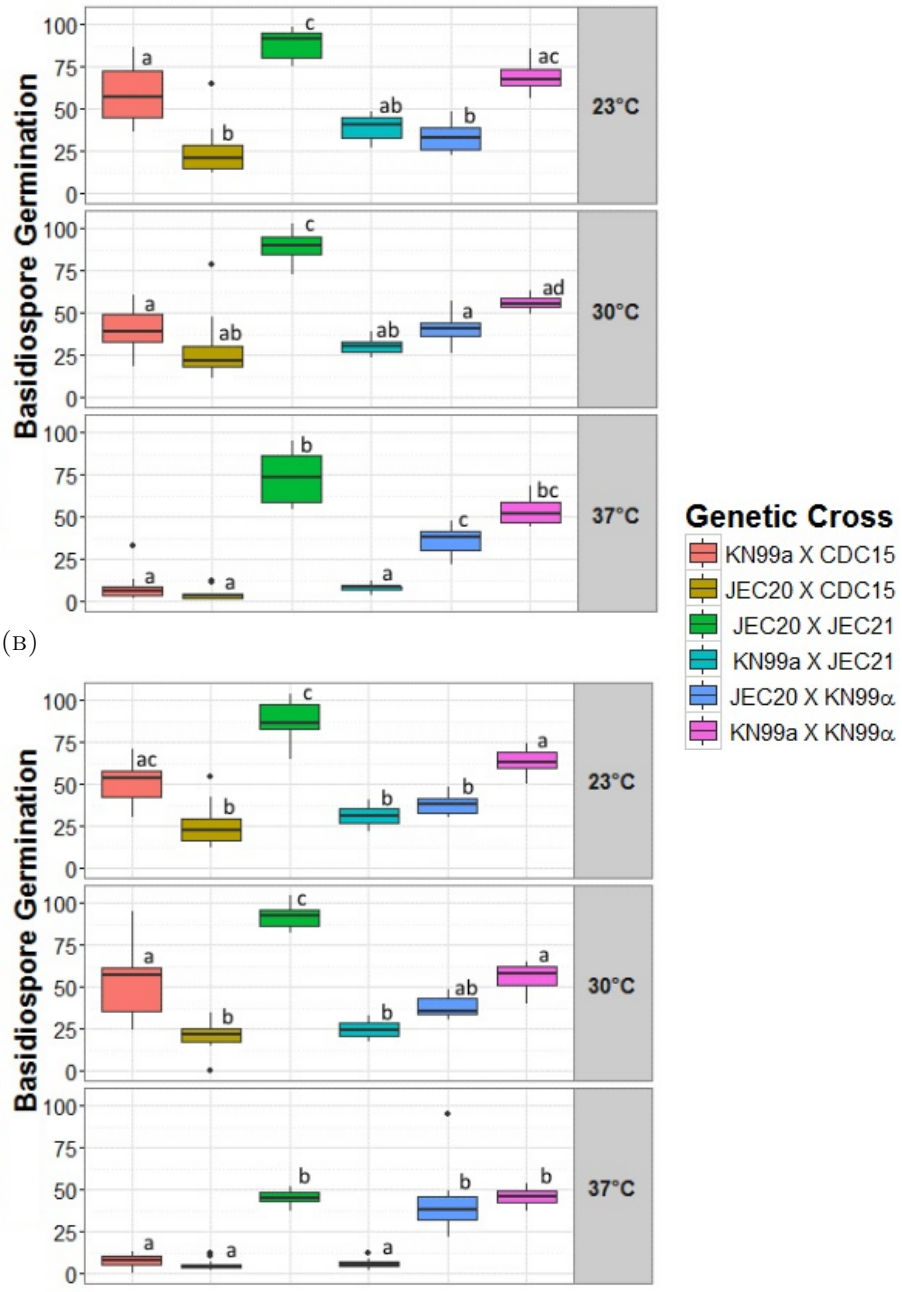
2.4 Results

In this study, we examined the rates of basidiospore germination from six crosses between strains in the human fungal pathogen *C. neoformans* species complex. The basidiospores were plated on two different media (a nutrient-rich YEPD medium and a nutrient poor minimal medium) and incubated at three different temperatures (23°C, 30°C and 37°C). For most of the crosses and treatments, only one method was used to obtain basidiospores and to estimate germination. However, for two of the crosses, an additional method using a micromanipulator was used to isolate basidiospores, though these basidiospores were only incubated at one temperature and on one medium. A summary of basidiospore germination is presented in 2.1 and statistical significance of their differences is shown in 2.1. Below we describe the influences of the examined genetic and environmental factors on basidiospore germination among our crosses.

TABLE 2.1: Mean proportion of basidiospore germination after day seven for various genetic crosses of *Cryptococcus* strains under varying environmental conditions. † and ‡ are from microdissection experiment.

Genetic Cross	Temperature	Medium	Percent of Basidiospores Germinated
KN99a X CDC15α	23°C	MM	59.55 ± 16.98
		YEPD	55.51 ± 20.79
	30°C	MM	39.60 ± 12.36
		YEPD	52.78 ± 19.93
	37°C	MM	8.67 ±8.57
		YEPD	7.74 ± 3.76
JEC20a X CDC15α	23°C	MM	25.00 ± 14.82
		YEPD	26.15 ± 12.46
	30°C	MM	28.21 ± 18.68
		YEPD	21.30 ± 8.98
	37°C	MM	4.52 ± 3.76
		YEPD	5.47 ± 3.18
JEC20a X JEC21α	23°C	MM	88.24 ± 9.07
		YEPD	90.55 ± 13.61
	30°C	MM	94.66 ± 13.90
		YEPD	92.83 ± 7.94
	37°C	MM	73.39 ± 15.60
		YEPD	45.19 ± 4.66
KN99a X JEC21α	23°C	MM	39.24 ± 7.54
		YEPD	31.70 ± 6.17 29.15†
	30°C	MM	30.80 ± 5.14
		YEPD	24.85 ± 4.65
	37°C	MM	8.12 ± 2.76
		YEPD	6.08 ± 2.80
JEC20a X KN99α	23°C	MM	33.50 ± 8.85
		YEPD	38.49 ± 6.04 17.00‡
	30°C	MM	40.36 ± 8.18
		YEPD	38.49 ± 6.29
	37°C	MM	36.46 ± 8.14
		YEPD	42.47 ± 18.68
KN99a X KN99α	23°C	MM	69.26 ± 8.85
		YEPD	64.50 ± 7.23
	30°C	MM	56.43 ± 3.79
		YEPD	55.76 ± 8.42
	37°C	MM	54.36 ± 8.26
		YEPD	46.09 ± 4.61

(A)



(B)

FIGURE 2.1: The germination of hybrid *Cryptococcus* basidiospores on MM (A) and YEPD (B) agar media, separated by incubation temperature. Tukey groups, as denoted by the characters above each box, demonstrate significant differences between measures of germination. Offspring of cross between isogenic parents generally had a higher germination potential than those of non-isogenic parents – especially at higher temperatures.

2.4.1 Comparison of Spore Dissecting Methods

In this study, the sexual spores of CNSC were isolated by either directly picking basidiospores using a micromanipulator and placing them on fresh agar plates, or through gently washing sexual hyphae/basidiospores with a surfactant Tween 20 solution and then spread them on fresh agar plates. Basidiospore germination in two crosses (JEC20a X KN99α and KN99a X JEC21α) were assessed using both methods (1.1). Using the micromanipulator, a total of 547 and 415 basidiospores were dissected from crosses JEC20a X KN99α and KN99a X JEC21α, respectively. The dissected basidiospores were placed on the rich YEPD medium and incubated at 23°C. In one cross, JEC20a X KN99α, the germination of micro-dissected basidiospores (17.0%) was significantly lower than that obtained using the spread-plating method (38.48%; $p < 0.005$). However, there was no significant difference between the two spore isolation methods for basidiospore germination from the other cross, KN99a X JEC21α (29.16% vs. 31.7%; $p > 0.05$). Interestingly, as shown in Table 2, there was a wide variation in germination among basidiospores from different basidia within each of the two crosses.

2.4.2 Effects of Temperature

Our analyses showed that temperature had a notable influence on basidiospore germination and that the effects differed among the crosses (Table 2.1; Table 2.2). The highest germination potential was found for basidiospores from the intra-species cross, JEC20a X JEC21α at 23°C and 30°C, followed by that of another intra-specific cross KN99a X KN99α at 23°C. Both of the above two crosses involved isogenic strain pairs. Interestingly, in the other intra-specific cross, KN99a X CDC15α, while basidiospore germination rates at 23°C were comparable to that of KN99a X KN99α, there was a significant reduction in germination rates at 37°C. Specifically, less than 10% of the

basidiospores germinated at 37°C, over a five-fold reduction compared to germination at the other temperatures.

Of the three inter-specific crosses, one (JEC20a X KN99α) showed relatively little change among the three temperature treatments while the other two crosses (JEC20a X CDC15α and KN99a X JEC21α) showed significant reduction in germination at 37°C compared to 23°C and 30°C. The lowest germination potential (~5%) was recorded for progeny from the inter-specific cross JEC20a X CDC15α under 37°C.

TABLE 2.2: The results of a multiple ANOVA demonstrate the individual and combined effects of experimental factors on basidiospore germination. As a correction for large sample sizes, η^2 has been included as a measure of effect size (N = 360)

Genetic Cross	**Source of Variance**	**Degrees of Freedom**	**Germination**		**Change in Germination**	
			p − value	η^2	**p − value**	η^2
KN99a X CDC15α	Temperature	2	***	0.67	***	0.39
	Media	1	0.43	0.003	***	0.05
	Temperature:Media	2	0.12	0.02	0.28	0.01
	Residuals	66	-	0.31	-	0.54
JEC20a X CDC15α	Temperature	2	***	0.41	***	0.25
	Media	1	0.57	0.003	0.13	0.01
	Temperature:Media	2	0.41	0.02	0.27	0.01
	Residuals	66	-	0.57	-	0.72
JEC20a X JEC21α	Temperature	2	***	0.56	***	0.15
	Media	1	**	0.05	0.17	0.01
	Temperature:Media	2	***	0.11	0.06	0.03
	Residuals	66	-	0.29	-	0.81
KN99a X JEC21α	Temperature	2	***	0.82	***	0.45
	Media	1	***	0.04	0.07	0.01
	Temperature:Media	2	0.17	0.01	0.87	0.001
	Residuals	66	-	0.14	-	0.54
JEC20a X KN99α	Temperature	2	0.41	0.03	0.77	0.01
	Media	1	0.21	0.02	0.43	0.004
	Temperature:Media	2	0.36	0.03	0.65	0.006
	Residuals	66	-	0.92	-	0.99
KN99a X KN99α	Temperature	2	***	0.47	0.28	0.02
	Media	1	*	0.05	0.4	0.005
	Temperature:Media	2	0.19	0.02	0.7	0.005
	Residuals	66	-	0.46	-	0.97

Overall, our statistical analyses showed that temperature had significant effects on basidiospore germination in five of the six crosses (Table 2.2). The values of η^2 in Table 2.2 show the amount of variance in basidiospore germination that could be attributed to changes in temperature. In three of the six crosses (KN99a X CDC15α, JEC20a X JEC21α, KN99a X JEC21α), temperature explained more than 50% (67%, 55.6%, 81.7%, respectively) of the observed variance in basidiospore germination rates. For two of the three remaining crosses, JEC20a X CDC15α and KN99a X KN99α, temperature showed smaller but still significant effects, contributing to 40.9% and 46.7% of the total variance respectively. The only cross that temperature did not show a significant effect on basidiospore germination was JEC20a X KN99α, for which η^2 was 2.5%.

The above comparisons were based on total basidiospore germination over the seven-day period. Interestingly, in addition to influencing the proportion at which basidiospores germinate at day seven, temperature was also found to affect the timing of spore germination (Figure 2.2; Table 2.2). At 37°C, most germinated basidiospores formed visible colonies within two days of incubation. However, in several of the crosses, a significant number of basidiospores germinated only after more than two days of incubation (Figure 2.2). For example, at 23°C, a relatively small number of basidiospores germinated in the inter-species cross JEC20a X KN99α within two days of incubation. In contrast, in JEC21α X KN99a, a cross with parental strains having the same genome structures as those of JEC20a X KN99α except at the mating types that were from alternative species, we found that most germinated basidiospores formed visible colonies within two days of incubation under the same conditions (Figure 2.2; Table 2.2). Taken together, these results suggest that temperature had a cross-specific temporal effect on basidiospore germination.

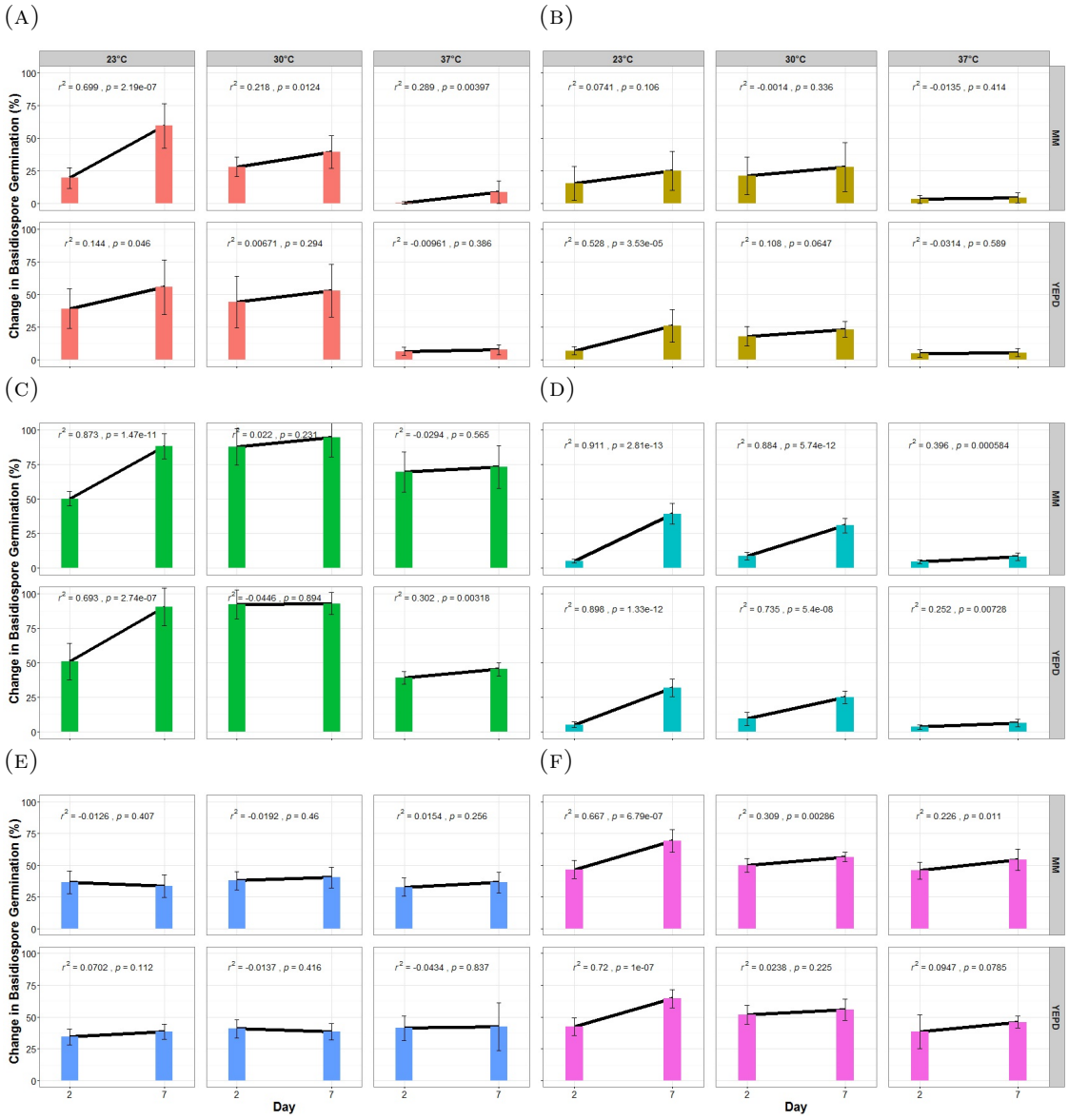


FIGURE 2.2: The change in basidiospore germination from day 2 to day 7 following inoculation for all crosses: (A) KN99a X CDC15α (B) JEC20a X CDC15α (C) JEC20a X JEC21α (D) JEC20a X KN99α (E) KN99a X JEC21α (F) KN99a X KN99α. Individual panels correspond to different treatment conditions. Statistical analysis from individual cases are included with error bars indicating standard deviation.

2.4.3 Effects of Medium on Basidiospore Germination

In contrast to the large effects that temperature had on basidiospore germination, relatively minor differences were observed between the two medium. Specifically, there was no difference between YEPD and MM on basidiospore germination at each of the three temperatures for three (KN99a X CDC15α, JEC20a X CDC15α, and JEC20a X KN99α) of the six crosses. For the remaining three crosses (JEC20a X JEC21α, KN99a X KN99α, KN99a X JEC21α), while a statistically significant contribution of media to basidiospore germination differences was observed, media only accounted for about 5% or less of the total observed variance (Table 2.2). Interestingly, in all three of these crosses, germination proportions generally higher on the minimal medium than on the rich YEPD medium (Table 2.1; Table 2.2). The largest difference contributed by the media treatment was found for the isogenic cross of JEC20a X JEC21α at 37°C for which, basidiospore germination was 73% on MM and only 45% on YEPD ($p = 0.00373$). In addition, there was a significant temperature - media interaction effect on basidiospore germination among progenies from this cross ($p = 0.0005$). However, such an interaction effect was not observed in other crosses.

Unlike the notable effects of temperature on the temporal patterns of basidiospore germination among some of the crosses, the effect of medium was again relatively minor. Progeny from most crosses showed a similar pattern of basidiospore germination between days two and seven on the two media (Figure 2.2). The only significant difference was observed for the cross KN99a X CDC15α where progeny showed a significantly delayed germination on MM as compared to the rich YEPD medium (Figure 2.2).

2.4.4 Effects of Genome Structural Differences

The chromosomal structural differences between pairs of strains were calculated based on information presented in Sun and Xu (Sun and Xu, 2009). In our analyses of the effect of genome structural differences on germination potential, the lengths of all known rearrangements were were compared between parental genomes resulting in a ratio of syntenic regions/non-syntenic regions. Thus, both isogenic crosses JEC20a X JEC21α and KN99a X KN99α were considered to have syntenic ratios of 1. The third intra-specific cross KN99a X CDC15α had a syntenic ratio ~0.94, with the notable difference between these two strains coming from the translocation between Chromosomes 3 and 11 (Sun and Xu, 2009). The hybrid crosses, KN99a X JEC21α and JEC20a X KN99α, differed at every genomic rearrangement shown in Sun and Xu (2009) and they have a syntenic ratio of ~0.76. Finally, the last strain pair CDC15α and JEC20a has a syntenic ratio of 0.82.

Because of the significant influences of temperature and medium on basidiospore germination in *C. neoformans*, we used a linear model analysis for each of the six temperature - media combinations to determine the relationship between basidiospore germination rates and the syntenic ratios of parent strains among the crosses (Figure 2.3). Under 23°C, basidiospore germination was strongly correlated with the ratio of shared syntenic ratio (r^2 = 0.788 and 0.73 on MM and YEPD media respectively; p <0.05). However, the strength of this correlation was reduced at higher temperatures, with r^2 = 0.441 (on MM; p=0.09) and 0.614 (on YEPD; p=0.04) at 30°C; and 0.275 (on MM; p=0.164) and 0.0213 (on YEPD; p=0.352) at 37°C respectively. Taken together, our results suggest that the relationship between genome structure similarity and basidiospore germination was highly dependent on environmental conditions

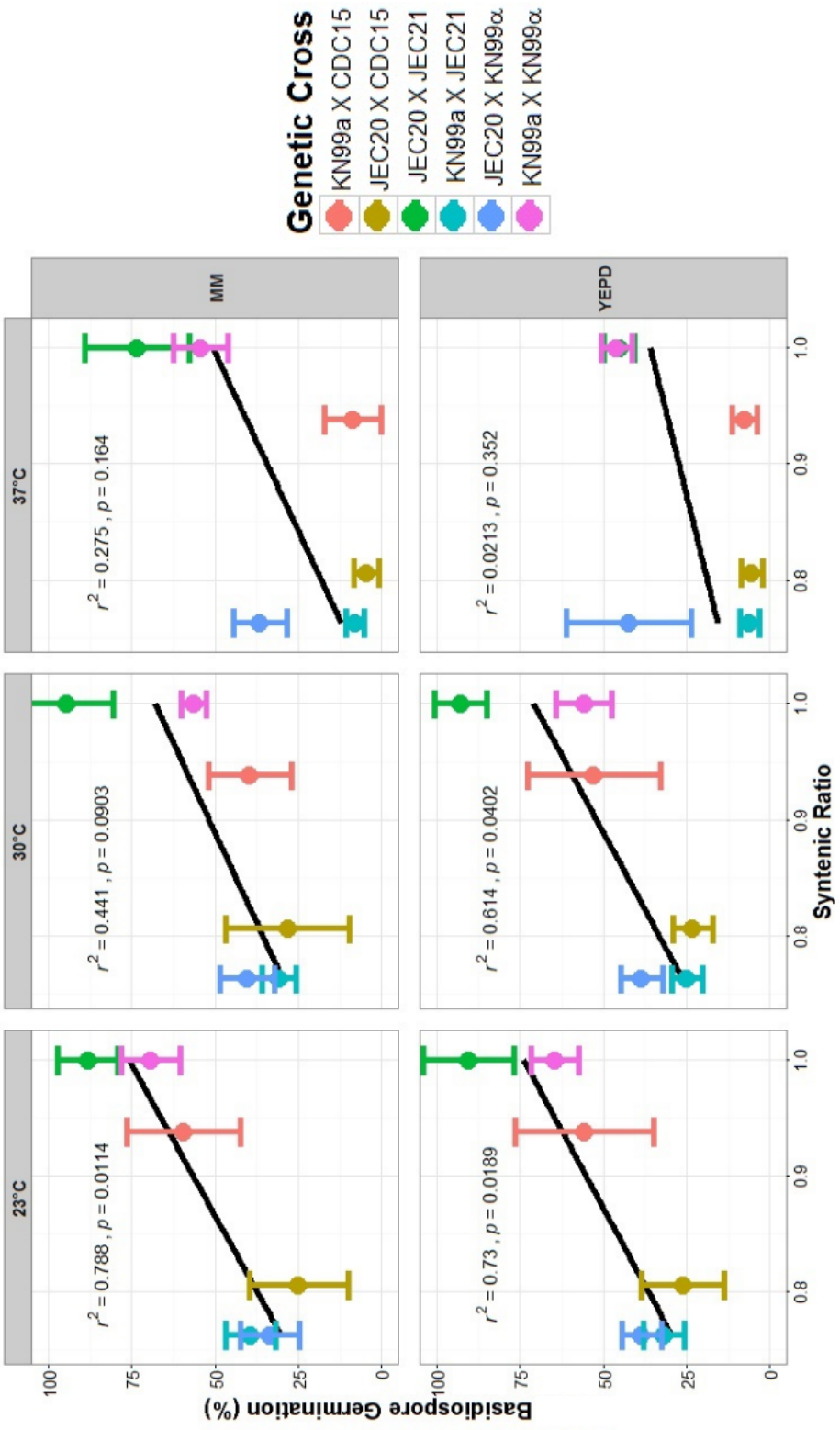


FIGURE 2.3: Ratio of non-syntenic to syntenic blocks between parental strains used in germination experiments. The panels in this figure correspond with the different experimental conditions that basidiospore germination was facilitated under. Within each panel, individual statistical results are presented with standard deviation error bars.

2.5 Discussion

Prior to this study, there have been several reported estimates of basidiospore germination in the *Cryptococcus neoformans* species complex. These estimates have shown to be highly variable, from about 5.5% (Lengeler et al., 2001) and 19% (Vogan et al., 2013 to 69% (Idnurm, 2010). Basidiospores that germinated in low proportion (5.5%-19%) were found in progeny from hybrid crosses between *C. neoformans* and *C. deneoformans* while the high germination potential was found in an intra-specific cross. All these studies used microdissection to examine basidiospore germination. In this study, the manual dissection of basidiospore chains allowed for tracking and indexing of individual basidiospores in two crosses, providing fine germination estimates of basidiospore germination from individual basidia. Our results showed that basidiospores from different basidia of the same cross could have very different germination, from 0% to 100% (2.3). Thus, large numbers of basidia and basidiospores need to be dissected in order to accurately estimate basidiospore germination rates. On the other hand, due to its ease of operation, spread-plating of a large number of basidiospores in spore suspensions can overcome the problem of large differences in germination among basidia within a cross. As a result, it could potentially provide an overall more robust estimate of basidiospore germination for each cross under each test condition for a broad set of conditions.

TABLE 2.3: Detailed germination proportions for spores from individually dissected basidia in two crosses. Nt: the total number of dissected spores from the specific basidia; Ng: the number of spores germinated from the specific basidia

Basidia Number	**JEC20a X KN99α**		**KN99a X JEC21α**	
	Nt	**Ng(%)**	**Nt**	**Ng(%)**
1	29	7 (24.14%)	16	0
2	1	0	1	0
3	8	8 (100%)	11	1 (9.09%)
4	23	3 (13.04%)	29	0
5	4	0	7	0
6	26	0	3	0
7	37	0	19	0
8	3	1 (33.33%)	27	0
9	26	6 (23.08%)	2	0
10	19	4 (21.05%)	22	0
11	5	2 (40%)	34	24 (70.59%)
12	15	3 (20%)	24	0
13	2	0	12	6 (50%)
14	23	8 (34.78%)	31	21 (67.74%)
15	30	13 (43.33%)	32	16 (50%)
16	16	0	8	7 (87.5%)
17	24	0	3	0
18	24	1 (4.17%)	24	0
19	19	15 (78.94%)	20	10 (50%)
20	42	9 (21.43%)	22	11 (50%)
21	26	0	39	24 (61.54%)
22	3	0	11	1 (9.09%)
23	19	0	18	0
24	23	0	-	-
25	29	0	-	-
26	47	13 (27.66%)	-	-
27	24	0	-	-
Total:	547	93 (17%)	415	121 (29.2%)

At present, the reason(s) for the difference between these two methods for cross JEC20aa X KN99α is not known. One potential reason for the relatively higher basidiospore germination potential for the spread-plated basidiospores was that these basidiospores were submerged in Tween 20 solutions before plating. A previous study showed that submerging basidiospores of the pine rust pathogen *Cronartium quercuum f. sp. fusiforme* in water before plating on agar significantly enhanced the spore germination, likely due to the release of inhibitory compounds from spore surfaces (Spaine and Kaneko, 1993). In addition, Tween 20 is a nonionic surfactant and mild detergent that can help solubilize basidiospore surface molecules and may enhance their germination. However, since basidiospore germination by the two methods for the other cross KN99a X JEC21α did not show a significant difference, our results could also be explained by the stochastic effects based on which basidia were picked using the microdissection method. As shown in 2.3, there was a big variation among basidia within each of the two crosses in the percentages of spores that were germinated. Below we mainly discuss the results obtained using the spread-plating method.

2.5.1 Influence of Temperature on Basidiospore Germination

For human pathogens, tolerance to high temperatures is crucial for sustained infection of an endothermic host. In this study, we found that high temperature had a significant inhibitory effect on the germination of basidiospores from both intra-specific and inter-specific crosses. This result is different from that in the coprophilous fungus *Coprinus radiatus* where a significantly greater proportion of basidiospores germinated at the high temperature of 45°C than at the low temperatures of 30-35°C (Mills and Eilers, 1973). Interestingly, the inhibitory effect of 37°C on basidiospores of CNSC was greater on progeny from inter-specific crosses (*e.g.* as shown in two of the three examined inter-specific crosses) than those from intra-specific crosses (*e.g.* as shown in one of the three

intra-specific crosses) (2.1).

Currently, the reasons for the divergent proportions of basidiospore germination among the different temperature conditions and among crosses are largely unknown. Using both site-directed and random mutagenesis, a recent study identified about 50 genes essential for basidiospore germination in *C. neoformans* (Ianiri and Indrum, 2015. The genes belong to diverse functional categories and include those involved in mitochondrial maintenance and function, cell division and cell cycle control, and the syntheses of membrane ergosterol and the cell wall. However, all those essential genes are found in the genomes of both *C. neoformans* and *C. deneoformans* and most of these genes are also present in other fungi (Ianiri and Indrum, 2015). Regardless, understanding their expression patterns and the detailed molecular and cellular processes that these genes exert in controlling basidiospore germination may help us reveal the divergent proportions of basidiospore germination among the crosses and temperature conditions observed here. Since all five strains in our study are wild type and contain all the functional copies of these genes, our results suggest that other factors such as gene-gene interactions, strain-strain interactions, and/or genetic-environment interactions likely play important roles in determining basidiospore germination potential in CNSC.

Most previous studies of basidiospore germination have examined only one cross each and thus the potential genotype-environment as well as strain-strain interaction effects between crosses could not be identified (Ianiri and Indrum, 2015; Idnurm, 2010; Lengeler et al., 2001; Mills and Eilers, 1973; Sia et al., 2000; Spaine and Kaneko, 1993; Vogan et al., 2013). Interestingly, the large differences in basidiospore germination at 37°C between reciprocal inter-specific crosses (KN99a X JEC21α and JEC20a X KN99α) suggest that mating type combinations likely play a role, with the combination of MATα from *C. neoformans* and MAT**a** from *C. deneoformans* generating basidiospores that can germinate more easily at 37°C than the parental combination of MAT**a** from *C.*

neoformans and MATα from *C. deneoformans*. This observation is also consistent with the predominance of *C. neoformans* MATα strains in clinics and human patients (Yan et al., 2002).

Alternatively, the progeny mitochondrial type might also have played a role. Progeny mitochondrial DNA in inter-specific crosses in CNSC are predominantly inherited from the MAT**a** parent (Xu et al., 2000a; Yan and Xu, 2003). Consistent with previous findings, our results indicated that progeny from the KN99a X JEC21α cross inherited the KN99a mitochondrial DNA while those from the JEC20a X KN99α inherited the JEC20a mitochondrial DNA (data not shown). Thus, it's possible the higher germination potential of progeny from the JEC20a X KN99α cross than the KN99a X JEC21α cross at 37°C could have been due to the mitochondrial genotype from the JEC20a parent being able to better support basidiospore germination than the KN99a mitochondrial genotype. However, such a mitochondria-specific effect from the JEC20a parent was not observed for progeny from cross JEC20a X CDC15α where a low basidiospore germination potential was observed at 37°C. Similarly, despite inheriting the KN99a mitochondrial genotype, progeny from cross KN99a X KN99α showed a high germination potential at 37°C. Thus, if there were an effect of mitochondrial genotype on basidiospore germination, such as an effect were likely exerted through interacting with other genetic factors in the nuclear genome and seemed environment-specific.

Such incompatibility has been observed among cytonuclear-hybrids ("cybrids") of *Saccharomyces cerevisiae*, in which the nuclear and cytonuclear genome are inherited independently and are incompatible. These incompatibilities can be directly examined through transplanting mitochondrial genes into a mutant strain that lacks mtDNA, and observing the level of post-zygotic reproductive isolation in progeny created through hybridization with a separate strain. The genus *Saccharmoyces* contains multiple species that have become adapted to survive in varying environments, and inter-specific crosses

often result in inviabilities that are due to multiple large-scale chromosomal rearrangements. Between these species, nuclear-mitochondrial incompatibilities are unidirectional, such that *S. paradoxus* mtDNA was found to cause loss-of-function in *S. cerevisiae*. While inviability occurring in the opposite direction, with mtDNA from *S. cerevisiae* present in a compatible *Saccharomyces* strains resulted in tolerance of 37°C conditions and growth on non-fermentable carbon sources; both traits were not observed in other cybrids that did not contain *S. cerevisiae* mtDNA (Špírek et al., 2015). The patterns of mitochondrial inheritance in Cryptotcoccus resulting in cytonuclear incompatibility could be sufficient to explain the specific parental-effects that we have observed among our crosses.

Similar to the patterns of temperature effects on basidiospore germination overall, we observed little consistent effects of individual parental strains on the temporal pattern of basidiospore germination. Instead, most of the temporal variations could be attributed to strain-pair specific effects. Similarly, the reciprocal congenic crosses JEC20a X KN99α and KN99a X JEC21α showed large differences in their temporal patterns at all three temperatures, consistent with a role for the mating type locus combination in determining the timing of basidiospore germination under specific conditions.

2.5.2 Effects of Media on Spore Germination

Overall, our results showed that media could have a significant effect on basidiospore germination in certain crosses. Interestingly, in crosses where significant differences in basidiospore germination were found, the minimum medium tended to support a greater germination potential than the rich YEPD medium (Table 2.1). Our results suggest that limiting the supply of carbohydrates and amino acids can enhance the germination of basidiospores in the *C. neoformans* species complex. A slightly different but similar phenomenon has been reported for a variety of oligotrophic microorganisms, where limiting nutrient supplies can lead to enhanced microbial growth (Borlestean et

al., 2015; Hirsch, 1986). Given that the natural conditions that allow for hybridization within the CNSC likely have limited supply of free carbohydrates and free amino acids, our results are consistent with the hypothesis that the CNSC might have adopted a basidiospore germination strategy that required limited amount of organic compounds. Further experiments may lead to identify an optimal medium/media that will support a greater germination potential than the two media we tested here.

Within the human host, the basidiospore germination condition likely differs from those on artificial media and in nature. In addition to the high temperature (at 37°C) and an environment different from their natural ecological niche, the pathogen basidiospores have to face host defenses. Furthermore, these conditions can change as cryptococcal infection progresses from the respiratory tract to the bloodstream and the central nervous system (Davson et al., 1987). Thus, the basidiospore germination potential inside hosts may be much lower than what we observed here on artificial media at 37°C. The importance of environmental conditions on the fitness of CNSC was also demonstrated in an earlier study where transgressive hybrids showed greater fitness advantage at the high temperature and limiting nutrient environment (37°C and MM) than at the low temperature and high nutrient environment (23°C and YEPD) (Shahid et al., 2008). Together, these results call into question of the common assumption that basidiospores are the most important infectious propagules in CNSC. Instead, given the low germination potential of basidiospores at 37celsius, the roles of desiccated vegetative cells during infection could also be important.

2.5.3 Relationship between Genome Structural Differences

In general, we found that the more similar the parental strains are in their chromosomal structure, the greater the germination potential of their progeny. This result is consistent with our expectation that genome synteny contributes to basidiospore viability. Similar

results have been found in other organisms (Greig, 2009; Ranz et al., 2007). For example, in the *Saccharomyces cerevisiae* species complex, chromosomal structural differences contributed significantly to post-zygotic reproductive isolation among the closely related species (Greig, 2009; Xu and He, 2011). Such contributions were at least partly due to chromosome non-disjunction during meiosis I that generated spores without certain essential chromosomal regions. Indeed, evidence for non-disjunction has been reported in hybrid crosses in CNSC (Vogan et al., 2013). In addition, evidence for multiple genetic incompatibilities influencing hybrid basidiospore viability between *C. neoformans* and *C. deneoformans* has also been reported (Vogan and Xu, 2014).

Among the six crosses, of special note is the intra-specific cross KN99a X CDC15α. The two parental strains in this cross have an estimated syntenic ratio of ~94% but with a reciprocal translocation between Chromosomes 3 and 11 (Sun and Xu, 2009). Interestingly, progeny from this cross had comparable germination as the isogenic cross KN99a X KN99α at 23°C and 30°C environments but were significantly lower at the 37°C environment. The results suggest that the translocation might have a high-temperature specific effect on basidiospore germination in this cross. However, aside from the main reciprocal translocation, the translocated regions also contained several small-scale rearrangements between *C. neoformans* and *C. deneoformans* that could further contribute to low basidiospore viability at 37°C.

2.5.4 Genetic-Environmental Interaction Effects on Post-Zygotic Reproductive Isolation

As described above and shown previously (Sun and Xu, 2009; Vogan and Xu, 2014), neither chromosomal non-disjunction nor BDM genetic incompatibility (or a combination of both) were sufficient to explain the observed low basidiospore germination in several of our crosses and incubation conditions. In addition, the germination potential

for most crosses were highly dependent on environmental conditions, with temperature being a major factor in our analyses. These results suggest that basidiospore germination and post-zygotic reproductive isolation in the CNSC were highly dependent on environmental conditions. Environmental-specific, parental-genotype influences of basidiospore germination are of particular interest for research on genetic incompatibilities that arise from genomic rearrangements, as genes that flank breakpoints can have modified expression levels (Pérez-Ortín et al., 2002). Indeed, gene expression and repression under high temperature treatments have previously been described within CNSC (Kraus et al., 2004; Steen et al., 2002) and have been demonstrated to be crucial for sustained growth *in vivo* (Steen et al., 2003).

The expression of target genes such as those involved in basidiospore germination within a stressful environment (*e.g.* high temperature) may be impacted by the specific genetic incompatibilities that arise under these conditions. In our analyses, the correlation between syntenic ratio and germination rate was found to progressively decay as incubation temperature increased (Figure 2.2). In particular, KN99a X CDC15α basidiospores germinated in proportions similar to those of the KN99a X KN99α cross at 23°C and 30°C, consistent with the expectation based on their high syntenic ratios. However, in both crosses involving strain CDC15α, there was a large decrease in basidiospore germination at 37°C, suggesting that certain genetic feature(s) within CDC15α likely makes its sexual progeny particularly susceptible to low germination at high temperature. It should be noted that this phenomenon was not unique to strain CDC15α as progeny from cross KN99a X JEC21α also showed very low germination potential at 37°C.

In contrast to the observation for progeny from the intra-specific cross KN99a X CDC15α, we found that progeny from an inter-specific cross (JEC20a X KN99α) showed a high germination potential at 37°C (2.1). The high germination potential was especially notable when compared to results from a comparable cross KN99a X JEC21α (except

at the mating type locus) where relatively few basidiospores germinated at 37°C. In CNSC, the mating type loci contain genes that are crucial for sexual reproduction and pathogenicity (Wickes, 2002). These regions differ significantly in structure and gene arrangements between opposite mating types of the same species but the differences were greater between members of the two different species (Lengeler et al., 2002). Previous investigation of the inter-specific cross, JEC20a X CDC15α, did not identify BDM incompatibilities within the mating type regions (Vogan and Xu, 2014). However, along with the results from the cross KN99a X KN99α, the results from the above three crosses suggest that the MATα allele in KN99α might enable progeny to germinate more efficiently at high temperatures, either alone or in combination and interaction with other genes in the genome. Significant influences of genetic interactions between loci on post-zygotic reproductive isolation have been reported in fruit flies (Schaeffer et al., 2003). Further genetic analyses of the germinated basidiospores at 37°C between the two reciprocal hybrid crosses (JEC20a X KN99α and KN99a X JEC21α) in our study are needed in order to identify the genes and their interactions in influencing basidiospore germination.

Aside from the contributions of genome structure differences, nucleotide sequence divergence between parental strains could also play a significant role to basidiospore germination differences between the intraspecific and interspecific crosses. Specifically, *C. neoformans* and *C. deneoformans* exhibit ~10% sequence divergence at the nucleotide level (Sun and Xu, 2009). When homologous chromosomes with such divergent sequences pair with each other and cross over during meiosis, the highly conserved mismatch repair system will be frequently triggered, interfere with normal chromosome disjunction, and lead to faulty chromosome segregation and lethal chromosomal rearrangements/deletions (Myung et al., 2001; Reenan and Kolodner, 1992; Williamson et al., 1985). Hybrid crosses between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* have shown to result in spontaneous DNA lesions and chromosomal rearrangements, resulting in

vastly decreased spore viability compared to intraspecific crosses (Hunter et al., 1996). Furthermore, chromosomal rearrangements and nucleotide sequence divergence can act synergistically to increase the rate of non-disjunction and reduce spore viability in *S. cerevisiae* and *S. paradoxus* (Myung et al., 2001; Hunter et al., 1996; Greig et al., 2003). Whether a similar mechanism exists in CNSC awaits further investigation.

2.5.5 Conclusions and Perspectives

This study described the patterns of basidiospore germination among six crosses within and between the two closely related species *C. neoformans* and *C. deneoformans*. In addition to examining the potential effects of known genetic differences between pairs of strains on offspring basidiospore germination, we also examined the effects of three temperatures and two media on basidiospore germination. Our analyses revealed that all examined factors (individual parental strain, strain pair, temperature, and medium) could impact basidiospore germination. As expected, progeny from inter-specific crosses generally have a lower germination potential than those from intra-specific crosses. However, environmental factors can significantly impact the pattern. Importantly, there were notable interaction effects between the examined factors, with the 37°C causing a large reduction of basidiospore germination for two of the three inter-specific crosses.

Previous studies have suggested that basidiospores are the most likely infectious propagules in the pathogenic *Cryptococcus* species complex. Our results here suggest that the 37°C environment were not very conducive for basidiospore germination in the majority of the crosses. In addition, the low basidiospore germination potential at 37°C forms a stark contrast to the vigorous vegetative growths of all parental strains as well as their germinated progeny at this temperature (Shahid et al., 2008; Sia et al., 2000; Steen et al., 2003; Steen et al., 2002). Taken together, these results indicate that basidiospores might not be the most important infectious propagule as commonly assumed. Rather,

airborne, desiccated vegetative cells may also play an important role in initiating host infection.

Aside from the implications for pathogenesis, our results also have implications for the evolution and speciation research in these and other fungi. Evidence for both recent and potentially ancient hybridizations between *C. neoformans* and *C. deneoformans* have been reported (Xu et al., 2002; Xu et al., 2000b; Kavanaugh et al., 2006). In laboratory settings, there has been little evidence for pre-zygotic reproductive isolation between *C. neoformans* and *C. deneoformans* as mating can be easily induced between many strains of these two species. Instead, post-zygotic reproductive isolation is common, as shown here and in previous studies. However, the BDM genetic incompatibilities identified based on one basidiospore-germination condition (23°C on YEPD) likely explains only a subset of the incompatibilities between these two species. Additional incompatibilities that are unique to a specific environmental condition must also exist. For example, genic interactions between specific loci near translocated regions could play a significant role in offspring inviability during high temperature growth (Morrow et al., 2012). The locations of those genetic factors and how they interact with each other await further investigation.

Chapter 3

General Conclusion

Prior estimates of basidiospore germination in the *C. neoformans* species complex (CNSC) have been shown to be highly variable (Lengeler et al., 2001; Idnurm, 2010; Vogan et al., 2013) and the occurrence of mating and sexual reproduction between strains of two divergent lineages in this species complex suggests that reproductive isolation is incomplete (Giraud et al., 2008). In this thesis, I have provided estimates of basidiospore germination for multiple intra-specific and inter-specific crosses. In addition to describing these results, I discussed the impact that genetic incompatibilities have on offspring viability for a hybrid cross between two closely related species of *Cryptococcus*. Furthermore, I also described and discussed the effects of environmental factors on basidiospore germination. In this chapter the implications of my findings to hybridization and speciation within CNSC are discussed.

Past work in our group has identified large-scale genomic rearrangements between the two diverged lineages of the CNSC (Sun and Xu, 2009) and the Bateson-Dobzhansky-Muller (BDM) incompatible genomic regions were shown to contribute to hybrid basidiospore inviability. The presence of these genetic incompatibilities was recently used in part to justify the distinction of *C. neoformans* and *C. deneoformans* as separate species

(Hagen et al., 2015). However, reproductive barriers have not been completely established between these two species as they are still capable of producing viable hybrid offspring. Also, the genomic comparisons between two strains representing these two lineages provided evidence of ancient hybridization events (Kavanaugh et al., 2006).

Traditionally, species of *Cryptococcus* have been distinguished by a collection of genetic and morphological characteristics (Ellis et al., 2000; Kwon-Chung et al., 1982; Meyer et al., 1999). Genetic analysis and serotyping of progeny from a cross between H99 (*C. neoformans*) and JEC20a (*C. deneoformans*) reveals that 70% of all progeny are diploid/aneuploid and 15% carried genes from both parents. The ploidy and genotypes present hybrids suggest that they are the result mixing between MAT**a** and MATα strains, or fusion between strains of A or D serotype of the same mating type (Kwon-Chung and Varma, 2006). In this case, species of *Cryptococcus* are not easily distinguished using serotypes as they were found to be unstable (hybrid offspring could have serotypes D, A or AD) and do not always correspond with results of genotyping (Kwon-Chung and Varma, 2006). Phylogenetic analysis of CNSC mostly resolves serotypes A, D, and AD into monophyletic group, separate from species with B and C serotypes (Butler and Poulter, 2005; Diaz et al., 2005; Fan et al., 1995; Fell et al., 2000; Xu et al., 2000a). Yet, as per the biological species complex, it is clear that interactions between the members of the CNSC indicate that species distinctions are not fully established. The use of either the biological or phylogenetic species concepts alone may not be effective at reliably resolving species relationships in the CNSC.

Despite the affinity for hybridization between strains of the CNSC, sexual reproduction does not occur within all *Cryptococcus* populations in nature; some are strictly clonal (Brandt et al., 1996; Chen et al., 1995; Franzot et al., 1997). With the benefits that sexual reproduction brings, the presence of asexual reproduction may be due to a mating type bias. MAT**a** is rare in natural populations (Litvintseva et al., 2003), and

both clinical and environmental samples have shown a preference for the MATα mating type. Multiple studies of *Cryptococcus* populations conclude that roughly 90% of all samples tend to be MATα mating type (Hironaga et al., 1983; Jong et al., 1982; Kwon-Chung and Bennett, 1978; Madrenys et al., 1993). The dominance of one mating type could be explained by sampling within a niche where MATα strains outcompete those of MAT**a**, or MAT**a** strains are geographically restricted in their distribution.

Nutrients from the environment often dictate germination/reproductive events in fungi. Although many fungal species are capable of surviving in extreme environments, in the absence of key nutrients or suitable conditions, replication/reproductive events can be halted. This is commonly observed in fungal species with carbon, nitrogen, or phosphate deprivation (Freese et al., 1982); as well as high temperatures or pressure (Dijksterhuis et al., 2002; Dijksterhuis and Teunissen, 2004. In the absence of a sexual partner, MAT**a**/α *Cryptococcus* strains exhibit filamentous growth when starved of nitrogen (Lin and Heitman, 2006; Wickes et al., 1996). Several loci, including the MAT locus, are responsible for development of filamentous growth. Lin and Heitman (2006) described multiple quantitative trait loci for filamentation in *Cryptococcus*. These loci also coincided with pigment production and high temperature growth. Strains that are more susceptible to limiting conditions may be less able to produce hyphae, pigment, and withstand high temperatures. This has implications for clonal populations that may stay clonal in certain niches. If mating does not play a central role in the life cycle of *Cryptococcus*, then hyphal growth may serve a separate function aiding in the uptake of nutrients from the environment (Wickes, 2002).

The implications of mating type bias extend beyond environmental populations. Clinical strains are predominantly of a single mating type (MATα) and there is conflicting evidence on whether these infections occur through propagules created by sexual reproduction or from self-mating events (Brandt et al., 1996; Igreja et al., 2004; Lin and

Heitman, 2006; Meyer et al., 1999; Sorrell et al., 1996). Mating under pathogenic conditions has not been reported, yet hybridization prior to infection may be possible to generate hybrid offspring that are resistant to host environments (Cohen et al., 1982; Ellis and Pfeiffer, 1990).

Cryptococcal infections occur when infectious particles enter the respiratory tract and cross the alveolar membrane, invading the bloodstream. Once in circulation, infections crossing the blood-brain barrier can lead to encephalitis, while further invasion of the spinal column results in meningoencephalitis. As a prolific pathogen of immunocompromised individuals, there has been much focus on the study of virulence factors and their impacts on pathogenesis in *Cryptococcus* (Casadevall et al., 2003; Zhu et al., 2001). The functional propagule responsible for infections was originally proposed as the basidiospore (Cohen et al., 1982). However, *Cryptococcus* yeast cells could be equally as virulent as basidiospores and have also been suggested to play a role in infection (Velagapudi et al., 2009). Both basidiospores and vegetative yeast cells are small enough to enter the capillaries. However, as the smaller of the two, basidiospores are more likely to be resilient to environmental stressors when traveling between hosts (Ruiz et al., 1982; Wickes et al., 1996) and are employed in animal models to induce infection (Sukroongreung et al., 1998). As our results have shown, basidiospores created from interspecific or intraspecific genetic crosses have poor germination under conditions similar to the host environment. Indeed, our results suggest that hybrid basidiospores are unlikely to be efficient at germinating and causing diseases within hosts.

Over the last few decades, *Cryptococcus* has transitioned from a predominantly environmental microbe to a major human pathogen (UNAIDS, 2010), primarily due to the increasing number of immunocompromised hosts. In recent years, expansion of its distribution has also resulted in infections of seemingly healthy patients (Kidd et al.,

2004; Chen et al., 2008). This has been made possible by the development of multiple virulence traits that first allowed for environmental tolerance and host resistance in multiple protozoan and animal species, with the maintenance of traits contributing to pathogenicity in humans (Casadevall et al., 2003). Due to these traits, *Cryptococcus* displays relatively little host selectivity, allowing it to infect diverse types of hosts. However, *C. neoformans* and *C. deneoformans* are among the few species to consistently cause systemic infections and withstand host conditions in humans (Fromtling et al., 1982; Lin and Heitman, 2006; Polacheck et al., 1982).

The burden of fungal infections are increasing worldwide (Fisher et al., 2012). Understanding the fluidity of populations and clear species distinctions as they relate to pathogenesis/pathogenicity becomes even more crucial. Given the genetic isolation and environmental pressures that are acting on hybrids in the CNSC, the results presented in this thesis are of significance to species relationships in other pathogenic fungi. In recent years the Chytrid fungus (*Batrachochytrium dendrobatidis*) holds a significant threat to many frog species across the globe. Intraspecific hybrids of chytrid in Brazil have increased pathogenicity and host range (Farrer et al., 2013). In the case of *Cryptococcus*, hybrid genotypes appear to be of predominantly recent origins (Xu et al., 2002; Xu and Mitchell, 2003) and laboratory hybrids exhibit hybrid vigor in both *in vivo* and *in vitro* experiments using murine models (Lin et al., 2008). This could explain their wide abundance in nature despite the fact that hybrid offspring are largely inefficient at meiosis/sporulation and are trapped in a diploid state. With increased niche breadth, continued mixing and hybridization can erode species barriers, and lead to the future amalgamation of these now separate *Cryptococcus* species. In addition, the interaction between the sex-specific genes and environmental pressures can impact the barriers that separate the members of a species complex. Further investigation of environment-specific genetic incompatibility requires more in depth genetic studies of hybrid basidiospore incorporating the analyses of BDM loci and their virulence properties.

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