

**MATING SYSTEM AND MITOCHONDRIAL
INHERITANCE IN A BASIDIOMYCETE YEAST,
*CRYPTOCOCCUS NEOFORMANS***

PhD Thesis-Zhun Yan __ McMaster University- Biology

**MATING SYSTEM AND MITOCHONDRIAL
INHERITANCE IN A BASIDIOMYCETE YEAST,
*CRYPTOCOCCUS NEOFORMANS***

By
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ABSTRACT

In the majority of sexual eukaryotes, mitochondria are inherited predominantly from a single, usually the female, parent. Like the majority of higher plants and animals, the pathogenic yeast *Cryptococcus neoformans* has two mating types (sexes), however, these two sexes are morphologically similar. In this study, I examined the distribution of the mating types and how mating types influence the inheritance of mitochondria in *C. neoformans*. My survey of mating type alleles in 358 isolates collected from four geographic areas in the US showed a biased distribution of mating type alleles with most isolates containing mating type α alleles. To characterize the role of mating type locus on mitochondrial inheritance, I constructed two pairs of congenic strains that differed only at the mitochondrial genome and mating type locus. Mating between these two pairs of strains demonstrated that uniparental inheritance in *C. neoformans* was controlled by the mating type locus and progeny predominantly inherited mitochondria from the mating type α parent. Specifically, we identified two genes within the mating type locus, *SXI1 α* in mating type α strain and *SXI2 α* in mating type α strain, that control mitochondrial inheritance. Disruption of these two genes resulted in biparental mitochondrial inheritance in sexual crosses. These two genes are the first ones identified capable of controlling uniparental mitochondrial inheritance in any organism. In addition, we determined that the deletion of the *SXI1 α* gene enhanced the spread of mitochondrial introns in sexual crosses. This discovery is consistent with the hypothesis that uniparental inheritance might have evolved to prevent the spread of selfish cytoplasmic elements.

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LIST OF ABBREVIATIONS

A α	MAT α allele in serotype A strain
A α Da	MAT alleles A α and Da in serotype AD strain
Aa	MATa allele in serotype A strain
AaD α	MAT alleles Aa and D α in serotype AD strain
AFLP	Amplified Fragment Length Polymorphisms
AIDS	Acquired Immune Deficiency Syndrome
B α	MAT α allele in serotype B strain
CDC	Centers for Disease Controls and Prevention
CHEF	Contour-clamped Homogeneous Electrophoretic Field
cpDNA	Chloroplast DNA
D α	MAT α allele in serotype D strain
Da	MATa allele in serotype D strain
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
HIV	Human Immunodeficiency Virus
HS	Hypersuppressive petite
MAPK	Mitogen-activated protein kinase
MAT	Mating type
MATa	Mating type a
MAT α	Mating type α
mRNA	Messenger RNA
mtA	Type A mitochondrial genome
mtD	Type D mitochondrial genome
mtDNA	Mitochondrial DNA
ORF	Open Reading Frame
<i>Ori</i>	Replication origins
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
tRNA	Transfer RNA
UV	Ultraviolet

PREFACE

This thesis consists of seven chapters. The first chapter is a general introduction and part of its content was published as a review paper in a book. Chapters 2 to 6 have been written as manuscripts for publication in peer-reviewed scientific journals. At the time this thesis is submitted, chapters 2, 3 and 4 have been published. Chapter 5 and 6 are in preparation for submission to journals. Chapter 7 includes a general conclusion and future perspectives. Information about the title and authors to each chapter is listed below:

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CHAPTER 1: GENERAL INTRODUCTION,

Fungal mating system and mitochondria

Preface:

Part of this chapter was published as a review paper in a book.

Yan, Z. and Xu, J. 2005. Fungal mitochondrial inheritance and evolution. In *Evolutionary Genetics of Fungi* (J. Xu editor). Horizon Scientific Press, England. Pp. 221-252.

Dr. Xu and I discussed and organized the content. I gathered the information, prepared the figures, tables and wrote the manuscript. Dr. Xu helped interpret the data and edit the manuscript.

The fungal kingdom is one of the most abundant and diverse groups of organisms on earth. There are at least 100,000 known fungal species representing five principal fungal divisions: Ascomycetes, Basidiomycetes, Zygomycetes, Chytrids and Glomeromycetes (Moncalvo, 2005). These fungal species are found in virtually every terrestrial ecosystem as mutualists, pathogens, parasites or saprobes. The morphology of different fungal species are quite diverse ranging from unicellular organisms such as budding yeast to huge organisms as mushrooms in which a single individual can cover 600 hectares (Smith et al., 1992; Bollock, 1992). Despite their great diversity, relatively little is known about the processes that govern fungal population and evolutionary processes. In this thesis, I will examine the relationship between mating system and the mitochondrial genome in the model basidiomycete yeast *Cryptococcus neoformans* and how their relationships might have contributed to the natural population dynamics in this species.

1. Fungal mating system

Despite much debate on the precise benefit of sexual reproduction, sex is generally regarded as a process that can improve the fitness of an organism either by purging deleterious mutations or combining beneficial mutations. To allow efficient genetic exchange with unrelated individuals, many forms of genetic barriers exist to avoid self-crossing or inbreeding. The X-Y system is the most familiar sex-determining mechanism which ensures that the two mating partner are genetically different. Unlike the big X and Y chromosomes which determine the identity of the two mating partners in

more complex eukaryotic organisms, the identity of fungal sexual partner is established by a relatively short region in a chromosome, the mating type (MAT) locus (the region differs in DNA sequence between cells of opposite mating type). Here, the term “mating type” is used to differentiate individuals that are sexually compatible.

1.1 Classification of fungal mating system

Fungal mating systems are traditionally classified by two sets of criteria. First, based on the self-mating ability, the fungal mating system can be classified into homothallic and heterothallic to represent self-fertility and self-sterility respectively. Second, for the heterothallic species, based on the organization of the MAT locus, the fungal mating system can be classified as bipolar and tetrapolar. In organisms with a bipolar mating-type system, cell identity is dictated by a single MAT locus with two or more alternate alleles. For sexual reproduction to occur, mating partner cells must have different MAT alleles. In fungi with a tetrapolar system, by contrast, cell identity is determined by two unlinked regions of the genome. Both of these regions must differ for mating to occur. In many tetrapolar fungi, both mating-type loci are multiallelic, giving rise to potentially thousands of mating-types. In heterothallic ascomycetous fungi, only the bipolar mating-type system with only two alleles exists. In basidiomycetous fungi, both the bipolar and the tetrapolar mating systems exist and the number of alleles varies among species, from two alleles to dozens of alleles for each locus. Still in other fungi (about 20% of all known fungal species), no mating or meiosis has been observed. These fungi are traditionally grouped into the division of Fungi Imperfecti or Deuteromycota.

1.2 Structure and function of fungal mating type locus

In the last two decades, the MAT loci of many fungal species have been determined. The structure is quite diverse among different fungal species. Here, I will concentrate chiefly on four species *Saccharomyces cerevisiae* (a budding ascomycete yeast), *Neurospora crassa* (a filamentous ascomycete), *Cryptococcus neoformans* (a basidiomycetous yeast), and *Ustilago maydis* (the corn smut fungus, a dimorphic basidiomycete). These species are chosen here because they have been used as model organisms to study mating system structure and function in fungi and because they represent evolutionarily divergent systems.

1.2.1 The MAT locus in *S. cerevisiae*

The budding yeast *S. cerevisiae* has probably the best studied MAT locus. *S. cerevisiae* is a bipolar homothallic organism with two haploid mating types, a and α . Under nitrogen-limiting conditions, the haploid a and α cells can fuse and produce a diploid cell (a/ α) via a pheromone response pathway. The mating type of *S. cerevisiae* is determined by the MAT locus on chromosome III. The MAT locus of each mating type contains two genes: a1 and a2 in MAT_a locus; and α 1 and α 2 in MAT _{α} locus (Fig 1.1, A). Sequence analyses showed that α 1 (747 bp) and a1 (642 bp) are totally dissimilar genes, while the a2 and α 2 gene have partial homology (Nasmyth and Tatchell, 1980; Strathern et al., 1980; Astell et al., 1981). Except the function of a2 is unknown, the other genes encode three important cell fate determinants, the a1, α 1 and α 2 proteins. The a1 and α 2 proteins both contain a homeodomain DNA-binding motif which can homodimerize (α 2- α 2) or heterodimerize (a1- α 2) to repress the a-specific or haploid-specific genes

respectively. The third protein $\alpha 1$ is also a DNA-binding protein which can activate α -specific gene in mating type α cell (Herskowitz et al., 1992). Chromosome III also carries two other loci involved in mating type determination called HMR and HML. These two loci contain unexpressed or silent copies of MAT α and MAT α alleles respectively (Fig 1.1, A).

One unusual feature of the mating system in *S. cerevisiae* is the ability to switch mating types. Sometimes, the allele at the MAT locus can be excised and subsequently replaced in a process resembling transposition by the alternative MAT allele from one of the silent loci (HMR or HML). For example, the MAT α allele in the MAT locus can be replaced by MAT α allele from the silent HML locus which changes the original mating type a cell into mating type α . This process is mediated by a genetic recombination reaction that involves an endonuclease known as HO (Herskowitz et al., 1992). This unusual character of *S. cerevisiae* makes it a homothallic organism.

1.2.2 The MAT locus in *N. crassa*

N. crassa is a heterothallic bipolar organism with two alternate mating types, termed A and a. The MAT locus of type a strain is 3.2kb long with two transcription units (*mat a-1* and *mat a-2*). In contrast, the MAT locus of type A cell is about 5.3kb long and contains three genes, *mat A-1*, *mat A-2* and *mat A-3* (Fig.1.1, B). DNA sequence analysis showed that the two MAT loci were highly dissimilar and that they do not appear to have a common evolutionary origin. To better describe the MAT locus, a new term “idiomorph” was proposed to indicate sequences at the MAT loci which do not show homology between strains of opposite mating types (Metzenberg and Glass, 1990) in

contrast to the old term “allele” to denote homologous DNA sequences. Like genes in the MAT locus of *S. cerevisiae*, genes in MAT locus of *N. crassa* encode DNA binding proteins (Philly and Staben, 1994; reviewed by Coppin et al., 1997) which can regulate the expression of other sex-specific genes such as pheromone and pheromone receptor genes (Bobrowicz et al., 2002). Unlike in *S. cerevisiae*, only a single copy of mating type sequence exists in a haploid genome of *N. crassa*. The absence of DNA sequences of the opposite mating type in the same haploid genome explains that the mating type in *N. crassa* can not be switched.

1.2.3 The MAT locus in *C. neoformans*

C. neoformans has a heterothallic and bipolar mating system with haploid a and α cells. The MAT locus of *C. neoformans* is much bigger than that of the above two species. In total, the MAT locus in *C. neoformans* spans over 100 kb and contains about 21 genes (Lengeler et al., 2002; Fraser et al., 2004b). Each gene within the locus, with the exception of *SXI1 α* and *SXI2a*, has a counterpart allele that encodes a similar, but not identical, protein in the opposite mating type (Fig1.1, C). In contrast, *SXI1 α* and *SXI2a* are unique to their respective mating types. Both *SXI1 α* and *SXI2a* encode a homeodomain protein homologous to the $\alpha 2$ and $\alpha 1$ protein in *S. cerevisiae* respectively. Like the $\alpha 1$ and $\alpha 2$ proteins found in *S. cerevisiae*, *Sxi1 α* and *Sxi2a* proteins can form a heterodimer that can regulate sexual development. (Hull et al., 2002; 2005)

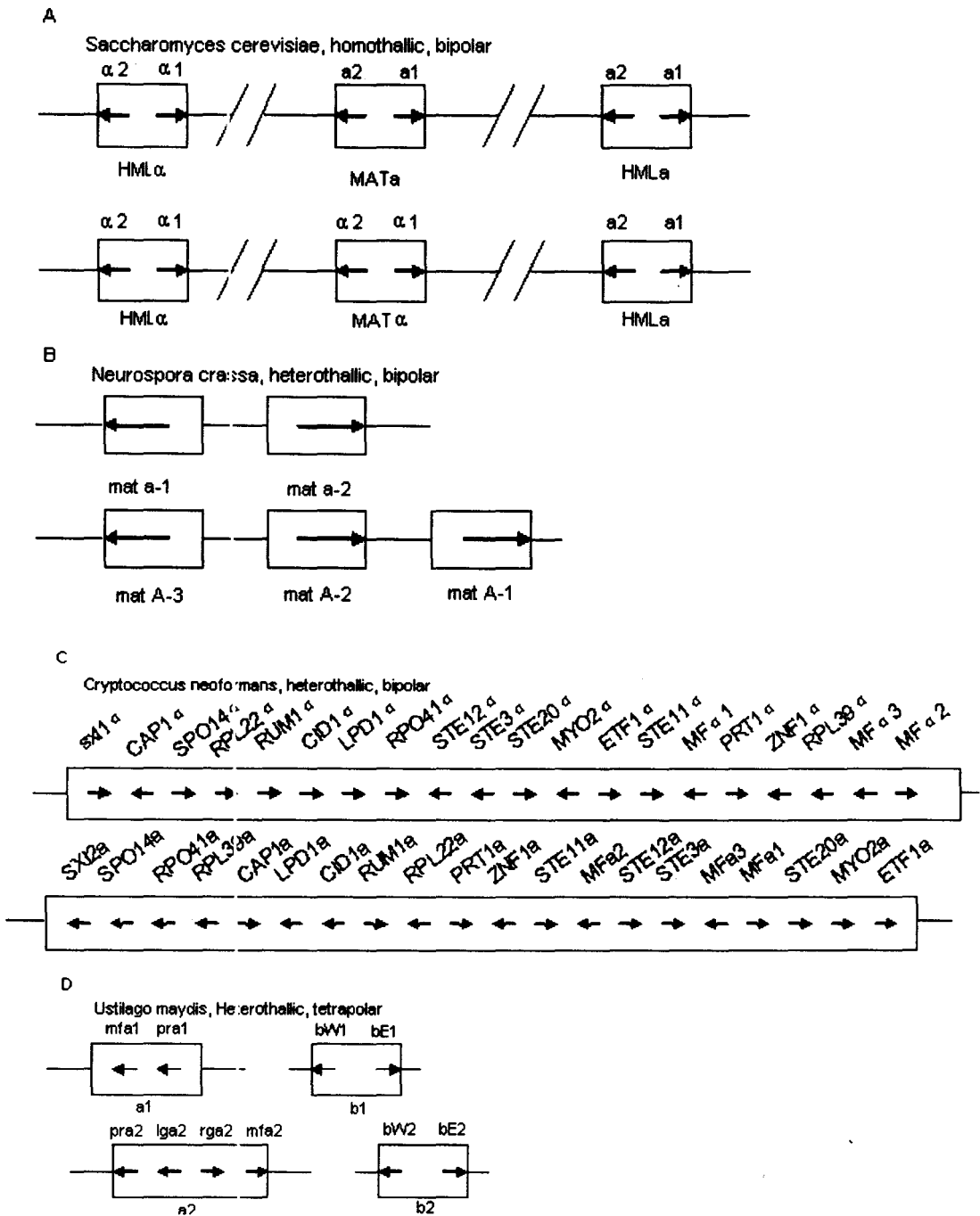


Figure 1.1 The structures of representative fungal mating type loci (revised from Fraser and Heitman, 2003 and Coppin et al., 1997).

Some of the other genes in the MAT locus encode proteins involved in pheromone-activated MAPK cascade that governs mating. There are also genes in the MAT locus whose role in mating, if any, is at present unknown. DNA sequence analysis indicated that recombination in the MAT locus was suppressed probably as a result of extensively gene inversion, rearrangement, divergence and the presence of transposons (Lengeler et al., 2002). The MAT locus of *C. neoformans* shares feature with both the traditional fungal MAT loci seen in *S. cerevisiae* and *N. crassa* and the larger, more complex sex chromosomes of animals. Indeed, the gene arrangement of MAT locus in *C. neoformans* has been suggested to represent an early step in the evolution of sex chromosome (reviewed by Fraser and Heitmain, 2004a).

The MAT locus of *C. neoformans* is also related to virulence. In an animal experiment, a MAT α strain was found to be more virulent than its congeneric MAT α strain (Kwon-Chung et al., 1992). However, later studies identified that this result is highly context dependent (Nielsen et al., 2005).

1.2.4 The MAT locus in *U. maydis*

U. maydis is a heterothallic basidiomycete with a tetrapolar mating system. The mating type in this species is determined by two loci, a and b, located on different chromosomes. For mating to occur, idiomorphs (alleles) at both of the loci must be different. The a locus is bi-allelic with two alternate idiomorphs termed a1 (4.5 kb) and a2 (8.0 kb) (Froeliger and Leong, 1991). Idiomorphs a1 and a2 each contains a gene for

pheromone (*mfa*) and a gene for pheromone receptor (*pra*), designated *mfa1* and *pra1* in a1 strain, and *mfa2* and *pra2* in a2 strain respectively (Bolker et al., 1992, Fig 1.1, D). In addition, the a2 idiomorph contains two other genes, *lga2* and *rga2* (Urban et al., 1996). The function of this pheromone receptor system in *U. maydis* is very similar to that in the budding yeast where the pheromone and its receptor are also expressed by the two mating partners that control the initial recognition and fusion of haploid cells. However, the pheromone receptor system in budding yeast is not a master regulator of sexual development as it is in *U. maydis* (Herskowitz, 1989). The other two genes in the a2 idiomorph (*lga2* and *rga2*) are not necessary for mating and their functions are largely unknown (Urban et al., 1996).

The *b* locus in *U. maydis* is multi-allelic and encode multi-version of homeodomain protein. In total, 25 different versions of *b* alleles have been discovered. Each version of the *b* alleles contains two genes termed *bE* and *bW* (Fig 1.1, D). The *bE* and *bW* genes have low sequence similarity but different version of *bEs* (e. g. *bE1* vs *bE2*) or *bWs* (e. g. *bW1* vs *bW2*) are nearly identical except in the amino-terminal region (Kronstad and Leong, 1990; Schulz et al., 1990; Gillissen et al., 1992). The amino-terminal region has been shown responsible for the allelic specificities of the proteins. In vitro substitutions of residues in this region can influence the specificity (Kamper et al., 1995; Yee et al., 1993). The DNA sequence variation at the amino-terminal region and the 260 bp intervening region between *bE* and *bW* genes were sufficient to prevent homologous recombination between different allelic versions of the *bE* and *bW* genes. In genetic crosses, these two genes inherit as a single unit. Like the homeodomain genes in

S. cerevisiae and *C. neoformans*, different version of bE and bW proteins (eg. bE1+bW2 or bE2+bW1) can form heterodimer which can regulate the development of dikaryotic filamentous mycelium and sexual reproduction. (Gillissen et al., 1992).

The tetrapolar mating system in *U. maydis* can promote out-crossing and decrease inbreeding. For example, in a mating between two haploid strains with genotype a1-b1 and a2-b2, progeny with four mating types may be produced, a1-b1, a1-b2, a2-b1 and a2-b2. However, random encounters among progeny may result in about only 25% of successful mating. Specifically, for example, the progeny with mating type a1-b1 can only mate with the a2-b2 sibling but not with siblings with a1-b1, a1-b2 or a2-b1 mating types. Another feature of the tetrapolar mating system in *U. maydis* is that the MAT locus is bi-allelic for the a locus and multi-allelic for the b locus. Recombination between the two loci can give rise to dozens of different mating types in *U. maydis*.

1.3 Evolution of fungal mating system

1.3.1 Sexual vs asexual

Asexual species also widely exist in fungi. The absence of sex in these species is not due to the lack of mating type genes. In all the asexual species which have been examined, mating type genes have been found (Coppin et al., 1997). Instead, the loss of sex in these species could be the result of mutations in the mating type gene(s) or the targets of mating type genes. For example, in the asexual ascomycetes, *Bipolaris sacchari*, a gene highly similar (with 98% identity) to the *MAT-2* gene of its related species, *Cochliobolus heterostrophus*, has been identified. Introduction of the *MAT-2*-like

gene from *B. sacchari* into the *C. heterostrophus* induced sexual development. In contrast, when the MAT genes of *C. heterostrophus* were transformed into *B. sacchari*, the recipient could undergo neither selfing nor crossing with other *B. sacchari* strains. The results suggest that the lack of sex in *B. sacchari* is not due to the lack of mating type genes but is probably due to a mutation on their target genes (Sharon et al., 1996). In the human pathogenic fungus, *C. neoformans*, the spontaneous mutation rate of loss of sex was estimated to be in excess of 0.0172 (per genome per asexual division) (Xu, 2002).

Another possible scenario to explain the existence of asexual species in fungi is that the 'asexual' species are in fact sexual. For instance, the pathogenic yeast *Candida albicans* had been thought to be asexual. However, recent researches found that this species contains MAT genes sharing striking features with those from *S. cerevisiae* (Hull and Johnson, 1999). Under certain conditions, *C. albicans* is still able to undergo mating although, despite multiple attempts, meiosis has not been convincingly demonstrated (Magee and Magee, 2000; Miller and Johnson, 2002).

1.3.2 Homothallism vs heterothallism

Sexual fungi have adopted two different reproduction strategies: homothallic (self-fertile) and heterothallic (self-sterile). This difference raises the problem of evolutionary relation between different mating systems: is homothallism ancestral to heterothallism or vice versa?

By comparing the structure of MAT loci from homothallic and heterothallic *Cochliobolus* species, Yun and his colleagues (1999) found that homothallic species carry

both MAT genes closely linked in a single nucleus, in contrast to heterothallic species, which have alternate MAT genes in different nuclei (Fig 1.2). This result suggests that the homothallic reproductive mode may be converted from heterothallic mode through a recombination event between different MAT genes (Fig 1.2). The expression of a fused MAT gene from a homothallic species confers self-fertility on a MAT-null strain of a heterothallic species. These results therefore provided strong evidence supporting the hypothesis that heterothallism is ancestral.

Yun's experiment (1999), however, can not completely exclude the possibility that homothallic is the ancestral to heterothallic. A recent research demonstrated that the reproductive mode can also be shifted from homothallic to heterothallic by deleting one of the linked MAT genes of the homothallic ascomycetous fungal, *Gibberella zeae* (Lee et al., 2003). The evolutionary relation between homothallic and heterothallic is reminiscent of the story about the chicken and egg problem.

In the genus of *Neurospora*, the homothallic species tend to occur at extremes of *Neurospora*'s ecological niches suggesting that the homothallic mating system could be adaptations to ensure sexual reproduction when encounters between unlike genotype are rare (Kronstad, 1997).

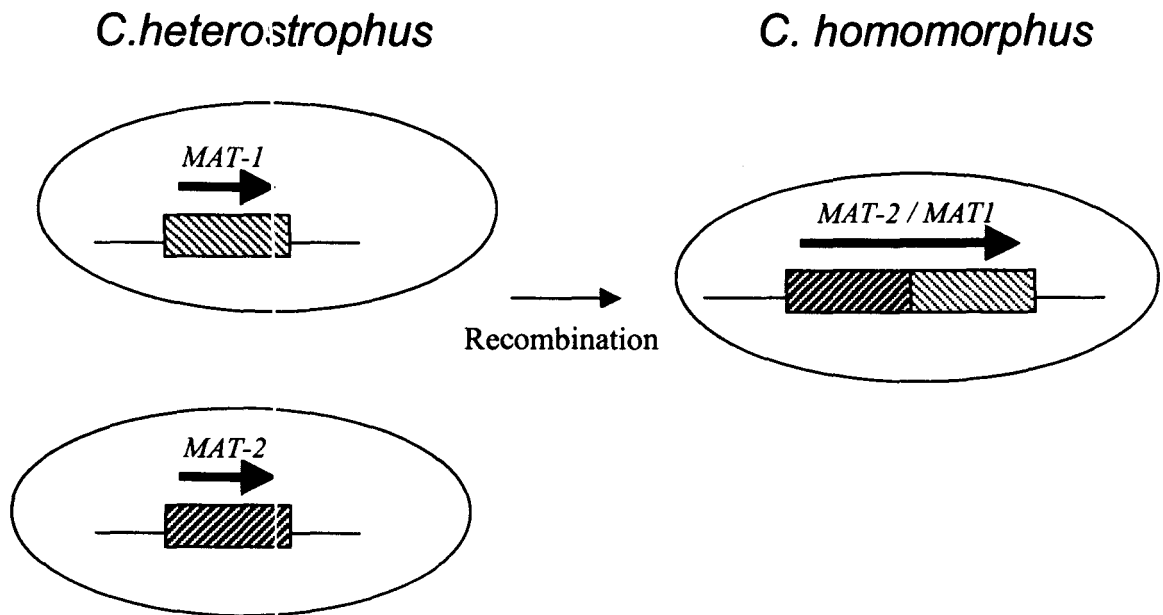


Figure 1.2 A demonstration of conversion from heterothallic to homothallic organisms. The *MAT-1* and *MAT-2* loci in *Cochliobolus homomorphus* corresponding to the *MAT* loci in its heterothallic relative (*C. heterostrophus*) fused, which resulted a homothallic mating system (revised from Yun et al., 1999).

1.3.3 Bipolar vs tetrapolar mating system

In heterothallic basidiomycete fungi, both bipolar and tetrapolar mating system exists. By comparing the *MAT* loci between two species in the same genus *Ustilago*, Bakkeren and Kronsad (1993; 1994) proposed a molecular explanation for the difference between bipolar and tetrapolar mating system. They found that the *a* and *b* loci are physically and genetically linked in the bipolar organism, *Ustilago hordei*, in contrast to the arrangement of *a* and *b* loci in the tetrapolar organism, *U. maydis*, where the two loci were located on different chromosomes (Fig 1.3). Further experiments determined that the *MAT* locus of *U. hordei* spans 500 kb in a *MAT-1* strain and 430 kb in a *MAT-2* strain and recombination was suppressed within the *MAT* region (Lee et al., 1999).

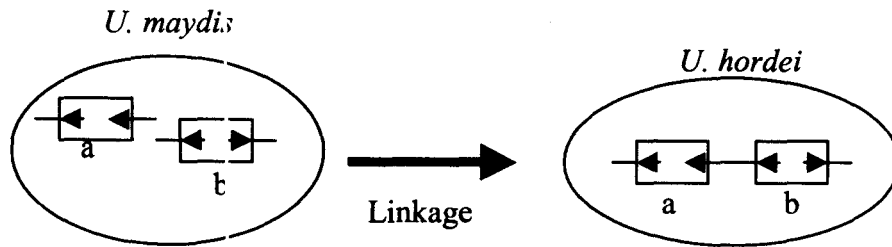


Figure 1.3 A demonstration of conversion from tetrapolar to bipolar organisms. The a and b MAT loci in *U. hordei* corresponding to the MAT loci of its tetrapolar relative, *U. maydis*, are linked, which result in a bipolar mating system (revised from Bakkeren and Kronstad, 1993).

1.4 Population structure of mating type alleles

Unlike many animals and plants, fungal species can usually reproduce both sexually and asexually. As a result, a fungal species may not undergo sexual reproduction very often even though it contains functional MAT alleles. A straightforward method to determine if a fungal species reproduce sexually in natural environment is to detect the population structure, including the distribution of MAT alleles. A sexually reproductive fungal would present a near-equal frequencies of different MAT alleles as a result of negative frequency-dependent selection that selects for rare MAT alleles (May *et al.* 1999). In contrast, a primarily asexually reproductive fungal may present a biased MAT allelic ratio. This same selective pressure tends to equilibrate allele frequencies among populations (Wright, 1939; Zambino *et al.* 1997).

Many studies have attempted to determine population structure of MAT alleles for various heterothallic fungi in the past. The results shows that the frequency of MAT alleles are near equal in some fungi species, such as *Schizophyllum commune* (Raper *et al.*, 1958), *Rhynchosporium secalis* (Linde *et al.*, 2003), *Tapesia acufiformis* and *T.*

yallundae (Douhan et al., 2002), *Mycosphaerella graminicola* (Zhan et al., 2002), *Ascochyta rabiei* (Armstrong et al., 2001), some *Cryphonectria parasitica* populations (Liu et al., 1996), and *Cochliobolus carbonum* (Welz and Leonard, 1995). These results suggest that sexual reproduction may be a regular feature in populations of these species. There were also some fungal species with skewed MAT allelic ratios, consistent with infrequent or a lack of sexual reproduction. Examples of fungi with highly biased MAT alleles include, *Magnaporthe grisea* (Dayakar et al., 2000 ; Mekwatanakarn et al., 1999 ; Notteghem and Silue, 1992 ; Viji and Uddin, 2002; Consolo et al., 2005) and *Ascochyta fabae* f. sp. *lentis* in Canada (Ahmed et al., 1996).

2. Fungal mitochondrial genome

The mitochondrion as an organelle related to respiration and energy production is unique due to its monophyletic origin and non-Mendelian pattern of inheritance. In recent years, the fungal mitochondrion has attracted special attention because of its influence on virulence, mycelial growth, senescence (Esser and Tudzynski, 1977; de la Bastide *et al.*, 1997; Olson and Stenlid, 2001) and its role in the studies of phylogenetics.

2.1 Structure and size of fungal mitochondrial genome

2.1.1 Forms of fungal mitochondrial genome

Compared to the nuclear genome, the mitochondrial genome is much smaller, yet it contains some essential genes. Table 1.1 listed 40 fungal mitochondrial genomes available in NCBI (National Center for Biotechnology Information) and FMGP (Fungal

Mitochondrial Genome Project). Most fungi species in Table 1.1 have a circular mitochondrial genome. It should be noted that not all of the mitochondrial genomes in these species are circular as we had expected. In contrast, the mitochondrial genomes in some of these species have been shown to consist predominantly of linear, multimeric head-to-tail concatamers in vivo (Bendich 1993; 1996). This observation is not in contradiction to the fact that most of the species in Table 1.1 were mapped as circular molecules considering the rolling-circle replication mechanisms (Maleszka, 1991) which can produce a lot of linear molecules. The mitochondrial genomes of five fungal species (*Candida parapsilosis*, *Hanseniaspora uvarum*, *Candida metapsilosis*, *Kluyveromyces thermotolerans* and *Hyaloraphidium curvatum*) in Table 1.1 are considered as true linear because they appear to have specific telomeric structures that protect the ends from degradation and ensure that sequence information is not lost during replication (Forget *et al.* 2002; Nosek *et al.* 1995; 1998).

The physical forms of the mitochondrial genome may vary in closely related organisms or even among strains within the same species. For example, an extensive survey of *C. parapsilosis* isolates uncovered that nearly all strains from groups I and III possessed linear mtDNA molecules terminating with arrays of tandem repeat units, while most of the group II strains have a circular mitochondrial genome. In addition, it was found that strains belonging to different groups appear to have similar genes in the same gene order (Rycovska *et al.*, 2004). The highly conserved genetic organization and homology of the coding regions among linear- and circular-mapping mitochondrial genomes found in *C. parapsilosis* strains indicate that both forms originated from a

common ancestor via a relatively simple mechanism. Up to date, the origin of mitochondrial telomeres is still unknown. Nosek & Tomaska (2002; 2003) proposed that mitochondrial telomeres might have evolved from mobile genetic elements (e.g. transposons, plasmids, telomeric minicircles) that invaded mitochondria, integrated into a circular-mapping mtDNA and eventually resulted in the formation of linear mtDNA molecules of defined length, terminating with specific telomeric structures. This view was supported by the observations found in other eukaryotes that integration of linear mitochondrial plasmid into circular-mapping mitochondrial genomes of *Zea mays* (Scharidl *et al.* 1984, 1985) and *Physarum polycephalum* (Takano *et al.* 1996; Sakurai *et al.* 2000) resulted in the formation of linear mtDNA molecules terminating with the sequences of plasmid telomeres.

2.1.2 Size and composition of fungal mitochondrial genome

Compared to the mitochondrial genome in animals, the fungal mitochondrial genome is much less compact and its size is highly variable range from 19 kb to more than 100 kb (Table 1.1). The size difference is mainly due to the variation in the number of genes and the number and length variations in introns and intergenic regions. The genes encoded by mtDNA fall into three groups. The first group encode hydrophobic subunits of respiratory chain complexes including apocytochrome b; cytochrome oxidase subunits 1, 2, and 3; NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5 and 6; and ATPase subunits 6, 8 and 9. The second group of genes are involved in mitochondrial protein synthesis, including genes encoding the large and small ribosomal RNA (rRNA), a set of tRNAs, a ribosomal protein associated with the small rRNA (Rp), and the RNA

component of RNase P (*rnpB*). The third group of genes consists of intron-encoded ORFs, maturases as well as unidentified ORFs etc. Table 1.1 shows that the gene complement is quite variable among different fungal species. For example, the *NAD* genes encoding NADH dehydrogenase subunits are absent from 12 fungi species in Ascomycota (e. g. *Saccharomyces cerevisiae*). The *ATP9* gene exists in the mitochondrial genome of most sequenced fungal species but it is absent from *Podospora anserina* mitochondrial genome. Another case of difference is the number of tRNAs among different species. More than 20 tRNAs are present in all ascomycete, basidiomycete and zygomycete mitochondrial genomes. In contrast, six out of seven chytridiomycetes have only 7-9 tRNAs. *Allomyces macrogyns* is the only species in chytridiomycetes with a complete set of tRNAs. These missing genes in the mitochondrial genomes must have been transferred from ancestral mitochondria to nucleus, where they acquired proper expression and re-imported into the organelle. Ricchetti and his co-workers (1999) found that fragments of mitochondrial DNA, in single or tandem array, could be transferred to yeast chromosomes under natural conditions during the repair of double-strand breaks in haploid mitotic cells, which suggested that the DNA transfer from mitochondria to nucleus may be an ongoing process in yeast. The gene transfer from ancestral mitochondria to nucleus is also confirmed in many other eukaryotes by genome sequence analysis (The Rice Chromosome 10 Sequencing Consortium, 2003; Stupar *et al.* 2001; Mourier *et al.*, 2001).

Table 1.1 also shows that some genes are maintained in all fungi species, including *ATP6*, *ATP8*, *COX1*, *COX2*, *COX3*, *RNL*, *RNS* and several *tRNAs*. The

maintenance of these genes but not other genes leads to the question as to why these genes were left in mitochondria at all species? A possible explanation was put forward by Allen (2003). His argument was simple: the expression of some organellar genes is required to be under the direct regulatory control of the redox state of their gene products so that they can synthesize those components as they are needed to maintain redox balance, thus avoiding the production of reactive oxygen species, which are exceedingly toxic. Another possible explanation is that particularly hydrophobic proteins cannot be imported by organelles, and hence must be synthesized *de novo* in the mitochondria.

Other differences among fungal mitochondrial genomes include the number of ORFs, introns and non-coding intergenic regions. For instance, there are 39 ORFs in the *P. anserina* mitochondrial genome but only 1 in the *Saccharomyces castellii* mitochondrial genome (Table 1.1). The variation in the number of introns is another important factor influencing genome size, even among strains within the same species. For example, Zimmer et al (1987) demonstrated that the mitochondrial genome sizes of 26 different *Schizosaccharomyces pombe* strains vary between 17.6 and 24.6 kb primarily due to the presence or absence of introns. The length of intergenic regions can also vary considerably among closely related species. This was demonstrated in many genera, such as *Saccharomyces* in which mitochondrial genomes vary from 25kb to 85kb (Petersen *et al.* 2002), *Schizosaccharomycetes* in which mitochondrial genomes vary from 17 to 80kb (Bullerwell *et al.*, 2003b) and *Monoblepharidales* in which mitochondrial genomes vary from 19 to 60kb (Bullerwell *et al.*, 2003a). The intergenic regions are the main reasons of genome size variation among species within these three genera.

S. punctatus is the only species found so far in fungi with three divided mitochondrial circular molecules (Table 1.1). The largest molecule spans about 58830 bps and includes most of the genes necessary for mitochondrial function. The other two molecules span 1381 bps and 1136 bps respectively. One of the smaller molecules contains the *ATP9* gene which encodes ATPase subunit 9, whereas the other has no identifiable gene. The organization of mitochondrial genome in *S. punctuatus* is very similar to those of plants whose mitochondrial genomes are commonly divided into several molecules, called “subgenomic” mtDNAs.

2.2 Fungal mitochondrial inheritance

The first case of fungal mitochondrial inheritance was reported in *Neurospora crassa* (Mitchell and Mitchell, 1952). In this species, the slow growth phenotype “poky” was transmitted to virtually all the progeny of crosses between poky female (the strain contributes a large gamete called protoperithecium) and normal males (the strain contributes a relatively smaller gamete called microconidium), very similar to the maternal mtDNA inheritance pattern found in typical plants and animals. The following studies on mitochondrial inheritance in other filamentous fungi species demonstrate many different inheritance patterns. However, due to the inaccurate definition of mitochondrial inheritance and complicated fungal mating process, some of the interpretations contradict each other. For example, the formation of the heterokaryotic mosaic of two mitochondrial genotypes by reciprocal nuclear migration (Fig 1.4, A, see below, the fertilization mechanism) in some basidiomycetes has been considered as uniparental inheritance by

some researchers (Eaptista-Ferreira *et al.*, 1983; Specht *et al.* 1992), whereas this same phenomenon was interpreted as biparental inheritance by others (Hintz *et al.*, 1988; Smith *et al.*, 1990).

2.2.1 The traditional mtDNA inheritance classification system

In typical textbooks, uniparental mitochondrial inheritance is defined as a pattern of inheritance in which only one parent provides mitochondrial genes to the progeny. Based on this definition, organisms are traditionally classified into two types: biparentally inherited organisms and uniparentally inherited organisms, depending on if their sexual progeny possess mtDNA from both parents. This classification system comes mainly from typical animals and plants in which mating happens by the fertilization of eggs in a specialized sexual structure. The zygotes then develop in this structure. Once mature, the zygote leaves the body of the female parent and grows independently. Typically the progeny will possess the same mtDNA as their female parent, hence these organisms are classified as having a uniparentally mitochondrial inheritance. However, this division system is not perfect, especially when used to classify mitochondrial inheritance of fungi, for the following two reasons. First, classifying organisms into mtDNA biparentally or uniparentally inherited is somewhat artificial. It is now known that there are rare purely biparentally or uniparentally inherited organisms. On the one hand, for organisms thought to have mtDNA biparentally inherited, their mtDNA transmission is not completely biparental. For example, the most thoroughly studied organism with extended biparental inheritance is the isogamous yeast *Saccharomyces cerevisiae*. Genetic analysis indicates that mitochondrial transmission in this organism is not purely biparental. There is a

mechanism to ensure that part of the progeny inherited mtDNA from only one parent (see below, Zygotic mechanisms). On the other hand, for organisms thought to have mtDNA uniparentally inherited, their mtDNA transmission is not absolutely uniparental. This phenomenon was found in many fungi where occasional mtDNA recombination was discovered although these fungi are traditionally considered as having uniparental mtDNA inheritance (Baptista-Ferreira *et al.*, 1983; Barroso *et al.*, 1997; Chung *et al.*, 1996; Fukuda *et al.*, 1995; Matsumoto and Fukumasa-Nakai, 1996). Furthermore, the lack of recombination of mtDNA in the animal kingdom has been questioned (Gylensten *et al.*, 1991; Kraytsberg *et al.*, 2004). As a result, Birky (1995) argued that it is more appropriate to treat mtDNA inheritance as a quantitative trait, and hence should present the frequency distribution of biparentally and uniparentally inherited progeny when describing mitochondrial inheritance pattern in a certain organism. Second, the traditional classification system did not consider the complexity of fungal mating process. Unlike mating in plants and animals in which the two parents are separated from each other after mating and the progeny leaves the body of their female parent after maturity, mating in fungi (especially filamentous fungi) usually results in physical connection between the two parents through an intermediate or conjunction zone and form a united “super” colony. This physical connection raises a problem on how to treat the super colony. If we take the super colony as just one progeny (zygote), this fungus should be classified as having a biparentally mtDNA inheritance because the super colony contains mtDNA from both parents. Otherwise, if we only treat some of the cells in this super colony as one progeny, this filamentous fungus would be classified as uniparental mtDNA

inheritance because each cell inherited mtDNA only from one parent. A good example to explain this complicated mating process is the basidiomycete *Coprinus cinereus*. In this species, mating occurs between vegetative mycelia with compatible mating types (i.e. different mating type alleles). Following hyphal anastomosis, there is a reciprocal exchange of nuclei with migration of donor nuclei through the established cells of each recipient monokaryon. This establishes cells with the dikaryotic pair of nuclei but because there is no accompanying exchange of mitochondria, the dikaryon which grows out has exclusively the cytoplasm of recipient mycelium. Cytoplasmic mixing is limited to the narrow conjunction zone where hyphae fuse between parental mycelium. Thus, the mating in *Coprinus cinereus* results in a united super colony composed of sectors which differ in mtDNA (Fig 1.4, A, May and Taylor, 1988). Clearly, in this case, if we treat the super colony as one progeny, *Coprinus cinereus* should be classified as a biparentally inherited organism because the progeny contains mtDNA from both parents although cells with different mtDNA are located in different regions. Otherwise, it should be classified as a uniparentally inherited organism. To avoid confusion, in the following discussions, we treated individual cells from the super colony instead of the whole colony as one progeny.

2.2.2 Possible mechanisms of fungal mitochondrial uniparental inheritance

The underlying mechanisms of uniparental inheritance can act at different stages of the sexual cycle: at the prezygotic, fertilization and zygotic stages.

2.2.2.1 Prezygotic mechanism

Prezygotic mechanism refers to the differential number of mitochondrial genomes among gametes generated during gametogenesis. It mainly applies to ascomycetes (eg. *Neurospora crassa*) in which sexual cross can be achieved by nuclei from conidia (male) entering trichogynes and traveling to female nuclei in the protoperithecia. In this case, the paternal and maternal contributions of mtDNA are widely different, with most mtDNA coming from the maternal parent. The consequence is that mtDNA from the paternal parent will be difficult to be detected in the progenies (Fig 1.5).

2.2.2.2 Fertilization mechanism

Fertilization mechanism refers to mtDNA of one gamete failing to enter the other gamete and thus being excluded from the zygote. In some fungi, the nuclei and mitochondria have different mechanisms for movement (Oakley and Rinehart, 1985). As a result, the nuclear migration from one parental cell to the other parental cell may not be accompanied by its mitochondria. This nuclear migration mode may play a major role in controlling mitochondrial inheritance in many fungi. In basidiomycetes, sexual crosses are usually made first by placing two plugs of monokaryotic mycelium some distance from each other on a plate. Based on the distribution of nuclear and mitochondrial genetic markers in the mating plate, different nuclear and mitochondria migration modes have been identified. For example, by placing two *Coprinus cinereus* inocular cubes 3cm apart on YpSs/2 agar, May and Taylor (1988) found that the nuclei migration manifest three different patterns followed by hyphal fusion (via herterokaryosis). In most cases, the pattern of migration was scored as bilateral (both parental strains donate and accept nuclei) because both original colonies were dikaryotized (Fig 1.4, A). In several other

cases, the migration pattern scored as unilateral (one strain would donate but not accept nuclei) (Fig 1.4, B). There were also cases recorded as no nuclei migration because only in the junction zone were dikaryons recovered (Fig 1.4, C). Because the mitochondria do not migrate at all, the newly formed dikaryon will contain only the mtDNA resident in the mated monokaryon.

2.2.2.3 Zygotic mechanism

The mitochondrial uniparental inheritance may also be determined after zygote formation by stochastic transmission, selective replication and/or destruction of mitochondria from one parent. The stochastic transmission process probably plays an important role in determining uniparental mtDNA inheritance in the yeast *Saccharomyces cerevisiae*. Figure 1.6 shows how the process works in *S. cerevisiae*. After formation of zygote, mtDNA from the two parents didn't mix well. The incomplete mixing of mtDNA was confirmed by cytological observations (Nunnari *et al.*, 1997). The subsequent stochastic budding produces three types of buds, the end zygotic buds inherited mtDNA predominantly from the parent which give rise to that end of the zygote while buds arising from the middle position have mitochondria from both parents (Strausberg and Perlman 1978; Zinn *et al.*, 1987).

In the zygote, selective replication of mtDNA from one parent is another possible mechanism to generate uniparental mtDNA inheritance. A good example of this mechanism is the mating between hypersuppressive petite (HS) and wild type yeast strains (Blanc and Dujon, 1980; de Zamaroczy *et al.*, 1981). A HS mitochondrial genome consists of small segments of the wild type genome, repeated to produce a molecule of

approximately normal size. Recent study revealed that the HS mitochondrial genome consists, in fact, many replication origins (MacAlpine, et al., 2001). The high density of *ori* sequences in the HS mitochondrial genome relative to wild type genome may account for hypersuppressiveness because the mtDNAs would replicate more efficiently for components of the replication apparatus (MacAlpine *et al.*, 2001).

Other zygotic deterministic mechanisms on uniparental mtDNA inheritance involve targeted destruction of mitochondria from one parent after forming zygote. This mechanism is supported by the evidence found in many other eukaryotes. For example, in the model green alga *Chlamydomonas reinhardtii*, the selective degradation of chloroplast DNA from the mt- parent was supported by the discovery that a mt+ gamete-specific nuclease targets mt- chloroplast during sexual reproduction (Nishimura et al., 2002). In mammals, selective destruction of sperm mitochondria inside fertilized cow and monkey eggs were supported by the discovery that sperm mitochondria are tagged by the recycling marker protein ubiquitin. Ubiquitin is a protein that binds to other proteins and marks them for degradation by the 26S proteasome or engulfment and lysis by lysosome or other vacuoles in the cell (Sutovsky et al., 1999). Up till now, there is no report that selective degradation of mitochondria happens in fungi. In many fungal species, the mechanism of uniparental mitochondrial inheritance remains a mystery. For example, in *Agaricus bisporus*, the formation of the heterokaryon only occurs at the junction zone by hyphal anastomosis between the parental homokaryons. No evidence of nuclear migration was observed. As a result, the uniparental mtDNA inheritance cannot be explained by prezygotic or fertilization mechanisms as did in many other fungi (Jin et al.,

1992). In *Neurospora tetrasperma*, 3 days after hyphal fusion, the mtDNA of nuclear acceptor strain replaced the nuclear donor's mtDNA throughout the entire colony. Clearly, the prezygotic and fertilization mechanisms cannot be used to explain the disappearance of the nuclear donor's mtDNA (Lee and Taylor, 1993). It is possible that selective degradation or replication mechanisms worked in these cases with one mtDNA type selectively destroyed or replicated.

2.2.3 Mode of mtDNA inheritance in fungi

To better describe fungal mtDNA inheritance, we classified fungi species into the following four different types: (1) yeast-like, (2) mussel-like; (3) plant-like and (4) *Agaricus bisporus*- like types.

Three species in Table 1.2 were classified having a yeast-like mitochondrial inheritance pattern. All these three species are yeast, including the most thoroughly studied species, *S. cerevisiae*. Mating in these three species begins with the fusion of yeast cells and usually results in a high frequency of progeny with mtDNA from both parents.

The only species belongs to the mussel-like pattern is *Ustilago violacea* (Table 1.2). Mussel (*Mytilus edulis*) is an animal belonging to the bivalve family. The mtDNA inheritance in this animal is very unique and depends upon the sex of the offspring. In this species, male progeny receive mtDNA from both the father and mother. Female progeny, however, receive mtDNA only from the mother (Skibinski *et al.*, 1994a; Skibinski *et al.*, 1994b; Zouros *et al.* 1994). Similar to the mtDNA inheritance mode in mussel, the transmission of mtDNA in *U. violacea* is influenced by the sex (the mating

type) of the progeny. After mating cells of opposite mating types (a1 and a2), Wilch and his co-workers (1992) found that mtDNA was inherited differently between a1 and a2 progeny. For progeny with the a1 allele, mtDNA from either parent was observed equally frequently. However, in progeny with the a2 mating type, the mtDNA was predominantly (94%) inherited from the a2 parent.

Table 1.2 shows that most fungal species are classified as plant-like type. In a typical cross in plants, both parents can act as female and male parent through reciprocal fertilization (hermaphrodite), which gives rise to progeny with two different mitochondrial types depending on the sampling location. For example, assuming there are two plants, parent 1 and parent 2. After mating these two plants through reciprocal fertilization, seeds can be sampled from either of the two parents. Seeds sampled from parent 1 will inherit mtDNA from parent 1, whereas seeds sampled from parent 2 will inherit mtDNA from parent 2. As a result, the mtDNA inheritance mode in plant ensures that the mtDNA from both parent are transmitted to their progeny. In fungi, a good example of the plant-like mtDNA inheritance type is seen in *C. cinereus*. Sexual cross in *C. cinereus* results in a mitochondrial mosaic through bilateral nuclei migration without accompanying mitochondria migration (Fig 1.4, A). In this case, mtDNA from both parents are transmitted to their progeny and the mtDNA in the progeny varies depend on where the progeny is sampled. In some mating combinations in *C. cinereus*, the nuclei migration is unilateral with one strain donating nuclei while the other only accepting nuclei (Fig 1.4, B). This asymmetric mating behavior is similar to the cross between a male sterile plant and a wild type plant in which the male sterile plant only accepts pollen

and the wild type plant can both accept and donate pollen. It should be noted that the mtDNA inheritance pattern in some fungal species classified as having a plant-like type may not be completely the same as that of plant. For instance, in *Pleurotus ostreatus*, some progenies sampled from the junction zone inherited mtDNA from both parent (Matsumoto and Fukumasa-Nakai, 1996), which is different from the mtDNA inheritance mode in plant. However, considering that these progeny can only be found in a very narrow region, we still classified *P. ostreatus* as having a plant-like mitochondrial inheritance pattern.

The mtDNA inheritance in *Agaricus bisporus* appears different from that of other fungal species (de la Bastide and Horgen, 2003). After mating, the progeny inherited mtDNA predominantly from one parent (hence different from the inheritance of yeast-like species in which has a high-frequency-biparentally inherited progenies). This inheritance mode is also independent of sex and sample location (hence different from mussel-like and plant-like mtDNA inheritance). In addition, the mtDNA inheritance in *Agaricus bisporus* is strain dependent. For example, the mtDNA of strain n97 is predominantly transmitted to the progeny, when strain n97 was mated with some strains (e. g. b70/5 × n97, b55/1 × n97, c40/1 × n97, and b67/11 × n97), but not when it was mated with others (e. g. 129/1 × n97 and b69/6 × n97).

It is worth noting that mtDNA inheritance in fungi (see above) is different from that in a typical animal (Giles et al., 1980). In a typical animal (e.g. in humans), mtDNA inheritance is sex independent. In another word, no matter what sex the progeny is and

where you sample it, the progeny inherits mtDNA predominantly from female parent (maternal inheritance).

2.2.4 Factors influence mtDNA inheritance pattern

Up to date, genes controlling mitochondrial inheritance are still largely unknown. However, two genes (*MAT3* and *EZY1*) found in *Chlamydomonas* are involved in uniparental transmission of chloroplast genes (Gillham, 1987; Armbrust, 1993). *MAT3* is a MAT-linked gene and mutation in *mat3* disrupted the uniparental transmission of chloroplast in *Chlamydomonas*. Unfortunately, the *MAT3* gene is still not isolated and its identity is unknown. Armbrust (1993) found the second MAT-linked gene, *EZY1* that participates in the uniparental inheritance of the chloroplast genome in *Chlamydomonas*. This finding was supported by the fact that *EZY1* was expressed within minutes of zygote formation and its gene product localized to chloroplast nucleoids. Importantly, *EZY1* expression displayed the same sensitivity to UV irradiation as uniparental chloroplast inheritance, suggesting that *EZY1* potentially plays a role in the destruction of mt-chloroplast.

Beside genetic factors, non-genetic factors can also influence mtDNA inheritance. For example, ammonium chloride treatment can influence mitochondrial behavior in animals (Sutovsky, 2000). In *Chlamydomonas*, many other environmental factors, e.g. UV irradiation and blue light exposure (Sager and Ramanis, 1967; Beckers et al., 1991), have been identified influencing the chloroplast inheritance. To our knowledge, there is no similar experiment done in fungi up to date.

2.2.5 Why uniparental inheritance?

In the majority of eukaryotes, mitochondrial genomes are inherited almost exclusively from a single parent (Birky, 1983). The widespread uniparental inheritance pattern is surprising considering that a biparental inheritance could avoid Muller's Ratchet (Muller, 1964) and compete with the potentially fast evolving parasites (Red Queen hypothesis). Birky (1995) explained that the advantage of biparental inheritance on nuclear genomes may not apply to mitochondrial genomes and the efficiency of natural selection may be only slightly reduced by uniparental inheritance for the following reasons: First, recombination in biparental inheritance may facilitate the repair of chromosome damage; but mitochondrial genome usually present many copies per cell, hence biparental mtDNA inheritance may provide little additional advantage. Second, the number of genes in mitochondrial genome is much smaller than that in the nuclear genome, thus the contribution of biparental inheritance to linkage disequilibria between mitochondrial genes is much smaller than that between nuclear genes. It is possible that the complete loss of biparental inheritance of mitochondrial genes will have the same effect on selection as a very modest decrease in recombination frequency of nuclear genes. For this reason, the loss of biparental inheritance in mitochondrial genome may only slightly decrease the fitness of their host organisms. Third, the uniparental inheritance is not absolute, the existence of low levels of biparental mitochondrial inheritance and recombination could offset part of their host's fitness losses and slow the ratchet. In fungi, more or less biparental mitochondrial inheritance and recombination was demonstrated in either laboratory crosses or through phylogeny analysis (Baptista-

Ferreira et al., 1983; Barroso et al., 1997; Chung et al., 1996; Fukuda et al., 1995; Matsumoto and Fukumasa-Nakai, 1996; Saville et al., 1996; 1998; Anderson et al., 2001). Fourth, the mitochondrial genome does not possess parasite resistance genes. Thus, the biparental inheritance of mitochondrial genes would contribute little to their host in competing with the fast evolving of parasites.

Moreover, several models have been put forward to explain the evolution of uniparental inheritance of cytoplasmic elements, including mtDNA. One of the models explains that uniparental inheritance was evolved to prevent the spread of selfish deleterious cytoplasmic element (Grun, 1976; Coleman, 1982; Hastings, 1992). The selfish deleterious cytoplasmic elements could be a gene in an organelle genome, a deletion in an organelle genome (e.g, the hypersuppressive petite mitochondrial genome in the yeast), a deleterious vertically transmitted bacterium, virus or related pathogen. This model is reasonable in that the deleterious selfish cytoplasmic elements have been found in many eukaryotes, such as the petite mitochondrial genome found in yeast (Dujon, 1981), the cytoplasmic incompatibility factor *Wolbachia* found in *Drosophila simulans* (Turelli and Hoffmann, 1991) and mitochondrial mutation causing cytoplasmic male sterility in plants (Saumitou-Laprade et al., 1994).

There is also a model emphasizing the importance of other features (eg. mating success through evolution of anisogamy) and suggests that uniparental inheritance was a byproduct of selection on those features (Birky, 1983). The mating success rate will increase with the increase of size differences between gametes, for the following reasons.

First, when sperm is limited, a larger egg will have an increased chance of being fertilized, either because its own mass offers a larger target for sperm or because larger eggs can produce a greater quantity of attraction pheromone and nutrition for zygote develop. Second, a small sperm will have motility and number advantage to ensure a greater mating success (Randerson and Hurst, 1999; 2001). In this aspect, the evolution to ensure mating success by increasing gamete size differences would bias cytoplasmic contribution from the two parents, eventually leading to uniparental inheritance.

Other models have been proposed to explain the uniparental inheritance of organelle genomes. For example Sears & VanWinkle-Swift (1994) proposed that uniparental inheritance of chloroplasts in *Chlamydomonas reinhardtii* is an adaptation to combat nitrogen starvation. This model proposes that cpDNA from the mt-gamete is degraded to act as nutritional source for the developing zygote. However, this explanation cannot be applied to anisogamy, which implies a mechanism whereby the female gamete eliminates its own cytoplasm.

3. *Cryptococcus neoformans*

C. neoformans is a pathogenic basidiomycetous yeast mainly infecting immunocompromised patients. Its importance as an opportunistic pathogen has increased in the past two decades, largely as a result of AIDS, cancer chemotherapy, and immunosuppression for organ transplants.

3.1 Serotypes

Based on reactions to monoclonal antibodies, five distinct serotypes have been identified: serotype A was classified as *C. neoformans* var *grubii*, serotype D as *C.*

neoformans var *neoformans* and serotype B and C as *C. neoformans* var *gattii*. Serotype AD is the hybrid between serotype A and serotype D. Among these serotypes, serotype A is the most prevalent. These serotypes differ in their ecology, molecular and morphological characteristics, epidemiology, pathogenicity, physiology and geographical distribution (Casadevall and Perfect, 1998). DNA sequence analysis has revealed that serotype A strains have diverged from serotype D strains for about 18 million years. Serotype B and C have diverged from serotype A and D strains for about 40 million years (Xu et al. 2000).

3.2 Life cycle of *C. neoformans*

C. neoformans has a defined life cycle that involves vegetative growth as budding yeast combined with the ability to undergo filamentous dimorphic transitions (reviewed by Hull and Heitman, 2002). The organism exists in the environment as haploid budding yeast with two mating types: α and a. Under nutrient-limiting environment, two haploid cells with different mating types can fuse. In the fused cell, the two nuclei would not fuse immediately. Instead, the cell with two nucleus will replicate and forming a dikaryotic filamentous hypha. In each cell of the hypha, there are two nuclei, one from each parent. Ultimately, a basidium is formed at the tip of the hypha. Typically, only in this structure, these two nuclei fuse and then undergo meiosis to produce four chains of sexual haploid spores.

Laboratory crosses of *C. neoformans* have demonstrated that the α - and a- mating type progenies are produced in equal numbers (Kwon-Chung, 1976). However, in natural populations, the α - mating type outnumbered the a- mating type by 30:1 and 40:1 in

isolates of clinical and environmental origin respectively (Kwon-Chung and Bennett, 1978). The biased distribution of different mating types in natural populations has been attributed to two features of mating type α strains. First, cells of the α mating type can undergo a dimorphic transition from haploid yeast cell phase to the hyphal phase during nutrient-limiting condition, in a phenomenon known as haploid fruiting (Wickes et al., 1996). Like in sexual crosses, haploid fruiting can form basidia in the tips of the hyphae and produce spores. However, the hyphae produced by haploid fruiting is monokaryotic with only one nucleus in each cell of the filaments. The nucleus in the basidium also does not undergo meiosis. Second, a more recent experiment found that two α mating type cells can also mate with each other and undergo meiosis, in a process known as same-sex mating (Lin et al., 2005). The same-sex mating may have contributed to the recent fungal meningoencephalitis outbreak on Vancouver Island, Canada (Fraser et al., 2005).

3.3 Mitochondrial genotypes in *C. neoformans*

Based on two molecular markers, four mtDNA haplotypes were identified among *C. neoformans*. The mitochondria of serotype A strains had mtDNA haplotype I mitochondria, strains of serotype D had haplotype II mitochondria, and strains of serotype B and C had haplotypes III and IV mitochondria (Xu, 2002). Comparing the genetic maps of serotype A and serotype D mitochondrial genomes, no difference in the order of genes between these two serotypes was found. However, they differed significantly in the sizes of their mitochondrial genomes measuring 32.6kb and 24.1 kb for serotype D and serotype A strain respectively. Several regions contributed to the size difference. The greatest difference was observed in the size and number of introns within

the *COX1* and *COB* genes. The size of *COX1* gene in the two sequenced serotype A strains was 1587bp without any intron, while in serotype D strain the gene was 6004bp and contained five introns (Litter et al., 2005).

3.4 *C. neoformans* as a model organism

C. neoformans is an excellent organism for the study of biological process.

Compared to many other eukaryotes, it has the following nice features: (i) it is small and can be easily manipulated in the lab; (ii) its life cycle is very short. It takes only one week to produce sexual progeny; (iii) its genome is small (about 20Mb). Both the nuclear and mitochondrial genomes are sequenced and available in the Genbank (Loftus et al., 2005); (iv) two transformation techniques have been developed with the biolistic transformation used successfully in both serotypes A and D strains (Edman and Kwon-Chung, 1990; Toffaletti, et al., 1993); (v) A pair of congenic isolates, differing only at the mating type locus, was created by consecutive backcrossing. Several auxotrophs have been derived from these strains, which facilitated classical genetic analysis (Heitman et al., 1999).

4. Objectives of this thesis

In this thesis, I mainly focus on two genetic components of *C. neoformans*, the MAT locus and the mitochondrial genome. In chapter 2, I determined the geographic distribution of MAT alleles across four geographic areas in the US. In chapters 3-6, I attempted to answer two questions about uniparental mitochondrial inheritance: (i) What are the genes controlling mitochondrial inheritance? (ii) Why is uniparental mitochondrial inheritance so prevalent? Below is a brief description of these chapters.

Chapter 2 in this thesis focused on the geographic distribution of MAT alleles. Prior to this study, mating has only ever been observed in the laboratory, never in nature. In particular, the mating type a (MATa) allele in serotype A strains had been thought to be extinct (Lengeler et al., 2000). In this chapter, I investigated the constitution of MAT alleles in natural population collected in four areas of the US. One of the main objectives of this study is to determine the possible area with fertile serotype A and mating type a strains.

In the following several chapters, I mainly focus on mitochondrial inheritance and how mating type locus might contribute to mitochondrial inheritance. It is well known that mitochondrial inheritance is uniparental in most sexual eukaryotes. However, no gene controlling mitochondrial inheritance has been found in any organism. Furthermore, although many hypotheses have been proposed to explain the prevalence of uniparental organelle inheritance, experimental evidence is still scarce. In chapters 3-5, I tested the role of MAT locus in controlling mitochondrial inheritance. In chapter 6, I then determined whether a mating type specific gene (*SXII α*) at MAT locus can inhibit the spread of a selfish element in the mitochondrial genome.

References:

- Ahmed, S., Morrall F.A.A., and Kaiser W.J. (1996). Distribution of mating types of *Ascochyta fabae* f. sp. *Lentis*. Can. J. Plant Pathol. 18: 347-353.
- Ainsworth, A.M., Beaching, J.R., Broxhollme, S.J., Hunt, B.A., Rayner, A.D.M., and Scard, P.T. (1992). Complex outcome of reciprocal exchange of nuclear DNA

- between two members of the basidiomycete genus *Stereum*. *J Gen Microbiol* 138:1147-1157.
- Allen, J.F. (2003). The function of genomes in bioenergetic organelles. *Philos Trans R Soc London B* 358: 19-37.
- Anderson, J.B., Wickens, C., Khan, M., Cowen, L.E., Federspiel, N., Jones, T., and Kohn, L.M. (2001). Infrequent genetic exchange and recombination in the mitochondrial genome of *Candida albicans*. *J Bacteriol* 183: 865-872.
- Armbrust, E.V., Ferris, P.J., and Goodenough, U.W. (1993). A mating type-linked gene cluster expressed in *Chlamydomonas* zygotes participates in the uniparental inheritance of the chloroplast genome. *Cell* 74:801-811.
- Armstrong, C.L., Chongo, G., Gossen, B.D., Duczek, L.J. (2001). Mating type distribution and incidence of the teleomorph of *Ascochyta rabiei* (*Didymella rabiei*) in Canada. *Can. J. Plant Pathol.* 23: 110-113
- Astell, C.R., Ahlstrom-Jonasson, L., Smith, M., Tatchell, K., Nasmyth, K.A. and Hall, B.D. (1981). The sequence of the DNAs coding for the mating-type loci of *Saccharomyces cerevisiae*. *Cell* 27: 15-23.
- Bakkeren, G., Kronstad, J.W. (1993). Conservation of the b mating-type gene complex among bipolar and tetrapolar smut fungi. *Plant Cell.* 5(1):123-36.
- Bakkeren, G., Kronstad, J.W. (1994). Linkage of mating-type loci distinguishes bipolar from tetrapolar mating in basidiomycetous smut fungi. *Proc Natl Acad Sci U S A.* 91(15):7085-9.

- Baptista-Ferreira, J.L.C., Economou, A., and Casselton, L.A (1983). Mitochondrial genetics of *Coprinus*: recombination of mitochondrial genomes. *Curr Genet* 7: 405-407.
- Barroso, G., Labarene, J. (1997). Genetic evidence for nonrandom sorting of mitochondria in the basidiomycete *Agrocybe aegerita*. *Appl Environ Microbiol* 63:4686-4691.
- Beckers, M.C., Muriat, C., Minet, A., and Matagne, R.F. (1991). The fate of mitochondrial DNAs of mt⁺ and mt⁻ origin in gametes and zygotes of *Chlamydomonas*. *Curr Genet* 20:239-243.
- Belcour, L., and Beigel, O. (1977). Mitochondrial genes in *Podospora anserina*: recombination and linkage. *Mol Gen Genet* 153:11-21.
- Bendich, A.J. (1993). Reaching for the ring: the study of mitochondrial genome structure. *Curr Genet* 24:279-290.
- Bendich, A.J. (1996). Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. *J Mol Biol* 255: 564-588.
- Birky, C.W. Jr. (1983). Relaxed cellular controls and organelle heredity. *Science* 222: 468-475.
- Birky, C.W. Jr. (1995). Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc Natl Acad Sci USA* 92:11331-11338.

- Blanc, H., and Dujon, B. (1980). Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. *Proc Natl Acad Sci USA* 77: 3942-3946.
- Bobrowicz, P., Pawlak, R., Correa, A., Bel-Pedersen, D., and Ebbole, D.J. (2002). The *Neurospora crassa* pheromone precursor genes are regulated by the mating type locus and the circadian clock. *Mol. Microbiol.* 45: 795-804.
- Bolker, M., Urban, M., and Kahmann, R. (1992). The a mating type locus of *U. maydis* specifies cell signaling components. *Cell.* 68(3):441-50.
- Bollock, J. (1992). Huge organisms. *New Scientist* 30: 54
- Brasier, C.M., and Kirk, S.A. (1986). Maternal inheritance of chloramphenicol tolerances in *Ophiostoma ulmi*. *Trans Br Mycol Soc* 87: 460-462.
- Bullerwell, C.E., Forget, L., and Lang, B.F. (2003a). Evolution of *monoblepharidalean* fungi based on complete mitochondrial genome sequences. *Nucleic Acids Res* 31:1614-1623.
- Bullerwell, C.E., Leigh, J., Forget, L., and Lang, B.F. (2003b). A comparison of three fission yeast mitochondrial genomes. *Nucleic Acids Res* 31:759-768.
- Casadevall, A., and Perfect, J.R. (1998). *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology Press.
- Chung, K.R., Leuchmann, A., and Schardl, C.L. (1996). Inheritance of mitochondrial DNA and plasmids in the ascomycetous fungus, *Epichloe typhina*. *Genetics* 142: 259-265.

- Coenen, A., Croft, J.H., Slakhorst, M., Debets, F., and Hoekstra, R. (1996).
Mitochondrial inheritance in *Aspergillus nidulans*. *Genet Res* 67:93-100.
- Coleman, A.W. (1982). Sex is dangerous in a world of potential symbionts or the basis of
selection for uniparental inheritance. *J. Theor. Biol.* 97: 367-369.
- Consolo, V.F., Cordo, C.A., and Salerno, G.L. (2005). Mating-type distribution and
fertility status in *Magnaporthe grisea* populations from Argentina.
Mycopathologia. 160(4):285-90.
- Coppin, E., Debuchy, R., Arnaise, S., and Picard, M. (1997). Mating types and sexual
development in filamentous ascomycetes. *Microbiol Mol Biol Rev.* 61(4):411-28..
- Cosmides, L.M., and Tooby, J. (1981). Cytoplasmic inheritance and intragenomic
conflict. *J Theor Biol* 89: 83-129.
- Dayakar, B.V., Narayanan, N.N., and Gnanamanickam, S.S. (2000). Cross-compatibility
and distribution of mating type alleles of the rice blast fungus *Magnaporthe
grisea* in India. *Plant Dis.* 84: 700-704.
- de la Bastide, P.Y., and Horgen, P.A. (2003). Mitochondrial inheritance and the detection
of non-parental mitochondrial DNA haplotypes in crosses of *Agaricus bisporus*
homokaryons. *Fungal Genet Biol* 38:333-342.
- de la Bastide, P.Y, and Sonnenberg, A.S.M., Van Griensven, L.J.L.D., Anderson, J.B.,
and Horgen, P.A. (1997). Mitochondrial haplotype influences mycelial growth of
Agaricus bisporus heterokaryons. *Appl Environ Microbiol* 63: 3426-3431.

- de Zamaroczy, M., Marotta, R., Faugeron-Fonty, G., Goursot, R., Mangin, M., Baldacci, G., and Bernardi, G. (1981). The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity. *Nature* 292:75-8.
- Douhan, G.W., Murray, T.D., and Dyer, P.S. (2002). Species and mating-type distribution of *Tapesia yallundae* and *T. acuformis* and occurrence of apothecia in the US Pacific Northwest. *Phytopathology*. 92: 703-709
- Dujon, B. (1981). Mitochondrial genetics and functions. In Strathern, JN, Jones EW and Broach JR (eds), *The Molecular Biology of the Yeast Saccharomyces*. Cold Spring Harbor: Laboratory Press, Cold Spring Harbor, New York 505–635.
- Edman, J. C., and Kwon-Chung, K. J. (1990). Isolation of the *URA5* gene from *Cryptococcus neoformans* var. *neoformans* and its use as a selective marker for transformation. *Mol. Cell. Biol.* 10:4538-4544.
- Esser, K., and Tudzynski, P. (1977). Prevention of senescence in the ascomycete *Podospora anserine* by antibiotic tiamulin. *Nature* 265: 454-456.
- Forget, L., Ustinova, J., Wang, Z., Huss, V.A., and Lang, B.F. (2002). *Hyaloraphidium curvatum*: a linear mitochondrial genome, tRNA editing, and an evolutionary link to lower fungi. *Mol Biol Evol* 19:310-319.
- Frank, S.A. (1996). Host-symbiont conflict over the mixing of symbiotic lineages. *Proc R Soc Lond B* 263: 339-344.

- Fraser, J.A., and Heitman, J. (2004). Evolution of fungal sex chromosomes. *Mol Microbiol.* 51: 299—306
- Fraser, J.A., Diezmann, S., Subaran, R.L., Allen, A., Lengeler, K.B., Dietrich, F.S., and Heitman, J. (2004). Convergent evolution of chromosomal sex-determining regions in the animal and fungal kingdoms. *PLoS Biol.* 2(12):e384.
- Fraser, J.A., Giles, S.S., Wenink, E.C., Geunes-Boyer, S.G., Wright, J.R., Diezmann, S., Allen, A., Stajich, J.E., Dietrich, F.S., Perfect, J.R., and Heitman, J. (2005). Same-sex mating and the origin of the Vancouver Island *Cryptococcus gattii* outbreak. *Nature.* 437(7063):1360-4.
- Froeliger, E.H., and Leong, S.A. (1991). The a mating-type alleles of *Ustilago maydis* are idiomorphs. *Gene.* 100:113-22.
- Fukuda, M., Harada, Y., Imahori, S., Fukumasa-Nakai, Y., and Hayashi, Y. (1995). Inheritance of mitochondrial DNA in sexual crosses and protoplast cell fusions in *Lentinula edodes*. *Curr Genet* 27: 550-554.
- Giles, R.E., Blanc, H., Cann, H.M., Wallace, D.C. (1980). Maternal inheritance of human mitochondrial DNA. *Proc Natl Acad Sci USA* 77: 6715-6719.
- Gillham, N.W., Boynton, J.E., Johnson, A.M., and Burkhart, B.D. (1987). Mating type linked mutations which disrupt the uniparental transmission of chloroplast genes in *chlamydomonas*. *Genetics.* 115(4):677-84.

- Gillissen, B., Bergemann, J., Sandmann, C., Schroeer, B., Bolker, M., and Kahmann, R. (1992). A two-component regulatory system for self/non-self recognition in *Ustilago maydis*. *Cell*. 68(4):647-57.
- Grun, P. (1976). Cytoplasmic genetics and evolution. Columbia University Press. New York.
- Gylensten, U., Wharton, A., and Wilson, A.C. (1991). Maternal inheritance of mitochondrial DNA during backcrossing of two species of mice. *Nature* 352: 255-257.
- Hastings, I.M. (1992). Population genetic aspects of deleterious cytoplasmic genomes and their effect on the evolution of sexual reproduction. *Genet Res.*59(3): 215-25.
- Heitman, J., Allen, B., Alspaugh, J.A., and Kwon-Chung, K.J. (1999). On the origins of congenic MAT α and MATa strains of the pathogenic yeast *Cryptococcus neoformans*. *Fung. Genet. Biol.* 28:1-5.
- Herskowitz, I. (1989). A regulatory hierarchy for cell specialization in yeast. *Nature*. 342(6251):749-57.
- Herskowitz, I., Rine, J., and Strathern, J. (1992). Mating type determination and mating-type interconversion in *Saccharomyces cerevisiae*. In: *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*, E.W. Jones, J.R. Pringle and J.R. Broach, eds. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NYk, pp. 583-656.

- Hintz, W.E.A., Anderson, J.B., and Horgen, P.A. (1988). Nuclear migration and mitochondrial inheritance in the mushroom *Agaricus bitorquis*. *Genetics* 119: 35–41.
- Hull, C.M., Boily, M.J., and Heitman, J. (2005). Sex-specific homeodomain proteins Sxi1alpha and Sxi2a coordinately regulate sexual development in *Cryptococcus neoformans*. *Eukaryot Cell*. 4(3):526-35.
- Hull, C.M., Davidson, R.C., and Heitman, J. (2002). Cell identity and sexual development in *Cryptococcus neoformans* are controlled by the mating-type-specific homeodomain protein Sxi1alpha. *Genes Dev*. 16(23):3046-60.
- Hull, C.M., and Heitman, J. (2002). Genetics of *Cryptococcus neoformans*. *Annu Rev Genet*. 36:557-615.
- Hull, C.M., and Johnson, AD. (1999). Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science*. 285(5431):1271-5.
- Hurst, L.D. (1990). Parasite diversity and the evolution of diploidy, multicellularity and anisogamy. *J Theor Biol* 144: 429-443.
- Hurst, L.D., and Hamilton, W.D. (1992). Cytoplasmic fusion and the nature of sexes. *Proc R Soc Lond B* 247: 189-194.
- Jin, T., Sonnenberg, A.S.M., Van Griensven, L.J.L.D., and Horgen, P.A. (1992). Investigation of mitochondrial transmission in selected matings between homokaryons from commercial and wild collected isolates of *Agaricus bisporus* (= *Agaricus brunnescens*). *Appl Environ Microbiol* 58: 3553-3560.

- Kamper, J., Reichmann, M., Romeis, T., Bolker, M., Kahmann, R. (1995). Multiallelic recognition: nonself-dependent dimerization of the bE and bW homeodomain proteins in *Ustilago maydis*. *Cell*. 81(1):73-83.
- Kraytsberg, Y., Schwartz, M., Brown, T.A., Ebralidse, K., Kunz, W.S., Clayton, D.A., Vissing, J., and Khrapko, K. (2004). Recombination of human mitochondrial DNA. *Science* 304: 981.
- Kronstad, J.W., and Leong, S.A. (1990). The b mating-type locus of *Ustilago maydis* contains variable and constant regions. *Genes Dev*. 4(8):1384-95.
- Kronstad, J.W., and Staben, C. (1997). Mating type in filamentous fungi. *Annu Rev Genet*. 31:245-76.
- Kwon-Chung, K.J., and Bennett, J.E. (1978). Distribution of alpha and alpha mating types of *Cryptococcus neoformans* among natural and clinical isolates. *Am J Epidemiol*. 108(4):337-40.
- Kwon-Chung, K.J., Edman, J.C., and Wickes, B.L. (1992). Genetic association of mating types and virulence in *Cryptococcus neoformans*. *Infect Immun*. 60(2):602-5.
- Kwon-Chung, K.J. (1976). Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. *Mycologia* 68:821-833.
- Lee, J., Lee, T., Lee, Y.W., Yun, S.H., and Turgeon, B.G. (2003). Shifting fungal reproductive mode by manipulation of mating type genes: obligatory heterothallism of *Gibberella zeae*. *Mol Microbiol*. 50(1):145-52.

- Lee, N., Bakkeren, G., Wong, K., Sherwood, J.E., and Kronstad, J.W. (1999). The mating-type and pathogenicity locus of the fungus *Ustilago hordei* spans a 500-kb region. *Proc Natl Acad Sci U S A*. 96(26):15026-31.
- Lee, S.B., and Taylor, J.W. (1993) Uniparental inheritance and replacement of mitochondrial DNA in *Neurospora tetrasperma*. *Genetics* 134: 1063-1075.
- Lengeler, K. B., Wang, P., Cox, G. M., Perfect, J. R., and Heitman, J. (2000). Identification of the MATa mating-type locus of *Cryptococcus neoformans* reveals a serotype A MATa strain thought to have been extinct. *Proc. Natl. Acad. Sci. USA* 97:14455-14460.
- Lengeler, K.B., Fox, D. S., Fraser, J. A., Allen, A., Forrester, K., Dietrich, F.S., and Heitman, J. (2002). Mating-type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. *Eukaryot. Cell* 1:704-718.
- Lin, X., Hull, C.M., and Heitman, J. (2005). Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. *Nature*. 434(7036):1017-21.
- Linde, C.C., Zala, M., Ceccarelli, S., and McDonald, B.A. (2003). Further evidence for sexual reproduction in *Rhynchosporium secalis* based on distribution and frequency of mating-type alleles. *Fungal Genet Biol.* 40(2):115-25.
- Litter, J., Keszthelyi, A., Hamari, Z., Pfeiffer, I., and Kucsera, J. (2005). Differences in mitochondrial genome organization of *Cryptococcus neoformans* strains. *Antonie Van Leeuwenhoek*. 88(3-4):249-55.

- Liu, Y.C., Cortesi, P., Double, M.L., MacDonald, W.L., and Milgroom, M.G. (1996). Diversity and multilocus genetic structure in populations of *Cryphonectria parasitica*. *Phytopathology*. 86: 1344-1351.
- Loftus, B.J., Fung, E., et al. (2005). The genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. *Science*. 307(5713):1321-4.
- MacAlpine, D.M., Kolesar, J., Okamoto, K., Butow, R.A., and Perlman, P.S. (2001). Replication and preferential inheritance of hypersuppressive petite mitochondrial DNA. *EMBO J* 20:1807-1817.
- Magee, B.B., and Magee, P.T. (2000). Induction of mating in *Candida albicans* by construction of MTL α and MTL α strains. *Science*. 289(5477):310-3.
- Maleszka, R., Skelly, P.J., and Clark-Walker, G.D. (1991). Rolling circle replication of DNA in yeast mitochondria. *EMBO J* 10:3923-3929.
- Matsumoto, T., and Fukumasa-Nakai, Y. (1996). Mitochondrial DNA inheritance in sexual crosses of *Pleurotus ostreatus*. *Curr Genet* 30: 549-552.
- May, G., and Taylor, J.W. (1988). Patterns of mating and mitochondrial DNA inheritance in the agaric Basidiomycete *Coprinus cinereus*. *Genetics* 118: 213-220.
- May, G., Shaw, F., Badrane, H., and Vekemans, X. (1999). The signature of balancing selection: fungal mating compatibility gene evolution. *Proc. Natl. Acad. Sci. USA* 96:9172-9177.

- Mekwatanakarn, P., Kositratana W., Phromraksa, T., and Zeigler, R.S. (1999). Sexually fertile *Magnaporthe grisea* rice pathogens in Thailand. *Plant Dis.* 83: 939-943.
- Metzenberg, R.L. and Glass, N.L. (1990). Mating type and mating strategies in *Neurospora*. *BioEssays.* 12: 53-59.
- Milgroom, M.C., and Lipari, S.E. (1993). Maternal inheritance and diversity of mitochondrial DNA in the chestnut blight fungus, *Cryphonectria parasitica*. *Phytopathology* 83: 563-567.
- Miller, M.G., and Johnson, A.D. (2002). White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell.* 110(3):293-302.
- Mitchell, M.B., and Mitchell, H.K. (1952). A case of maternal inheritance in *neurospora crass*. *Proc Natl Acad Sci USA* 38: 442-449.
- Mourier, T., Hansen, A.J., Willerslev, E., and Arctander, P. (2001). The human genome project reveals a continuous transfer of large mitochondrial fragments to the nucleus. *Mol Biol Evol* 18: 1833-1837.
- Muller, H.J. (1964). The relation of recombination to mutational advance. *Mutat Res* 106: 2-9.
- Nasmyth, K.A. and Tatchell, K. (1980). The structure of the transposable yeast mating type loci. *Cell* 19: 753-764.

- Nielsen, K., Marra, R.E., Hagen, F., Boekhout, T., Mitchell, T.G., Cox, G.M. and Heitman, J. (2005). Interaction between genetic background and the mating-type locus in *Cryptococcus neoformans* virulence potential. *Genetics*. 171(3):975-83.
- Nishimura, Y., Misumi, O., Kato, K., Inada, N., Higashiyama, T., Momoyama, Y., and Kuroiwa, T. (2002). An mt(+) gamete-specific nuclease that targets mt(-) chloroplasts during sexual reproduction in *C. reinhardtii*. *Genes Dev* 16:1116-1128.
- Nosek, J., and Tomaska, L. (2002). Mitochondrial telomeres: alternative solutions to the end-replication problem. In: Krupp, G., and Parwaresch, R. eds. *Telomeres, Telomerases and Cancer*. New York: Kluwer Academic/Plenum. pp396-417.
- Nosek, J., and Tomaska, L. (2003). Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. *Curr Genet* 44: 73-84.
- Nosek, J., Dinouel, N., Kovac, L., and Fukuhara, H. (1995). Linear mitochondrial DNAs from yeasts: telomeres with large tandem repetitions. *Mol Gen Genet* 247: 61-72.
- Nosek, J., Tomaska, L., Fukuhara, H., Suyama, Y., and Kovac, L. (1998). Linear mitochondrial genomes: 30 years down the line. *Trends Genet*. 14: 184-188.
- Notteghem, J.L., Silue, D. (1992). Distribution of the mating type alleles in *Magnaporthe grisea* populations pathogenic on rice. *Phytopathology*. 82: 421-424.
- Nunnari, J., Marshall, W.F., Straight, A., Murray, A., Sedat, J.W., and Walter, P. (1997). Mitochondrial transmission during mating in *Saccharomyces cerevisiae* is

- determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol Biol Cell* 8:1233-1242.
- Oakley, B.R., and Rinehart, J.E. (1985). Mitochondria and nuclei move by different mechanisms in *Aspergillus nidulans*. *J Cell Biol* 101: 2392-2397.
- Olson, A., and Stenlid, J. (2001). Plant pathogens. Mitochondrial control of fungal hybrid virulence. *Nature* 411: 438.
- Petersen, R.F., Langkjaer, R.B., Hvidtfeldt, J., Gartner, J., Palmen, W., Ussery, D.W., and Piskur, J. (2002). Inheritance and organisation of the mitochondrial genome differ between two *Saccharomyces* yeasts. *J Mol Biol* 318: 627-636.
- Philly, M.L., Staben, C. (1994). Functional analyses of the *Neurospora crassa* MT a-1 mating type polypeptide. *Genetics*. 137(3):715-22.
- Randerson, J.P., and Hurst, L.D. (1999). Small sperm, uniparental inheritance and selfish cytoplasmic elements: a comparison of two models. *J Evol Biol* 12:1110-1124
- Randerson, J.P., and Hurst, L.D. (2001). A comparative test of a theory for the evolution of anisogamy. *Proc R Soc Lond B Biol Sci* 268: 879-884.
- Raper, J.R., Krongelb, G.S., Baxter, M.G. (1958). The number and distribution of incompatibility factors in *Schizophyllum commune*. *Am. Nat.* 92:221-232.
- Ricchetti, M., Fairhead, C., and Dujon, B. (1999). Mitochondrial DNA repairs double-strand breaks in yeast chromosomes. *Nature* 402: 96-100.

- Robinson, H.L., Ridout, C.J., Sierotzki, H., Gisi, U., and Brown, J.K. (2002). Isogamous, hermaphroditic inheritance of mitochondrion-encoded resistance to Qo inhibitor fungicides in *Blumeria graminis* f. sp. *tritici*. *Fungal Genet Biol* 36: 98-106.
- Ross, I.K. (1976). Nuclear migration rates in *Coprinus congregatus*: a new record? *Mycologia* 68: 418-422.
- Rycovska, A., Valach, M., Tomaska, L., Bolotin-Fukuhara, M., and Nosek, J. (2004). Linear versus circular mitochondrial genomes: intraspecies variability of mitochondrial genome architecture in *Candida parapsilosis*. *Microbiology* 150:1571-1580.
- Sager, R., and Ramanis, Z. (1967). Biparental inheritance of nonchromosomal genes induced by ultraviolet irradiation. *Proc Natl Acad Sci USA* 58: 931-937.
- Sakurai, R., Sasaki, N., Takano, H., Abe, T., and Kawano, S. (2000). In vivo conformation of mitochondrial DNA revealed by pulsed-field gel electrophoresis in the true slime mold, *Physarum polycephalum*. *DNA Res* 7: 83–91
- Saumitou-Laprade, P., Cuguen, J., and Vernet, P. (1994). Cytoplasmic male sterility in plants: molecular evidence and the nucleocytoplasmic conflict. *Trends Ecol Evol* 9: 431-435.
- Saville, B.J., Kohli, Y., and Anderson, J.B. (1998). mtDNA recombination in natural populations. *Proc Natl Acad Sci USA* 95: 1331-1335.

- Saville, B.J., Yoell, H., and Anderson, J.B. (1996). Genetic exchange and recombination in populations of the root-infecting fungus *Armillaria gallica*. *Mol Ecol* 5: 485-497.
- Schardl, C.L., Lonsdale, D.M., Pring, D.R., and Rose, K.R. (1984). Linearization of maize mitochondrial chromosomes by recombination with linear episomes. *Nature* 310: 292-296.
- Schardl, C.L., Pring, D.R., and Lonsdale, D.M. (1985). Mitochondrial DNA rearrangements associated with fertile revertants of S-type male-sterile maize. *Cell* 43: 361-368.
- Schulz, B., Banuett, F., Dahl, M., Schlesinger, R., Schafer, W., Martin, T., Herskowitz, I., and Kahmann, R. (1990). The b alleles of *U. maydis*, whose combinations program pathogenic development, code for polypeptides containing a homeodomain-related motif. *Cell*. 60(2):295-306.
- Sears, B.B., and VanWinkle-Swift, K. (1994). The salvage/turnover/repair (STOR) model for uniparental inheritance in *Chlamydomonas*: DNA as a source of sustenance. *J Hered* 85:365-376.
- Sharon, A., Yamaguchi, K., Christiansen, S., Horwitz, B.A., Yoder, O.C., and Turgeon, B.G. (1996). An asexual fungus has the potential for sexual development. *Mol Gen Genet*. 251(1):60-8.
- Skibinski, D.O., Gallagher, C., and Beynon, C.M. (1994) Mitochondrial DNA inheritance. *Nature* 368: 317-818.

- Skibinski, D.O., Gallagher, C., and Beynon, C.M. (1994). Sex-limited mitochondrial DNA transmission in the marine mussel *Mytilus edulis*. *Genetics* 138:801-819.
- Smith, M., Bruhn, J., and Anderson, J. (1992). The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. *Nature* 356:428-431
- Smith, M.L., Duchesne, L.C., Bruhn, J.N., and Anderson, J.B. (1990). Mitochondrial genetics in a natural population of the plant pathogen *Armillaria*. *Genetics* 126: 575-582.
- Specht, C.A., Novotny, C.P., and Ullrich, R.C. (1992). Mitochondrial DNA of *Schizophyllum commune*: restriction map, genetic map, and mode of inheritance. *Curr. Genet.* 22: 129-134.
- Strathern, J.N., Spatola, E., McGill, C., and Hicks, J.B. (1980). Structure and organization of transposable mating type cassettes in *Saccharomyces* yeast. *Proc. Natl. Acad. Sci. USA* 77: 2839-2843.
- Strausberg, R.L., and Perlman, P.S. (1978). The effect of zygotic bud position on the transmission of mitochondrial genes in *Saccharomyces cerevisiae*. *Mol Gen Genet* 163:131-144.
- Stupar, R.M., Lilly, J.W., Town, C.D., Cheng, Z., Kaul, S., Buell, C.R., and Jiang, J. (2001). Complex mtDNA constitutes an approximate 620-kb insertion on *Arabidopsis thaliana* chromosome 2: Implication of potential sequencing errors caused by large-unit repeats. *Proc Natl Acad Sci USA* 98: 5099-5103.

- Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C., and Schatten, G. (1999). Ubiquitin tag for sperm mitochondria. *Nature* 402: 371-372.
- Takano, H., Mori, K., Kawano, S., and Kuroiwa, T. (1996). Rearrangements of mitochondrial DNA and the mitochondrial fusion-promoting plasmid (mF) are associated with defective mitochondrial fusion in *Physarum polycephalum*. *Curr Genet.*29:257-264.
- The Rice Chromosome 10 Sequencing Consortium. (2003) In-depth view of structure, activity, and evolution of rice chromosome 10. *Science* 300:1566–1569.
- Thomas, D.Y., and Wilkie, D. (1968). Recombination of mitochondrial drug-resistance factors in *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun.* 30:368-372.
- Thraillkill, K.M., and Birky, C.W. Jr. (1980). Intracellular population genetics: evidence for random drift of mitochondrial allele frequencies in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Genetics.* 96:237-262.
- Toffaletti, D. L., Rude, T.H., Johnston, S.A., Durack, D.T., and Perfect, J.R. (1993). Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. *J. Bacteriol.* 175:1405-1411.
- Turelli, M., and Hoffmann, A.A. (1991). Rapid spread of an inherited incompatibility factor in California *Drosophila*. *Nature* 353:440-442.

- Urban, M., Kahmann, R., and Bolker, M. (1996). Identification of the pheromone response element in *Ustilago maydis*. *Mol Gen Genet.* 251(1):31-7.
- Urban, M., Kahmann, R., Bolker, M. (1996). The biallelic a mating type locus of *Ustilago maydis*: remnants of an additional pheromone gene indicate evolution from a multi-allelic ancestor. *Mol Gen Genet.* 250(4):414-20.
- Van Horn, R., and Clay, K. (1995). Mitochondrial DNA variation in the fungus *Atkinsonella hypoxylon* infecting sympatric *Danthonia* grasses. *Evolution* 49:360-371.
- Viji, G., and Uddin, W. (2002). Distribution of mating type alleles and fertility status of *Magnaporthe grisea* causing gray leaf spot of perennial ryegrass and St. Augustinegrass turf. *Plant Dis.* 86: 827-832.
- Welz, H.G., Leonard, K.J. (1995). Gametic phase disequilibria in populations of race-2 and race-3 of *Cochliobolus carbonum*. *Eur. J. Plant Pathol.* 101: 301-310.
- Wickes, B.L., Mayorga, M.E., Edman, U., and Edman, J.C. (1996). Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the alpha-mating type. *Proc Natl Acad Sci U S A.* 93(14):7327-31.
- Wilch, G., Ward, S., and Castle, A. (1992). Transmission of mitochondrial DNA in *Ustilago violacea*. *Curr Genet* 22:135-140.
- Wright, S., (1939). The distribution of self-sterility alleles in populations. *Genetics* 24:538-552.

- Xu, J. (2002). Estimating the spontaneous mutation rate of loss of sex in the human pathogenic fungus *Cryptococcus neoformans*. *Genetics*. 162(3):1157-67.
- Xu, J. (2002). Mitochondrial DNA polymorphisms in the human pathogenic fungus *Cryptococcus neoformans*. *Curr Genet*. 41:43-47
- Xu, J., Vilgalys, R., and Mitchell, T. G. (2000). Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Mol. Ecol*. 9:1471-1482.
- Yee, A.R., and Krønstad, J.W. (1993). Construction of chimeric alleles with altered specificity at the b incompatibility locus of *Ustilago maydis*. *Proc Natl Acad Sci U S A*. 90(2):664-8.
- Yun, S.H., Berbee, M.L., Yoder, O.C., and Turgeon, B.G. (1999). Evolution of the fungal self-fertile reproductive life style from self-sterile ancestors. *Proc Natl Acad Sci U S A*. 96(10):5592-7.
- Zambino, P., Groth, J.V., Lukens, L., Garton, J.R., and May, G. (1997). Variation at the b mating type locus of *Ustilago maydis*. *Phytopathology*. 87:1233-1239.
- Zhan, J., Kema, G.H.J., Waalwijk, C., McDonald, B.A. (2002). Distribution of mating type alleles in the wheat pathogen *Mycosphaerella graminicola* over spatial scales from lesions to continents. *Fungal Genet. Biol*. 36: 128-136.
- Zimmer, M., Welser, F., Orlaer, G., and Wolf, K. (1987). Distribution of mitochondrial introns in the species *Schizosaccharomyces pombe* and the origin of the group II intron in the gene encoding apocytochrome b. *Curr Genet* 12:329-336.

Zinn, A.R., Pohlman, J.K., Perlman, P.S., and Butow, R.A. (1987). Kinetic and segregational analysis of mitochondrial DNA recombination in yeast. *Plasmid*. 17:248-256.

Zouros, E., Ball, A.O., Saavedra, C., and Freeman, K.R. (1994). Mitochondrial DNA inheritance. *Nature* 368: 818.

Table 1.1 Fungi mitochondrial genome size and structure¹

Organism order Species	Acc. #	Struct	Size (bp)	Basic ²	rRNA ³	# of tRNA	Rp mpB	# of Other ORFs
Ascomycota								
<i>Aspergillus niger</i>	NC_007445	c ⁴	31103	all	y ⁷	23		2
<i>Aspergillus tubingensis</i>	NC_007597	c	33656	all	y	23		2
<i>Epidermophyton floccosum</i>	NC_007394	c	30910	all	y	25	Rp	9
<i>Penicillium marneffei</i>	NC_005256	c	35438	all	y	28	Rp	9
<i>Hypocrea jecorina</i>	NC_003388	c	42130	all	y	26	Rp	4
<i>Candida metapsilosis</i>	NC_006971	l ⁵	24152	all	y	24		1
<i>Candida orthopsilosis</i>	NC_006972	c	22528	all	y	24		1
<i>Hanseniaspora uvarum</i>	NC_007780	l	18844	-nad	y	23		1
<i>Podospora anserine</i>	NC_001329	c	94192	-atp9	y	27	Rp	39
<i>Lecanicillium muscarium</i>	NC_004514	c	24499	all	y	25	Rp	-
<i>Candida parapsilosis</i>	NC_005253	l	32744	all	y	24	-	6
<i>Candida stellata</i>	NC_005972	c	23114	-nad	y	25	Rp	7
<i>Ashbya gossypii</i>	NC_005789	c	23564	-nad	y	23	Rp	-
<i>Pichia Canadensis</i>	NC_001762	c	27694	all	y	25	Rp	3
<i>Saccharomyces castellii</i>	NC_003920	c	25753	-nad	y	23	Rp, mpB	1
<i>Saccharomyces cerevisiae</i>	NC_001224	c	85779	-nad	y	24	Rp	11
<i>Saccharomyces servazzii</i>	NC_004918	c	30782	-nad	y	23	Rp	4
<i>Yarrowia lipolytica</i>	NC_002659	c	47916	all	y	27	-	10
<i>Kluyveromyces lactis</i>	AY654900	c	40291	-nad	y	22	Rp	1
<i>Kluyveromyces thermotolerans</i>	NC_006626	l	23584	-nad	y	24	Rp mpB	3
<i>Candida albicans</i>	NC_002653	c	40420	all	y	30	-	-
<i>Candida glabrata</i>	NC_004691	c	20063	-nad	y	23	Rp mpB	3
<i>Schizosaccharomyces japonicus</i>	NC_004332	c	80059	-nad	y	25	-	3
<i>Schizosaccharomyces octosporus</i>	NC_004312	c	44227	-nad	y	25	Rp	6
<i>Schizosaccharomyces pombe</i>	NC_001326	c	19431	-nad	y	25	Rp mpB	3
<i>Aspergillus nidulans</i> (partial)	FMGP ⁶	c	31858	all	y	27	-	3
Basidiomycota								
<i>Crinipellis perniciosia</i>	NC_005927	c	109103	all	y	25	Rp	72
<i>Cryptococcus neoformans</i>	NC_004336	c	24874	-nad4L	y	-	-	1
<i>Schizophyllum commune</i>	NC_003049	c	49704	all	y	27	Rp	5
Chytridiomycota								
<i>Allomyces macrogynus</i>	NC_001715	c	57473	all	y	25	Rp	10
<i>Harpochytrium sp. JEL94</i>	NC_004760	c	19473	all	y	8	-	-
<i>Harpochytrium sp. JEL105</i>	NC_004623	c	24169	all	y	8	-	-
<i>Monoblepharella sp. JEL15</i>	NC_004624	c	60432	all	y	9	-	12
<i>Rhizophydium sp. 136</i>	NC_003053	c	68834	all	y	7	-	23
<i>Spizellomyces punctatus</i>	NC_003052	c	58830	all	y	8	-	18
	NC_003061	c	1381					
	NC_003060	c	1136					
<i>Hyaloraphidium curvatum</i>	NC_003048	l	29593	all	y	7	-	4
Zygomycota								
<i>Rhizopus stolonifer</i>	FMGP	c	54178	all	y	24	-	5
<i>Mortierella verticillata</i>	NC_006838	c	58745	all	y	26	Rp mpB	9
<i>Rhizopus oryzae</i>	NC_006836	c	54178	all	y	24	mpB	9
<i>Smittium culisetae</i>	NC_006837	c	58654	all	y	27	Rp mpB	16

¹The information are based on the annotated genes.

² including 14 genes common to fungal mitochondrial genome; *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad5*, *atp6*, *atp8*, *atp9*.

³ ribosomal RNA, including *rns* and *rnl*.

⁴ circular.

⁵ linear.

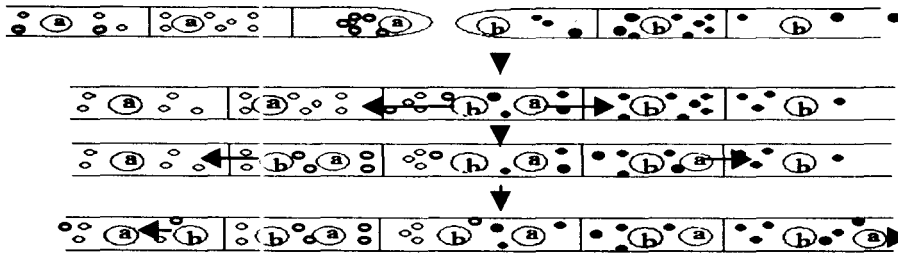
⁶Fungal Mitochondrial C enome Project
(<http://megasun.bch.umontreal.ca/People/lang/FMGP/seqprojects.html>).

⁴ exist.

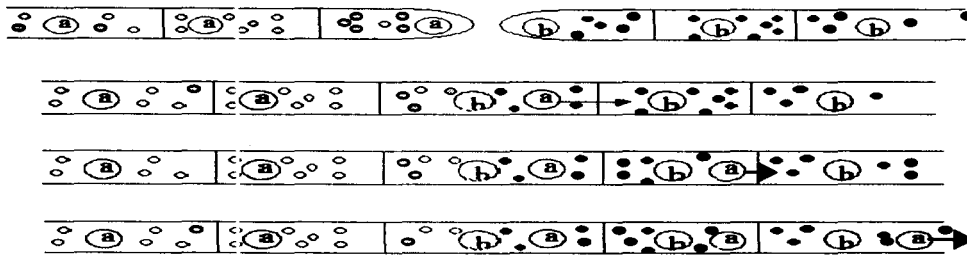
Table 1.2 Patterns of mitochondrial inheritance in fungi

Mitochondrial inheritance type	Characters	Fungal Species	Divisions	References
Yeast-like	High frequency of biparental progeny	<i>Saccharomyces cerevisiae</i>	Ascomycota	Thomas and wilkie, 1968
		<i>Saccharomyces castellii</i>	Ascomycota	Peterson <i>et al.</i> , 2002
		<i>Schizosaccharomyces pombe</i>	Ascomycota	Thraikill and Dirky, 1980
Mussel-like	Sex dependent	<i>Ustilago violacea</i>	Basidiomycota	Wilch <i>et al.</i> , 1992
Plant-like	Sample location dependent	<i>Aspergillus nidulans</i>	Ascomycota	Coenen <i>et al.</i> 1996
		<i>Atkinsonella hypoxylon</i>	Ascomycota	Van Horn and Clay, 1995
		<i>Blumeria graminis</i>	Ascomycota	Robinson <i>et al.</i> , 2002
		<i>Cryphonectria parasitica</i>	Ascomycota	Milgroom and Lipari, 1993
		<i>Epichloe typhina</i>	Ascomycota	Chung <i>et al.</i> , 1996
		<i>Neurospora crassa</i>	Ascomycota	Mitchell and Mitchell, 1952
		<i>Neurospora tetrasperma</i>	Ascomycota	Lee and Taylor, 1993
		<i>Ophiostoma ulmi</i>	Ascomycota	Brasier and Kirk, 1986
		<i>Podospora anserina</i>	Ascomycota	Belcour and Begel, 1977
		<i>Agaricus bitorquis</i>	Basidiomycota	Hintz <i>et al.</i> , 1988
		<i>Armillaria bulbosa</i>	Basidiomycota	Smith <i>et al.</i> , 1990
		<i>Coprinus cinereus</i>	Basidiomycota	Baptista- Ferreira <i>et al.</i> , 1983
		<i>Lentinula edodes</i>	Basidiomycota	Fukuda <i>et al.</i> , 1995
		<i>Pleurotus ostreatus</i>	Basidiomycota	Matsumoto and Fukumasa-Nakai, 1996
		<i>Coprinus congregatus</i>	Basidiomycota	Ross, 1976
		<i>Schizophyllum commune</i>	Basidiomycota	Specht <i>et al.</i> , 1992
<i>Stereum hirsutum X S.complicatum</i>	Basidiomycota	Ainsworth <i>et al.</i> , 1992		
<i>Agrocybe aegerita</i>	Basidiomycota	Barroso <i>et al.</i> , 1997		
<i>Agaricus bisporus</i> -like	Strain and dependent	<i>Agaricus bisporus</i>	Basidiomycota	Jin and horgen, 1992

A.



B



C

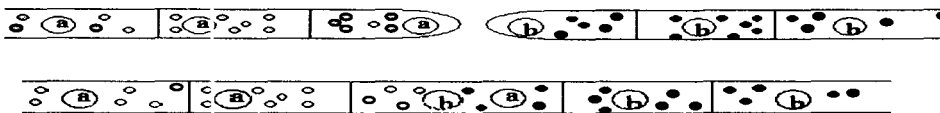


Figure 1.4 Demonstration of fertilization mechanism based on different nuclear migration mode in filamentous fungi. Open and filled small circles represent different mitochondria from two parents. Circles with letter a or b inside represent nuclear from the parent a and parent b respectively. Panel A represent a nuclei bilateral migration mode. After hyphae fusion, the nuclei of each parent can migrate through the established cells of each recipient monokaryon reciprocally without accompany the migration of mitochondria. This establishes cells with the dikaryotic pair of nuclei with exclusively the mitochondria of recipient mycelium. Mitochondria mixing is limited to the narrow conjunction zone of hyphal fusion between the two parents. Panel B represent nuclei lateral migration mode. After hyphae fusion, only parent a can donate nuclei. The other parent (parent b) only accepts nuclei. The donor nuclei migrate through the recipient monokaryon without accompany the migration of its mitochondria. This establishes cells with the dikaryotic pair of nuclei with exclusively the parent b mitochondria. Panel C represents a mode without nuclei migration.

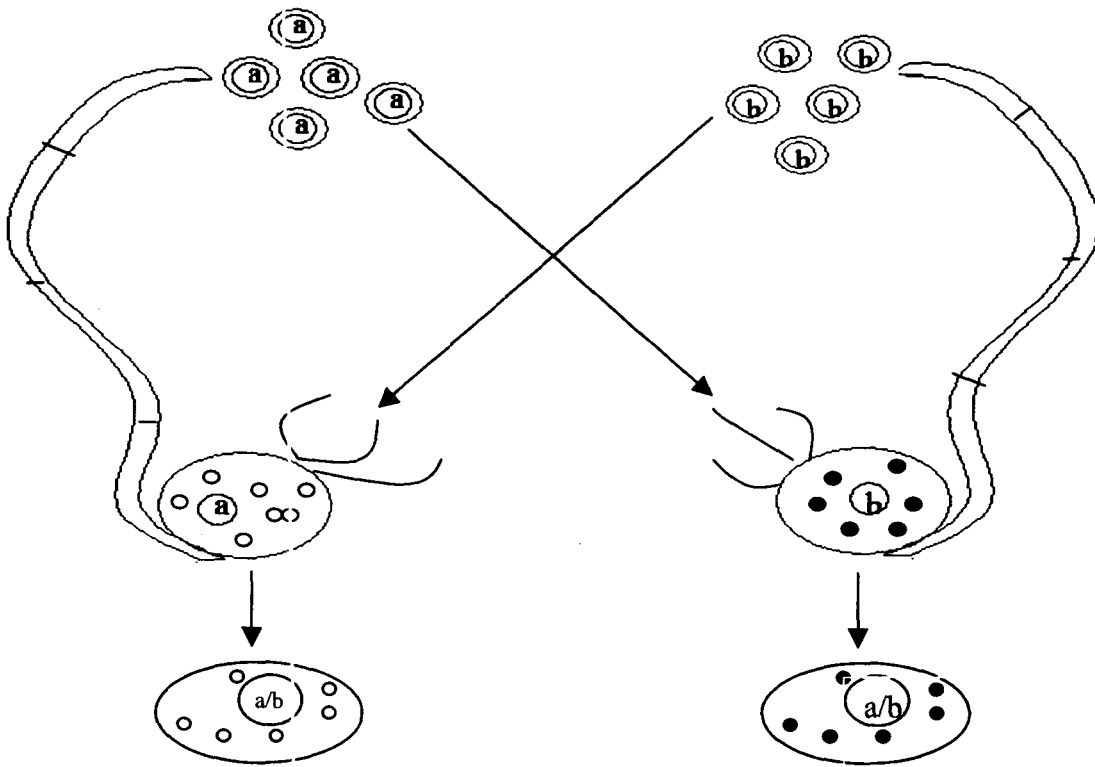


Figure 1.5 A demonstration of prezygotic mechanism in ascomycete. Filled and unfilled small circle represent different mitochondria in the two parents. Circles with letter a or b inside represent different nuclear in the cell of the two parents. The conidia (represent by a circle with only a nuclear inside contains fewer mitochondria than the egg cell (represent by a big circle with both mitochondria and nuclear inside) in the protoperithecia. The conidia nuclei from each parent can enter trichogyne (represent by curved single lines) of the other parent and travel to female nuclei and form a diploid zygote (its nuclear is represent by a circle with both letter a and b inside) with mitochondria only from one parent due to the widely different contributions of mitochondria between these two parents.

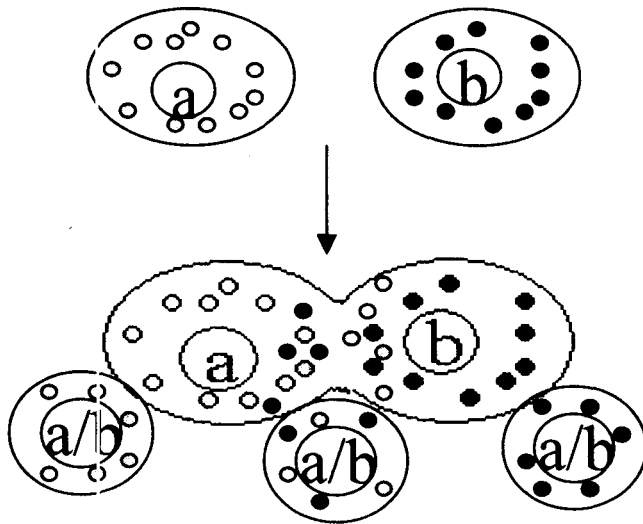


Figure 1.6 The zygotic mechanism of mitochondrial inheritance in *Saccharomyces cerevisiae*. Open and filled small circles represent different mitochondria from the two parents. Circles with letter a and b inside represent a diploid zygote nucleus. After formation of zygote by cell fusion, mitochondria (mtDNA) from the two parents didn't mix well. Following stochastic budding, three different diploid buds are produced. The end buds inherited mtDNA predominantly from the parent which gives rise to that end of the zygote. Buds arising from the middle position have mitochondria from both parents.

Chapter 2

Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States

Preface:

This chapter was published in a journal.

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This study was conducted under the guidance of my supervisor, Dr. Jianping Xu. I performed most of the experiments, prepared the figures, tables and wrote the manuscript. Xiaogang Li gave me many good suggestions. Dr. Xu performed part of the mating experiments, helped with interpreting the data and modifying the manuscript.

Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States

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Running title: Mating type alleles in *C. neoformans*

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Key words: infectious diseases, mating type, *STE20*, geographic structure.

ABSTRACT

To better understand the epidemiology and population structure of *Cryptococcus neoformans*, we determined mating types for 358 *C. neoformans* strains isolated through the active surveillance program from 1992 to 1994 in four geographic areas in the US: San Francisco (California), Georgia, Texas and Alabama. Two assays were used to determine mating types: (i) crossing with standard laboratory tester strains JEC20 and JEC21 on V-8 agar medium; and (ii) PCR with mating type α allele specific primer of the *STE12* gene and with serotype (A, D) and mating type (a, α) specific primers of the *STE20* gene. Using these two methods, we found that this sample consisted of: (i) 324 serotype A, mating type (MAT) α ($A\alpha$) strains; (ii) 12 serotype D, MAT α ($D\alpha$) strains; (iii) 14 serotype AD strains with mating type alleles Aa and $D\alpha$ ($AaD\alpha$); (iv) 2 serotype AD strains with mating type alleles $A\alpha$ and Da ($A\alpha Da$); (v) 3 serotype B, MAT α ($B\alpha$) strains; and (vi) 3 serotype AD strains but with only one mating type allele. No strain with MATa was found within serotypes A, B, or D in this collection. Interestingly, 14 of the 19 serotype AD strains contained the Aa allele at the *STE20* locus; 13 of these 14 were isolated in San Francisco. Our results suggest that the environment in San Francisco might contain Aa strains capable of mating with $D\alpha$ strains. In addition, our result demonstrate that the sample from San Francisco had a significantly higher proportion of self-fertile strains than those from other three areas.

INTRODUCTION

Cryptococcus neoformans is a basidiomycetous yeast that can cause significant morbidity and mortality in both immunocompromised patients and normal hosts (Casadevall and Perfect, 1998). In patients with the human immunodeficiency virus (HIV), a lifelong suppressive antifungal therapy is usually needed to reduce the likelihood of recurrent cryptococcal infections. However, long-term drug treatments can result in the development of drug resistance in *C. neoformans* (Paugam et al., 1993; Pfaller et al., 1998; Xu et al., 2001). A better understanding of the epidemiology, population structure and evolution of *C. neoformans* will enhance our ability to develop appropriate prevention and treatment strategies.

In response to commercial monoclonal antibodies, strains of *C. neoformans* manifest five distinct serotypes - A, B, C, D, and AD (Kabasawa et al., 1991). These serotypes differ in their ecological, molecular and morphological characteristics, epidemiology, pathogenicity, physiology and geographical distribution (Casadevall and Perfect, 1998; Xu et al., 2000). A small number of strains do not react with any of the four serotype-specific antibodies. Further classification of serotype groups into higher taxonomic groups remains controversial. However, because serotype has been used as the key feature in all currently proposed classification systems, to avoid confusion, we will restrict the strain affiliations to serotypes.

The mating system of *C. neoformans* is controlled by one locus with two alternative functional alleles, MAT α and MAT α . As in many other basidiomycete species, the mating type locus in *C. neoformans* is highly complex and contains a variety of genes essential for mating and morphogenesis, including the pheromone gene, *STE11*, *STE12*, *STE20* and others

(Clarke et al., 2001; Lengeler et al., 2000; Moore and Edman, 1993). The mating type locus in *C. neoformans* has also been identified as a potential virulence factor. In a mouse tail-vein injection model of systemic cryptococcosis, a MAT α strain was found to be more virulent than the congenic MAT α strain (Kwon-Chung et al., 1992). In addition, mating type genes can play important roles in the epidemiology and evolution of pathogens. For example, in an oomycetous fungus, *Phytophthora infestans*, which causes late blight of potato and tomato, non-Mexican populations of this species were dominated by a single clonal lineage of the A1 mating type before 1980. However, with the appearance of a compatible A2 mating type, the population became sexual, resulting in new genotypes displacing old clonal lineages and genotypes in only a few years (Fry and Goodwin, 1997).

One of the most significant findings in epidemiological surveys of *C. neoformans* was that there were far more MAT α strains (~95%) than MAT α strains (~5%) (Halliday et al., 1999; Kwon-Chung and Bennett, 1978; Madrenys et al., 1993). Mating types in *C. neoformans* have been traditionally determined by crossing with standard tester strains (Casadevall and Perfect, 1998; Halliday et al., 1999; Kwon-Chung and Bennett, 1978; Madrenys et al., 1993). In this method, cells of strains to be tested are mixed with those of individual tester strains [typically the congenic pair JEC20 (MAT α) and JEC21 (MAT α)] on medium under incubation conditions conducive for mating. Strains with different mating types can mate to form dikaryotic hyphae, which can undergo nuclear fusion and meiosis. The meiotic process can then restore haploidy in sexual progenies (i.e. basidiospores). Traditionally, the production of hyphae from mating mixtures was used as an indicator of compatible mating types. It should be emphasized, though, that hyphal production does not

imply the successful formation of stable dikaryons, nor normal meiosis or viable offspring from these crosses. This method has a few additional problems. First, the MAT α tester strain JEC21 and other MAT α strains could occasionally undergo haploid filamentation and fruiting, therefore, mating types could be erroneously assigned (Wickes et al., 1996). Second, mating is sensitive to a variety of environmental conditions, including temperature, nutrient availability and moisture level (Casadevall and Perfect, 1998). Third, many strains were unable to mate with either tester strains even after extensive manipulations of environmental conditions (e.g. Lengeler et al., 2000). Despite these caveats for the traditional crossing method, to be consistent with previous literature, the formation of hyphae from crosses will be considered compatible mating.

Recently, new methods have been developed for determining mating type alleles of *C. neoformans*. One such method uses mating type specific primers in Polymerase Chain Reaction (PCR) (Lengeler et al., 2001; Chaturvedi 2000; Halliday et al., 1999). Several mating type specific primers have been used to determine mating types. For example, the mating types of strains of serotype B were recently distinguished by two α -mating-type-specific primers for a population of *C. neoformans* from Australia (Halliday et al., 1999). A PCR-RFLP method has also been developed which permits the analysis of the mating type and ploidy based on the sequence divergence of *C. neoformans* within the mating pheromone gene (Chaturvedi 2000). In addition, Lengeler et al (2001). recently developed four pairs of serotype and mating type specific primers according to the different sequences within *STE20* locus.

To augment our understanding of the epidemiology and evolution of *C. neoformans*, the objective of this study was to determine mating type allele distributions using a combination of mating assay and the PCR method. We analyzed 358 strains obtained from the Centers for Disease Controls and Prevention in Atlanta, USA (CDC). This sample is one of the world's most comprehensive epidemiological collections of *C. neoformans*. It was obtained during the cryptococcal active surveillance program in four geographic areas in the US between 1992 and 1994 (Brandt et al., 1995; 1996). We were specifically interested in the frequency and distribution of the serotype A, MATa (Aa) allele at the *STE20* gene locus. Our analyses demonstrated that the Aa allele was widespread among strains of serotype AD.

Materials and Methods

Strains

The 358 isolates used in this study were collected by Centers for Disease Control and Prevention (CDC) from four regions in the United States from 1992 through 1994 (Brandt et al., 1995; 1996) and were generously provided by Dr. Mary E. Brandt. Strains were originally obtained through the Cryptococcal Active Surveillance program in Alabama, Georgia, Texas and San Francisco, California. Serotype and molecular subtypes based on multilocus enzyme electrophoresis (MLEE) for these strains were determined and described in earlier studies (Brandt et al., 1995; 1996).

Seven representative strains with different serotypes (A, B, C, and D) and mating types were tested to confirm the specificity of these primers (see below). These seven strains were E275, E312, B3181, B3184, JEC20, JEC21, and H99 (see Fig 2.1). Strains E275 and E312 were obtained from Dr. Dee Carter of the University of Sydney, Australia; strains

B3181 and B3184 were obtained from Dr. Mary E. Brandt of the CDC, Atlanta, USA; strains JEC20 and JEC21 were originally constructed by Dr. June Kwon-Chung of NIH, Maryland, USA, but were obtained through Dr. Joe Heitman of Duke University, Durham, USA; and strain H99 was obtained from Dr. John Perfect of Duke University.

Mating type determination based on laboratory crosses

The congenic strains JEC20 (Serotype D, MAT α) and JEC21 (Serotype D, MAT α) were used as testers. These two strains differ only at the mating type locus (Heitman et al., 1999). A loopful of 2-day-old yeast cells were mixed evenly with JEC20 or JEC21 respectively on V8 juice medium (5% V8 juice, 0.05% potassium phosphate monobasic, 4% agar, PH7.2). A negative control of each strain by itself was included. Strains JEC20 and JEC21 were mixed as positive controls. Plates were incubated at 25⁰C for 2~5 weeks and observed periodically by naked eye and by microscope. Following traditional interpretations, hyphal growth at the edge of the mating mixture was considered to be evidence of compatible mating (Kwon-Chung and Bennett, 1978; Madrenys et al., 1993; Halliday et al., 1999). Self-fertility was inferred if the negative control containing a single inoculum produced hyphae. For strains not able to form hyphae with either tester strain in the first round of the crossing experiment, up to two more rounds of crosses were performed. Positive controls of crossing between JEC20 and JEC21 always produced hyphae.

It should be noted that a compatible mating does not imply that the hyphae contains stable dikaryons, nor that all dikaryons will go through proper nuclear fusion and meiosis, nor that all meiotic progenies will be viable and fertile. Compatible mating types are a pre-

requisite but not sufficient for completing the sexual life cycle of *C. neoformans*, a multi-stage process involving cell-cell fusion, formation and maintenance of stable dikaryotic hyphae, nuclear fusion, meiosis and basidiospore formation in *C. neoformans* (Casadevall and Perfect, 1998; Lengeler et al., 2000).

Mating type determination through PCR

Genomic DNA was isolated from each strain by the method described by Xu et al. (2000). Five pairs of primers were used in this study to determine mating types (Table 2.1). *STE12 α* primer pair was MAT α specific (see Fig 2.1). The other four pairs of primers were designed from DNA sequences of the *STE20* gene and they were serotype (A, D) and mating type (a and α) specific (Lengeler et al., 2001). The specificity and selectivity of these primers were originally determined with a random sample of 65 strains of serotypes A, D, and AD by Lengeler et al. (2001). In addition, these primers were tested here using seven strains representing all four serotypes A, B, C, and D (see above). Specificity of the *STE12 α* primer pair was confirmed for strains in all four serotypes (Fig 2.1). However, cross amplifications were observed for *STE20Da* (for strains from serotypes B and C, Fig 2.1) and occasionally *STE20D α* primers (for strains from serotype A, see below).

Each PCR reaction contained 2 mM magnesium chloride (MgCl₂), 0.1 mM deoxyribonucleotide triphosphate, 0.75 pmol of each primer, 0.1 unit of Taq DNA polymerase (Promega) and 0.2 ng DNA in a total volume of 10 μ l. The annealing temperature, the number of cycles, and the expected fragment sizes are presented in Table 2.1 for each primer pair. Each of the five primer pairs was used individually for PCR for

each of the 358 strains. PCR products were run on 1% agarose gel, stained with ethidium bromide, visualized under ultraviolet light, photographed using FluoroChem 8800 (Canberra Packard Canada) and scored manually.

Mating type allele determination and analyses

Determination of serotype and mating type specific alleles was based on evidence from three sources: (i) crossing experiments; (ii) PCR with serotype and/or mating type specific primers at the two gene loci, *STE12* and *STE20*; and (iii) serotype identifications based on earlier published results for these strains (Brandt et al., 1995; 1996). In the case of cross amplification by PCR, ambiguities were resolved by comparing directly to crossing experiments and serotype identifications. Specifically, despite extensive searches for highly unique serotype and mating-type specific primers from the *STE20* sequences, cross amplification of serotype A strains was observed occasionally when the serotype D, MAT α specific primer pair was used in PCR (Lengeler et al., 2001, see also Results and Discussion). However, these cross amplifications did not affect our interpretation of mating type alleles in any of the 358 strains (see below).

Comparisons of mating type alleles and self-fertility rates among geographic areas were performed using standard Chi-square tests.

Confirmation of a single mating type allele in three serotype AD strains by Southern hybridization

Previous studies identified that strains of serotype AD were often diploid and contained genetic material from both serotypes A and D (Lengeler et al., 2001; Takeo et al., 1993; Viviani et al., 2001; Xu et al., 2002). Therefore, for serotype AD strains with only one mating type allele as determined by PCR, Southern hybridization was used to confirm that these strains indeed contained only one MAT allele (MAT α or MAT α). To perform Southern hybridization, genomic DNAs were first digested with two restriction enzymes *Xba*I and *Sac*I (MBI, Fermentas), separately. Digested samples were electrophoresed in 1% agarose gel and transferred to a nylon membrane (Hybond-N, Amersham). This membrane was separately probed with ³²P dCTP-labeled *STE20* gene fragments originally amplified from JEC20 (MAT α) and JEC21 (MAT α). Under high stringency conditions (e.g. 65°C washing), the *STE20D* α gene fragment can hybridize to D α and A α alleles, but not to A α and D α alleles. Similarly, *STE20Da* gene fragment can hybridize to Da and Aa alleles, but not to D α and A α alleles (Lengeler et al., 2001). Southern hybridization and detection of hybridized bands were performed according to the standard protocols (Sambrook, 1989).

RESULTS

Identification of mating types based on crossing experiments on V8 juice agar

All 358 strains were mixed with tester strains JEC20 and JEC21 on V8 juice medium. Among the 716 combinations, 111 produced hyphae and were scored as compatible with either JEC20 or JEC21. A summary of the crossing experiment is presented in Table 2.2.

Most of the strains (110/111) produced hyphae when crossed to JEC20 (MAT α), therefore these 110 strains were scored as MAT α . Only strain MAS94-0351 (a serotype AD strain) produced hyphae when crossed to JEC21. The mating success rate did not differ significantly among samples from different geographic areas. However, the rates varied significantly among serotypes (serotype A: 30.2%, serotype D: 66.7%, serotype AD: 26.3%, $P < 0.05$, Table 2.2). These mating results excluded the self-fertile strains described below.

Twenty-four strains produced hyphae on their own on V8 juice medium. These strains were considered self-fertile. The self-fertility rates varied among serotypes and geographic regions. Specifically, first, strains of serotype AD had a significantly higher self-fertility rate than those of serotypes A and D (serotype AD: 31.58%, serotype A: 5.25%, serotype D: 0, Chi-square test, $P < 0.01$, Table 2.2). Second, the sample from San Francisco had a higher rate of self-fertility than those from the other three regions (Alabama: 6.45%, Georgia: 1.74%, San Francisco: 12.77%, Texas: 2.81%, Chi-square test, $P < 0.01$, Table 2.2). Excluding the serotype AD strains in the analyses, the sample from San Francisco still had a significantly higher rate of self fertility than those from the other three areas ($P < 0.05$)

PCR identification of mating type alleles

In the initial descriptions of these primers, only strains of serotypes A, D, and AD were tested (Lengler et al., 2001). To confirm the initial observations and to examine whether these primers could be used to determine the mating types of strains of serotypes B and C, two strains each of serotypes B and C with known mating types were screened. Interestingly, the PCR with the STE12 α primer pair produced the expected DNA band

from MAT α strains from both serotypes B and C (Fig 2.1). The two MAT α strains (E312 and B3184) had no PCR product with the STE12 α primer. Furthermore, these two MAT α strains had similar sized DNA bands when a STE20Da primer pair was used during PCR (Fig 2.1). No other primer pair produced any band for the four strains with serotypes B or C (Fig 2.1). Therefore, these primer pairs could be used for determining the mating types of strains of serotypes B and C.

Using these five pairs of primers, the mating type alleles of all 358 strains were determined. In all 324 serotype A strains, the STE20A α primer pair amplified a 588bp DNA fragment and the STE12 α primer pair amplified a 379bp DNA fragment. Primer pairs STE20Aa and STE20Da could not amplify any DNA fragment from these serotype A strains. These results indicated that all serotype A strains in this collection had only the MAT α allele. Among these 324 A α strains, 116 also showed a PCR product of 443bp when the STE20D α primer pair was used in the PCR amplification. However, STE20A α primers did not amplify any PCR product from strains of other serotypes.

For the 12 strains of serotype D in this collection, all produced the expected DNA fragment with primers STE20D α and STE12 α . No DNA fragment was produced when primer pairs STE20Da, STE20Aa and STE20A α were used. Thus, these 12 strains were determined to be D α strains.

The primer pair STE12 α amplified a DNA fragment from each of the three strains of serotype B in this collection. However, none of the four primer pairs from the *STE20* gene amplified any PCR products from these strains. The PCR pattern from these three strains

was the same as the standard serotype B, MAT α strain E275, and unlike the pattern from the serotype B MAT α strain E312. Therefore, all three serotype B strains were MAT α . The PCR pattern of one of the three serotype B strains (MAS92-0221) is shown in Fig 2.1.

Of the 19 strains of serotype AD, two (MAS92-0022 and MAS92-0793) produced expected DNA fragments from PCR using primer pairs of STE12 α , STE20A α and STE20Da. These two strains were therefore identified as A α Da. For 14 of the 19 strains, the A α primer pair amplified an 855bp DNA fragment, the D α primer pair amplified a 443bp fragment, and the STE12 α primers amplified a 379bp fragment. Neither the STE20A α nor the STE20Da primers amplified any DNA from these 14 strains, therefore, these 14 strains were classified as A α D α .

For the remaining 3 strains of serotype AD, only one mating type allele at the *STE20* locus was detected for each strain (Fig 2.2). Specifically, strain MAS92-0224 carried only the D α allele at *STE20* locus but did not contain any other *STE20* alleles. However, STE12 α did produce the expected DNA fragment for MAS92-0224. Strain MAS92-0855 had the A α allele at the *STE20* locus but no other alleles. Similarly, STE12 α amplified an expected DNA fragment for this strain. Strain MAS94-0351 contained the Da at the *STE20* locus but no other allele (Fig 2.2). As expected, the STE12 α primer pair failed to amplify any PCR product from strain MAS94-0351.

Existence of only one mating type allele for the three serotype AD strains was confirmed by Southern hybridization. DNA from MAS92-0224 and MAS92-0855

hybridized to probe STE20D α but not to probe STE20Da. In contrast, DNA from MAS94-0351 hybridized to probe STE20Da but not to probe STE20D α (data not show).

Comparison between the two methods for determining mating type alleles

Overall, the PCR method was more successful in determining mating type alleles than the traditional crossing method. By the PCR method, mating type alleles for all 358 strains were identified (100%). In contrast, the crossing experiment unambiguously identified mating type alleles of only 111 strains (31%). For strains where mating types were unambiguously determined by both methods, the results were completely congruent. All strains which mated with JEC20 also contained alleles from *STE12* α and either the A α or the D α alleles at the *STE20* locus. Of particular notice was strain MAS94-0351. This serotype AD strain possessed only the Da allele at the *STE20* locus (Fig 2.2) and it mated successfully with the MAT α tester strain JEC21.

Geographic distributions of mating type alleles

Table 2.3 showed the distribution of mating type alleles in different geographic populations of *C. neoformans*. In all four populations, A α was the most common allele (Table 2.3; mean=90.50%). However, the ratio of mating type alleles varied among the four geographic areas. Specifically, first, San Francisco contained a significantly lower frequency of the A α allele than the other three areas (San Francisco: 84.39%, Georgia: 92.17%, Texas: 95.78%, Alabama: 100%, Chi square test, $P < 0.01$). Second, among the 19 serotype AD strains, 17 were collected from San Francisco and 13 (76.47%) contained the Aa allele.

Consequently, the frequency of the Aa allele was significantly higher in San Francisco than in the other geographic areas (San Francisco: 9.22%, Georgia: 0.87%, Texas: 0, Alabama: 0, Chi-square test, $P < 0.01$). Despite the high incidence of the Aa allele in strains of serotype AD from San Francisco, no serotype A strain with MATa was found in this sample.

DISCUSSION

This study identified mating type allele distribution for an epidemiologically comprehensive collection of the human pathogenic fungus *C. neoformans* (Brandt et al., 1995; 1996). Two methods were used in our identification: (i) crossing on V-8 juice agar medium; and (ii) PCR with serotype and mating-type specific primers. Overall, these two methods produced congruent results. Similar to previous analyses of other samples, this collection of *C. neoformans* had a significantly skewed distribution of mating type alleles in favor of MAT α . All MATa alleles were found in strains of serotype AD. Interestingly, most of the MATa alleles in serotype AD strains were Aa and most of these were from San Francisco.

Mating type allele identification methods

Similar to previous studies (e.g. Halliday et al., 1999; Kwon-Chung et al., 1992; 1978), the traditional crossing method was able to identify mating types of only a portion of the collection. In contrast, the PCR method determined the mating types of all 358 strains. The PCR method was highly efficient and less susceptible to environmental variables and was essential for determining strains containing Aa (Lengler et al., 2001). In addition, using

serotype and mating type specific primers at the *STE20* locus, we were able to distinguish AaD α from A α Da for strains within the serotype AD group.

Despite these advantages, the PCR method has its drawbacks as well. First, DNA fragments of about 30% of A α strains were amplified by the serotype D, MAT α specific primer pair at the *STE20* locus (STE20D α). Nucleotide identity between A α and D α alleles at the *STE20* locus was 93-95% (Lengler et al., 2000). Therefore, it was difficult to design primers that differed by more than a couple of bases within the *STE20* locus (Lengler et al., 2001). Similar results were obtained in genomic regions unrelated to the mating type locus (Xu et al., 2000). Primer pairs from all four genes used in the study by Xu et al. (2000) amplified genes from strains in all five serotypes A, B, C, D and AD. Secondly, because primers were designed based on only a few sequences from common laboratory strains, gene sequence variation at these primer sites among clinical or natural strains was generally unknown. Therefore, if mutations existed at these primer sites, standard PCR conditions could result in failure to detect the potential allele. Under these circumstances, changes of PCR conditions were needed to identify mating types. In our work, primer pair STE20 A α failed to amplify the expected product from 70 A α strains when the annealing temperature was set at 55°C as calculated from the primer sequences (data not shown). A touch-down protocol (annealing temperatures 55°C-47°C) was used to alleviate this problem. At present, the extent of sequence variation among strains within the same serotype group at the mating-related gene loci remains to be determined. However, these problems do highlight the necessity of a combination of methods with varying conditions involving both traditional (e.g.

crossing experiment and serotype identification) and molecular methods in epidemiological studies of *C. neoformans*.

Different self-fertility rate among serotypes

When nitrogen-starved, haploid MAT α strains can produce hyphae on their own. This phenomenon was termed haploid fruiting. In this analysis, 17 strains of A α and one strain of B α underwent haploid fruiting on V8 juice agar medium. None of the 12 D α strains underwent haploid fruiting on this medium. This rate of haploid fruiting (~5%) was much lower than a previous observation based on a small set of laboratory strains (Wickes et al., 1996).

Six of 19 serotype AD strains (31.6%) also produced hyphae on their own. All six self-fertile strains contained both a MAT α and a MATa allele in their genome. Previous studies demonstrated that strains of serotype AD were generally diploid or aneuploid (Lengler et al., 2001), and the results of recent hybridization between strains of serotypes A and D (Xu et al., 2000; 2002). Because most of the serotype AD strains analyzed here contained both mating type alleles MATa and MAT α , they were expected to be self-fertile. Though strains of serotype AD had a significantly higher self-fertility than those of serotypes A and D, not all strains of serotype AD were self-fertile.

Skewed ratio of mating type alleles

Among the 358 isolates, 357 contained a MAT α allele. Only the serotype AD strain MAS94-0351 lacked the MAT α allele. This strain contained the MATa allele from serotype

D (Fig 2.2). No strain with only a MAT_a allele was found in strains of serotypes A, B, and D in this sample. Such an imbalance was also reported in other studies of clinical and natural isolates (Madrenys et al., 1993; Kwon-Chung et al., 1978; Halliday et al., 1999). Several attributes of strains with MAT_α alleles could have contributed to its prevalence in this sample. First, a laboratory study identified that a MAT_α strain was more virulent than its congeneric MAT_a strain in a mouse model. Therefore, MAT_α strains might more readily infect and be maintained in humans (Kwon-Chung et al., 1992). All the strains analyzed here were from human hosts, not the environment. Second, unlike MAT_a strains, MAT_α strains could undergo haploid fruiting (Wickes et al., 1996). The ability to undergo haploid fruiting and the production of basidiospores could aid the dispersal and survival of MAT_α strains in the environment. Because of their small size (diameter of 1.8-2.0 μm vs. 5-10 μm for vegetative cells), basidiospores have been traditionally regarded as the infecting agent for humans (Casadevall, and Perfect, 1998).

Clonal population structure

The skewed ratio of mating type alleles could also contribute to the clonal population structure of this pathogen. In a previous study, Brandt et al. (1996) revealed a significant clonal structure in this set of strains. They found that a few MLEE genotypes dominated all four geographic populations. Wide distributions of one or few genotypes were consistent with clonal population structure (Xu et al., 2002). While asexual reproduction seemed to be the predominant mode of reproduction of *C. neoformans* in the US (Brandt et al., 1996), sexual reproduction could also play a role in the generation of genetic variation in this

species. Indeed, the isolation of both AaD α and A α Da strains from human hosts suggested that populations from certain geographic areas, e.g. San Francisco, might contain significant evidence for sexual mating and recombination.

Several studies have demonstrated evidence of recombination in *C. neoformans* (Franzot et al, 1997). For example, using multiple gene genealogy analysis, Xu et al. identified genealogical incongruences among different genes for a group of 34 strains collected from different parts of the world (Xu et al., 2000). More recently, using a gene genealogical analysis, Xu et al. (2002) identified that strains of serotype AD were the products of recent hybridizations between strains of serotypes A and D. At least three independent hybridization events were inferred among the 14 strains of serotype AD that they screened (2002). The co-existence of sexual and asexual reproduction could contribute to the long-term survival and success of *C. neoformans*.

High incidence of AaD α from San Francisco

Geographical differences in mating type allele distribution have been observed in environmental populations of serotype B strains from Australia (Halliday et al., 1999). Unlike that study, the majority of the allelic differences observed here was for the Aa allele found only in strains of serotype AD, and mostly from San Francisco. These results suggested that serotype A, MATa strains must exist in North America, and San Francisco should be a good candidate area for further investigations.

Compared to the other three regions, San Francisco is cooler in the summer than southern part of the US but warmer in the winter than Alabama and Georgia. Eucalyptus

trees are common in the San Francisco Bay area. These trees were identified as potential hosts for strains of serotypes B and C in both Southern California and Australia (Casadevall and Perfect, 1998). Both mating type alleles, MAT α and MAT β , were found in strains obtained from eucalyptus trees (Halliday et al., 1999). Whether these conditions were responsible for the high incidence of AaD α in San Francisco is not known. Indeed, the potential genetic diversity of environmental strains of *C. neoformans* is still largely unexplored. Aside from Eucalyptus trees, there are many other potential reservoirs for *C. neoformans* in the environment, including pigeon droppings (Casadevall and Perfect, 1998), bat guano (Lazera et al., 1993), wasp nests (Gezuele et al., 1993), slime flux of mesquite (Evenso and Lamb, 1964), and hollows formed by decaying wood in living trees (Lazera et al., 1996).

Origin and stability of strains of serotype AD

Several recent studies indicated that strains of serotype AD were generally diploid or aneuploid (Cogliati et al., 2001; Lengler et al., 2001) and typically contained genetic material from strains of both serotypes A and D (Lengler et al., 2001; Xu et al., 2001). If serotype AD strains were the results of hybridization between strains of serotypes A and D, these strains were expected to contain one mating type allele from each partner. However, in the present study, three serotype AD strains contained only a single *STE20* allele (Fig 2.2). The existence of a single *STE20* allele in these strains was likely the results of deletion and/or chromosome loss of one mating type allele after the hybridization. In *C. neoformans*, genetic changes have been reported for strains which underwent prolonged asexual propagation and

improper storage in the laboratory (Franzot et al., 1998). Lengeler et al (2001) also observed allelic losses for many of the loci examined in a small collection of serotype AD strains.

It should be noted, however, that an alternative hypothesis regarding the relationship between strains of serotype AD and those of serotypes A and D has not been critically examined and refuted. In this alternative hypothesis, strains of serotype AD could give rise to serotype A or D. Such a process could occur through the loss of alleles either sexually through meiosis or asexually through mitosis. Indeed, laboratory crosses between strains of serotypes A and D have produced progenies with serotype A, D, or AD (Lengler et al., 2001; J. Xu unpublished data). Unfortunately, our current data are not sufficient to unambiguously infer the frequency or the directions of these relationships among the 358 strains.

Conclusion

Using both traditional and molecular approaches, we identified the mating types of 358 strains of *C. neoformans* collected from four areas in the US. Among the four serotypes (A, B, D, and AD) identified, three (A, B, and D) contained strains of only MAT α . In the fourth serotype group AD, 14 of the 19 strains had the MAT α allele from serotype A and the MAT α from serotype D. Of these 14 AaD α strains, one was from Georgia and 13 were from San Francisco. Our results suggested that, as in Africa (Lengler et al., 2000) and Europe (Viviani et al., 2001) where serotype A and MAT α strains were recently found, these strains, once thought to be extinct, likely also exist in North America.

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REFERENCES

- Brandt, M.E., L.C. Hutwagner, R.J. Kuykendall, R.W. Pinner, and the Cryptococcal Disease Active Surveillance Group. 1995. Comparison of multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis for molecular subtyping of *Cryptococcus neoformans*. *J. Clin. Microbio.* 33:1890~1895.
- Brandt, M.E., L.C. Hutwagner, L.A. Klug, W.S. Baughman, D. Rimland, E.A. Graviss, R.J. Hamill, C. Thomas, P.G. Pappas, A.L. Reingold, R.W. Pinner, and the Cryptococcal Disease Active Surveillance Group. 1996. Molecular subtype distribution of *Cryptococcus neoformans* in four areas of the United States. *J. Clin. Microbiol.* 34: 912-917.
- Casadevall, A., and J.R. Perfect, 1998. *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology Press.
- Chaturvedi, S., B. Rodeghier, J. Fan, C.M. McClelland, B.L. Wickes, and V. Chaturvedi. 2000. Direct PCR of *Cryptococcus neoformans* MAT α and MAT α pheromones to determine mating type, ploidy and variety: a tool for epidemiological and molecular pathogenesis studies. *J. Clin. Microbiol.* 38: 2007-2009.
- Clarke, D.L., G.L. Woodlee, C.M. McClelland, T.S. Seymour, and B.L. Wickes. 2001. The *Cryptococcus neoformans* STE11 α gene is similar to other fungal mitogen-activated protein kinase kinase kinase (MAPKKK) genes but is mating type specific. *Molecular Microbiology.* 40:200-213.

- Cogliati, M., M. C. Esposto, D. L. Clarke, B. L. Wickes, and M. A. Viviani. 2001. Origin of *Cryptococcus neoformans* var. *neoformans* diploid strains. *J. Clin. Microbiol.* 39:3889-3894.
- Evenson, A., and J.W. Lamb. 1964. Slime flux of mesquite as a new saprophytic source of *Cryptococcus neoformans*. *J. Bacteriol.* 88:542.
- Franzot, S.P., J. Mukherjee, R. Cherniak, L.C. Chen, J.S. Hamdam, and A. Casadevall. 1998. Microevolution of a standard strain of *Cryptococcus neoformans* resulting in differences in virulence and other phenotypes. *Infect. Immun.* 66:89-97.
- Franzot, S.P., J.S. Hamdam, B.P. Currie, and A. Casadevall. 1997. Molecular Epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both local genetic differences and a global clonal population structure. *J. Clin. Microbiol.* 35: 2243-2251.
- Fry, W.E., and Goodwin, S.B. 1997. Re-emergence of potato and tomato late blight in the United States. *Plant Disease.* 81:1349-1357.
- Gezuele, E., L. Calegari, D. Sanabria, G. Davel, and E. Civila. 1993. Isolation in Uruguay of *Cryptococcus neoformans* var. *gattii* from a nest of the wasp *Polybia occidentalis*. *Rev. Iber. Micol.* 10:5-6.
- Halliday, C.L., T. Bui, M.Krockenberger, R. Malik, D.H. Ellis, and D.A. Carter. 1999. Presence of α and a mating types in environmental and clinical collections of *Cryptococcus neoformans* var. *gattii* strains from Australia. *J. Clin. Microbiol.* 37:2920-2926.

- Heitman, J., B. Allen, J.A. Alspaugh, and K.J. Kwon-Chung. 1999. On the origins of congenic MAT α and MAT α strains of the pathogenic yeast *Cryptococcus neoformans*. Fung. Genet. Biol. 28:1-5.
- Kabasawa, K., H. Itagaki, R. Ikeda, T. Shinoda, K. Kagaya, and Y. Fukazawa. 1991. Evaluation of a new method for identification of *Cryptococcus neoformans* which uses serologic tests aided by selected biological tests. J. Clin. Microbiol. 29:2873-2876.
- Kwon-Chung, K.J., B.L. Wickes, L. Stockman, G.D. Roberts, D. Ellis, and D.H. Howard. 1992. Virulence, serotype and molecular characteristics of environmental strains of *Cryptococcus neoformans*. Infect. Immun. 60:602-605.
- Kwon-Chung, K.J., J.C. Edman, and B.L. Wickes. 1992. Genetic association of mating types and virulence in *Cryptococcus neoformans*. Infect. Immun. 60:1869-1874.
- Kwon-Chung, K.J., and J.E. Bennett. 1978. Distribution of α and a mating types of *Cryptococcus neoformans* among natural and clinical isolates. Am. J. Epidemiol. 108: 337-340
- Lazera, M.S., B. Wanke, and M.M. Nishikawa. 1993. Isolation of both varieties of *Cryptococcus neoformans* from saprophytic sources in the city of Rio de Janeiro, Brazil. J. Med. Vet. Mycol. 31:449-454.
- Lazera, M.S., F.D.A. Pires, L. Camillo-coura, M.M. Nishikawa, C.C.F. Bezerra, L. Trilles, and B. Wanke. 1996. Natural habitat of *Cryptococcus neoformans* var. *neoformans* in decaying wood forming hollows in living trees. J. Med. Vet. Mycol. 34:127-131.

- Lengeler, K.B., G.M. Cox, and J. Heitman. 2001. Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. *Infect. Immun.* 69:115-122.
- Lengeler, K. B., R. B. Davison, C. D'Souza, T. Harashima , W-C Shen, P. Wang, X. Pan, M. Waugh, and J. Heitman. 2000. Signal transduction cascades regulating fungal development and virulence. *Microbiol. Mol. Biol. Rev.* 64:746-785.
- Lengeler, K.B., P. Wang, G.M. Cox, J.R. Perfect, and J. Heitman. 2000. Identification of the MATa mating-type locus of *Cryptococcus neoformans* reveals a serotype A MATa strain thought to have been extinct. *Proc. Natl. Acad. Sci. USA.* 97:14455-14460.
- Madrenys, N., C.De Vroey, C.R. Wuytack, and J.M. Torres-Rodriguez. 1993. Identification of the perfect state of *Cryptococcus neoformans* from 195 clinical isolates including 84 from AIDS patients. *Mycopathologia.* 123:65-68.
- Moore, T.D., and J.C. Edman. 1993. The alpha-mating type locus of *Cryptococcus neoformans* contains a peptide pheromone gene. *Mol. Cell. Biol.* 13:1962-1970.
- Paugam, A., J. Dupouy-Camet, P. Blanche, J.P. Gangneaux, C. Tourte-Schaefer, and D. Sicard. 1993. Increased fluconazole resistance of *Cryptococcus neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin. Infect. Dis.* 17:431-436.
- Pfaller, M., J. Zhang, S. Messere, M. Tumbeerland, E. Mbidde, C. Jessup, and M. Ghannoum. 1998. Molecular epidemiology and antifungal susceptibility of *Cryptococcus neoformans* isolates from Uganda AIDS patients. *Diagn. Microbiol. Infect. Dis.* 32:191-199

- Sambrook, E., F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning*. Cold Spring Harbor Press, Cold Spring Harbor, New York.
- Takeo, K., R. Tanaka, H. Taguchi, and K. Nishimura. 1993. Analysis of ploidy and sexual characteristics of natural isolates of *Cryptococcus neoformans*. *Can. J. Microbiol.* 39:958-963.
- Viviani, M. A., M. C. Esposto, M. Cogloati, M. T. Montagna, and B. L. Wickes. 2001. Isolation of a *Cryptococcus neoformans* serotype A MATa strain from the Italian environment. *Medical Mycology.* 39:383-386.
- Wickes, B.L., M.E. Mayorga, U. Edman, and J.C. Edman. 1996. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the α -mating type. *Proc. Natl. Acad. Sci. USA.* 93:7327-7331.
- Xu, J., G. Luo, R.J. Vilgalys, M.E. Brandt, and T.G. Mitchell. 2002. Multiple origins of hybrid strains of *Cryptococcus neoformans* with serotype AD. *Microbiology.* 148: 203-212.
- Xu, J., R.J. Vilgalys and T.G. Mitchell. 2000. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Molecular Ecology.* 9:1471-1481.
- Xu, J., A.R. Ramos, R.J. Vilgalys, and T.G. Mitchell. 2000. Clonal and spontaneous origins of fluconazole resistance in *Candida albicans*. *J. Clin. Microbiol.* 38:1214-1220.
- Xu, J., C. Onyewu, H.J. Yoell, R.Y. Ali, R.J. Vilgalys, and T.G. Mitchell. 2001. Dynamic and heterogeneous mutations to fluconazole resistance in *Cryptococcus neoformans*. *Antimicrobial. Agents Chemother.* 45:420-427.

Xu, J. and T. G. Mitchell, T. G. 2002. Strain variation and clonality in species of *Candida* and *Cryptococcus neoformans*. Chapter 33. pp. 739-750. in “Fungal Pathogenesis: Principles and Clinical Applications”. Editors: R. A. Calderone and R. L. Cihlar. Marcel Dekker, Inc.

Table 2.1 Primers used for identification of serotype and mating type specific alleles.

Primer name	Primer sequence (5'-3')	Annealing temp (°C)	No of cycles	Expected band size (bp)
STE20Aa	Forward: tccactggcaaccctgcgag Reverse: atcagagacagaggagcaagac	55	35	865
STE20A α	Forward: ccaaaagctgatgctgtgga Reverse: aggacatctatagcagat	55-45	38	588
STE20Da	Forward: gatctgtctcagcagccac Reverse: aatatcagctgccaggtga	60	32	~440
STE20D α	Forward: gatttatctcagcagccacg Reverse: aaatcggctacggcacgtc	61	28	443
STE12 α	Forward: ctgaggaatctcaaaccaggga Reverse: ccagggcatctagaacaatcg	55	35	379

Table 2.2 Distribution of mating types as determined by crossing experiments.

Geographic Location	Crossing	Serotype				Total (%)
		A	D	AD	B	
San Francisco, California	Mated ¹	39	3	4		46
	Self fertile (%)	11		6	1	18(12.77)
	Sterile	69	1	7		77
	Total	119	4	17	1	141
Georgia	Mated	26	4			30
	Self fertile (%)	2				2(1.74)
	Sterile	78	3	1	1	83
	Total	106	7	1	1	115
Texas	Mated	22	1	1		24
	Self fertile (%)	2				2(2.81)
	Sterile	44			1	45
	Total	68	1	1	1	71
Alabama	Mated	11				11
	Self fertile (%)	2				2(6.45)
	Sterile	18				18
	Total	31				31
Total	Mated (%)	98(30.2)	8(66.7)	5(26.3)		111(31.0)
	Self fertile (%)	17(5.25)		6(31.58)	1(33.33)	24(6.70)
	Sterile	209	4	8	2	223
	Total	324	12	19	3	358

1, “Mated” means hyphae were formed when strains were crossed to either of the two tester strains JEC20 or JEC21. Strains which produced hyphae on their own were not included in the “Mated” category, but the “Self fertile” category.

Table 2.3 Distribution of mating type alleles as determined by PCR with serotype- and mating type allele- specific primers in *C. neoformans*.

Loca- tion ¹	Serotype and mating type specific alleles, No. of strains (%)								
	A α	D α	AaD α	A α Da	AD α	A α D	ADa	B α	Total
SF	119(84.39)	4(2.84)	13(9.22)	2(1.42)	1(0.71)	1(0.71)		1(0.71)	141
GA	106(92.17)	7(6.09)	1(0.87)					1(0.87)	115
TX	68(95.78)	1(1.41)					1(1.41)	1(1.41)	71
AL	31(100)								31
Total	324(90.50)	12(3.35)	14(3.91)	2(0.56)	1(0.28)	1(0.28)	1(0.28)	3(0.84)	358

¹, SF: San Francisco; GA: Georgia; TX: Texas; AL: Alabama

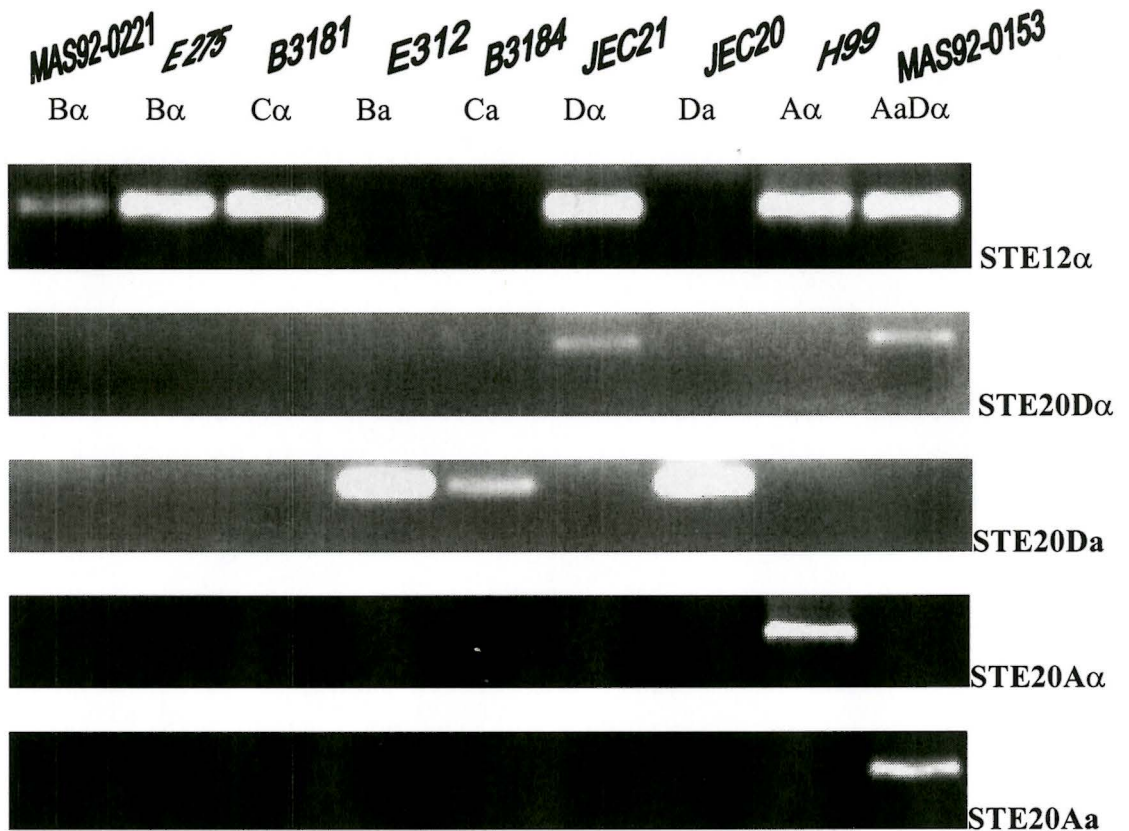


Figure 2.1 Confirmation of serotype and mating type specific primers using seven representative strains of *C. neoformans*. Two additional strains (MAS92-0221 and MAS92-0153) from this collection were also included in Fig 2.1. The following five primer pairs were tested: STE12α, STE20Aa, STE20Aα, STE20Da and STE20Dα (see Table 2.1 for details about these primer pairs). From left to right, MAS92-0221 (serotype B, MATα), E275 (serotype B, MATα), B3181 (serotype C, MATα), E312 (serotype B, MATa), B3184 (serotype C, MATa), JEC21 (serotype D, MATα), JEC20 (serotype D, MATa), H99 (serotype A, MATα) and MAS92-0153 (serotype AD, mating type Aa and Dα).

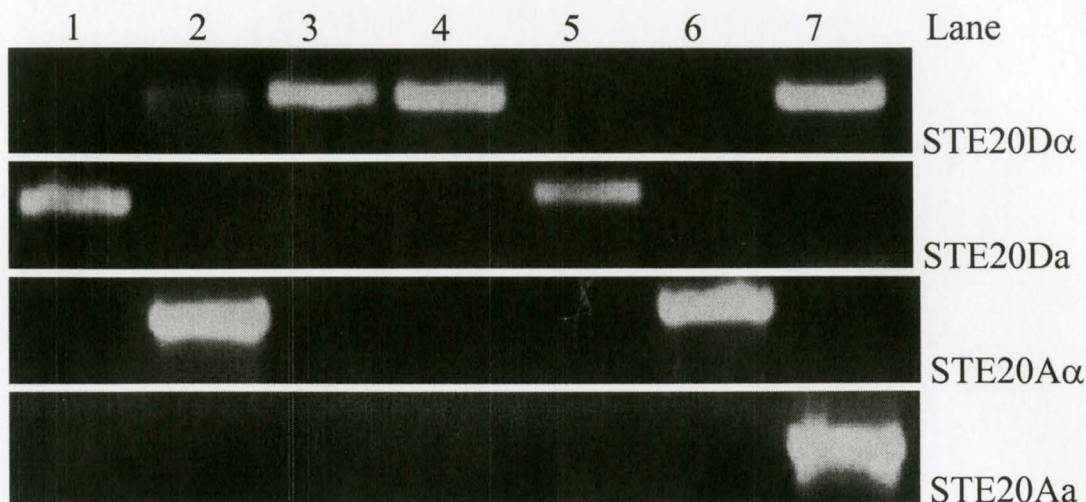


Figure 2.2 Three strains of serotype AD containing only one *STE20* allele were identified by PCR with four serotype and mating type specific primers (*STE20Aa*, *STE20Aα*, *STE20Da* and *STE20Dα*). From left to right, 1, MAS94-0351 (serotype AD, contained only the *STE20* Da allele but not the *STE20* Aα allele or other *STE20* alleles); 2, MAS92-0855 (serotype AD, possessed only the *STE20* Aα allele, but lacked *STE20* Da allele. A weak band was observed with primer pair *STE20Dα*, likely the result of a cross-reaction, see Results and Discussion); 3, MAS92-0224 (serotype AD, had only the *STE20* Dα allele, but lacked *STE20* Aa allele). Strains JEC21 (lane 4), JEC20 (lane 5), H99 (lane 6), and MAS92-0153 (lane 7) were used as controls and their serotypes and mating types were described in the text and the legend of Fig. 2.1.

**Chapter 3: Mitochondria are inherited from the MATa parent
in crosses of the basidiomycete fungus *Cryptococcus
neoformans***

Preface:

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This study was conducted under the guidance of my supervisor, Dr. Jianping Xu. I performed all the experiments, prepared the figures, tables and wrote part of the manuscript. Dr. Xu helped with designing the experiments and writing the manuscript.

Mitochondria are inherited from the MATa parent in crosses of the basidiomycete fungus *Cryptococcus neoformans*

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Running title: Mating type control of mtDNA inheritance in *C. neoformans*

Key words: Mating type, mitochondria, uniparental inheritance, isogamous mating.

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ABSTRACT:

Previous studies demonstrated that mitochondrial DNA (mtDNA) was uniparentally transmitted in laboratory crosses of the pathogenic yeast *Cryptococcus neoformans*. To begin understanding the mechanisms, this study examined the potential role of the mating type locus on mtDNA inheritance in *C. neoformans*. Using existing isogenic strains (JEC20 and JEC21) that differed only at the mating type locus and a clinical strain (CDC46) that possessed a mitochondrial genotype different from JEC20 and JEC21, we constructed strains that differed only in mating type and mitochondrial genotype. These strains were then crossed to produce hyphae and sexual spores. Among the total of 206 single spores analyzed from six crosses, all but one inherited mtDNA from the MATa parents. Analyses of mating type alleles and mtDNA genotypes of natural hybrids from clinical and natural samples were consistent with the hypothesis that mtDNA is inherited from the MATa parent in *C. neoformans*. To our knowledge, this is the first demonstration that mating type controls mtDNA inheritance in fungi.

INTRODUCTION

Compared to the nuclear genome, the relatively small mitochondrial genome is unique due to its symbiotic origin, its non-Mendelian pattern of inheritance, and the central role of mitochondrial oxidative respiration in cell metabolism and energy production (Gillham, 1994). In many species with gametes of different sizes (anisogamy), sexual crosses typically produce progeny with mitochondrial DNA (mtDNA) inherited from the larger gamete. This uniparental inheritance has been traditionally attributed to (i) failure of mitochondria from the smaller gamete (e.g., sperm) to enter the larger gamete (e.g. egg); or (ii) the relatively smaller number of organelles in the smaller gamete as compared to the larger gamete (Birky Jr., 1996). However, these cellular mechanisms cannot explain uniparental mtDNA inheritance in isogamous species with equal-sized and undifferentiated gametes.

Cryptococcus neoformans (Sanfelice) Vuillemin is an isogamous basidiomycete yeast. It is an important fungal pathogen of humans and other mammals throughout the world. Though diploid strains have been found, most natural strains of *C. neoformans* are haploid (Casadevall and Perfect 1998). Using commercial monoclonal antibodies to capsular epitopes, strains of *C. neoformans* manifest five distinct serotypes - A, B, C, D, and AD (Evans, 1950; Wilson et al., 1968; Ikeda et al., 1982; Kabasawa et al., 1991; Casadevall and Perfect, 1998). A small number of strains do not react with any of the four serotype-specific antibodies. These strains are called untypable or undefined. Recent studies showed that strains in different serotypes often exhibit significant divergence at the molecular level (e.g. Franzot et al., 1999; Xu et al., 2000c). Three varieties have been

proposed, in recognition of the divergence within this biological species: *C. neoformans* var. *neoformans* represents serotype D strains; *C. neoformans* var. *grubii* represents serotype A strains; and *C. neoformans* var. *gattii* represents serotypes B and C strains (Wilson et al., 1968; Kwon-Chung et al., 1982; Franzot et al., 1999). However, this three-variety classification system left serotype AD strains without any variety placement. More recently, based on evidence from amplified fragment length polymorphisms (AFLPs), Boekhout et al. (2001) proposed to divide *C. neoformans* into two separate species, with serotypes A, D and AD included in the species *Cryptococcus neoformans* (Sanfelice) Vuillemin, and strains of serotypes B and C comprising a new species, *Cryptococcus bacillisporus* Kwon-Chung. This nomenclature reverts to the original classification proposed for the teleomorphic species of *Filobasidiella* (Kwon-Chung, 1976; Aulakh et al., 1981). Because all proposed classification systems rely on serotype identifications, to avoid confusions only serotype identifications will be used for taxonomic descriptions of strains in this study. The original study reporting uniparental inheritance of mtDNA in *C. neoformans* was based on crosses between strains of serotypes A and D (Xu et al., 2000a).

In basidiomycete fungi, mating typically involves fusion of undifferentiated vegetative cells with compatible mating types (Raper, 1966). In typical laboratory crosses between two compatible homokaryotic mycelia, mating leads to the production of a mycelial mass with a uniform nuclear genotype containing genetic information from nuclei of both mating partners in each cell. However, this mycelial mass is mosaic in mtDNA distribution. The different patterns of nuclear and mtDNA distribution have been

traditionally attributed to the rapid migration of the nuclear genomes through recipient hyphae but limited or no migration for the mitochondrial genomes (May and Taylor, 1988).

Unlike most basidiomycete species, the predominant state of *C. neoformans* is yeast, not mycelial. Consequently, it was hypothesized that mtDNA inheritance in *C. neoformans* would be different from that of filamentous basidiomycete species but similar to that of the unicellular Baker's yeast *Saccharomyces cerevisiae* (Xu et al., 2000a). Interestingly, in laboratory crosses of strains of *C. neoformans*, mtDNA inheritance was different from those observed in both basidiomycete species and *S. cerevisiae*. In a given cross in *C. neoformans*, all progeny inherited mtDNA from only one parent (Xu et al., 2000a). Furthermore, unlike in *S. cerevisiae* or some basidiomycete species, among 570 progeny examined from six independent crosses, no progeny were heteroplasmic or contained recombinant mtDNA molecules (Xu et al., 2000a; Xu, 2002).

To understand the mechanism of mitochondrial inheritance, it is necessary to have easily distinguishable mtDNA genotypes within a common set of nuclear backgrounds. Because previous attempts to establish antibiotic resistance markers in mitochondria of *C. neoformans* failed (Xu et al., 2000a), we sought to utilize natural mtDNA variation (Xu, 2002) and the unique mating processes in *C. neoformans* to construct isogenic strains that differ only in mtDNA. *C. neoformans* has a heterothallic mating system with two alternative mating types, MAT_a and MAT_α (Kwon-Chung 1976). Typical mating experiments in *C. neoformans* are performed on nitrogen-limited medium. The predominant products of mating, dikaryotic hyphae, grow beyond the original mating yeast colony and extend to the surrounding medium, presumably for efficient foraging of

nutrients. This pattern of growth allows mated dikaryotic cells to be easily separable from original parental yeast cells. Along these dikaryotic hyphae, two types of spores are formed. The first type is sexual basidiospores. In the terminal cells (termed basidia) of dikaryotic hyphae, nuclear fusion and meiosis can occur and produce four chains of haploid basidiospores (Kwon-Chung, 1976). These basidiospores are recombinants containing genetic material from both mating partners. The second type of spores produced by dikaryotic hyphae are spontaneously generated asexual spores (called blastospore) (Erke, 1976). Blastospores typically contain only one nucleus. Because no karyogamy was involved to produce blastospores, nuclear material in these spores is typically from only one of the two parental nuclei, and is the product of neither karyogamy nor recombination. Because uniparental mitochondrial inheritance occurs very early in mating processes in *C. neoformans* (Xu et al., 2000a), it is therefore possible to obtain blastospores containing the nuclear genotype from one parent but the mitochondrial genotype from another parent. These spores can then be used in genetic crosses to study mechanism of mitochondrial inheritance in *C. neoformans*.

The objective of this study was to examine the potential role of the mating type locus in mtDNA inheritance in *C. neoformans*. We focused on this locus for two reasons. First, previous studies demonstrated that genes at the mating type locus controlled organelle inheritance in the model unicellular alga *Chlamydomonas reinhardtii* (Gillham, 1994). Second, isogenic strains (JEC20 and JEC21) in *C. neoformans* that differed only at the mating type locus are already available (Kwon-Chung et al., 1992; Heitman et al., 1999). To achieve our goal, we constructed strains with the same nuclear genotypes to

JEC20 and JEC21 but with different mitochondrial genotypes, utilizing the natural processes of blastospore genesis and micromanipulation. A series of genetic tests was performed to confirm their nuclear and mitochondrial genotype identity. Six such strains were obtained and these strains were crossed to either JEC20 or JEC21. A total of 206 meiotic progeny from these six crosses were analyzed at both the mating type locus and the mitochondrial genomes. All but one progeny inherited mtDNA from the MATa parent. Analyses of mating type alleles and mtDNA genotypes for natural hybrids in *C. neoformans* were also consistent with the hypothesis that progeny inherit mtDNA from the MATa parent.

MATERIALS AND METHODS

Strains: The following three initial strains were used to construct isogenic strains with different mtDNA genotypes: JEC20; JEC21; and CDC46. JEC20 and JEC21 were isogenic except at the mating type locus: JEC20 has the mating type a allele (MATa) and JEC21 the mating type α allele (MAT α) (Kwon-Chung et al., 1992; Heitman et al., 1999). Both JEC20 and JEC21 were serotype D and had the serotype D-specific mtDNA genotype (mtDNA haplotype II in Xu, 2002). Strain CDC46 was isolated from a patient in San Francisco, California, USA, during an epidemiological survey conducted by the Centers for Disease Control and Prevention of the U. S. (CDC) in 1992. CDC46 is serotype AD and contains both MATa and MAT α alleles at the mating type locus (Yan et al., 2002). It has a serotype A-specific mtDNA haplotype (haplotype I in Xu, 2002).

Natural and clinical samples of *C. neoformans* used to compare mating type alleles and mtDNA genotypes were obtained through the following two sources: (i) the Centers for Disease Control and Prevention of the U. S. (CDC) and kindly provided by Dr. Mary Brandt; and (ii) the Nagasaki University in Japan and generously provided by Dr. S. Maesaki. Serotypes of strains from the CDC were determined using monoclonal antibodies and for strains of serotypes AD and B, confirmed by indirect immunofluorescence with a combination of polyclonal and monoclonal reagents (Brandt et al., 1995). Serotypes for strains from Japan were determined by the Iatron commercial kit (Iatron Laboratories Inc., Co. Tokyo, Japan). Mating type alleles for strains from CDC were recently reported by Yan et al. (2002). Mating type alleles for strains from Japan were determined based on serotype and mating type specific PCR primers and followed the protocols described in Yan et al. (2002). Mitochondrial genotypes for strains from both CDC and Japan were determined and reported by Xu (2002).

Construction of isogenic strains with different mtDNA genotypes. Our initial screening demonstrated that CDC46 contained both MAT α and MAT α (Yan et al. 2002). This strain was found able to mate on V8 juice medium with both JEC20 and JEC21. In addition, it had a mtDNA genotype different from JEC20 and JEC21 (Xu, 2002). Therefore, we initiated two crosses, one between JEC20 and CDC46 and the other between JEC21 and CDC46, with the objective of obtaining progeny having the same nuclear genotype as JEC20 and JEC21, but the mtDNA genotype from CDC46. Mating was performed on V8-juice agar medium (Kwon-Chung 1976). After 2-3 weeks of incubation at room temperature (~22°C), extensive hyphae were produced and both basidiospores

(sexual spores) and blastospores (asexual spores) could be observed under microscope. Single-spore isolates (SSIs) were obtained by picking randomly germinated single spores as described previously by Xu et al. (2000a). As shown in a later section, SSIs derived from this process are not different from those derived by micromanipulation.

Genotypic identification: To identify strains with identical nuclear genotypes to JEC20 and JEC21 but with the mtDNA genotype from CDC46, genetic tests were performed in a stepwise manner. Genomic DNA was first extracted from each of the 123 SSIs according to the method described by Xu et al. (2000b). The following four types of DNA-based markers were used: (i) mating type allele-specific PCR products; (ii) restriction site polymorphisms of specific genes; (iii) randomly amplified polymorphic DNA (RAPD) bands; and (iv) chromosomal length polymorphisms. These markers are described separately in the following sections:

(I) Mating type allele-specific PCR: Four mating type-specific PCR primer pairs for the *STE20* locus were used to identify serotype and mating type-specific alleles among the single spore isolates. The effectiveness of these primer pairs for serotype specific mating type allele identification has been demonstrated in several laboratories using over 400 strains (Lengeler et al., 2001; Yan et al., 2002). In the three initial strains, JEC20 had the serotype D-specific MATa (abbreviated as Da); JEC21 had the serotype D-specific MAT α (abbreviated as D α); and CDC46 had both the serotype A-specific-MATa allele (abbreviated as Aa) plus the serotype D-specific MAT α allele (abbreviated as D α). Therefore, strain CDC46 had the mating type AaD α . Table 3.1 lists the sequences, annealing temperatures, and number of cycles for different primer pairs. Each PCR

reaction contained 0.4ng genomic DNA template, 0.5uM of each primer, 2mM magnesium chloride (MgCl₂), 0.1 mM deoxyribonucleotide triphosphate (dNTP) and 0.2 unit of Taq DNA polymerase in a total volume of 20ul. Typical PCR reaction conditions were: 3 min at 94°C, followed by 40 cycles of 30s at 94°C, 30s at the indicated annealing temperature, and 30s at 72 °C, and finally, 7 min of extension at 72°C. PCR products were run on 1% agarose gels, stained with ethidium bromide and revealed using ultraviolet light, and scored for the presence/absence of expected DNA fragments.

(II) Restriction site polymorphisms: Restriction site polymorphisms were examined for two genes: (i) the nuclear gene orotidine monophosphate pyrophosphorylase (*URA5*); and (ii) the mitochondrial NADH dehydrogenase subunit 2 (*ND2*) (<http://www-sequence.stanford.edu/group/C.neoformans/index.html>). Restriction site polymorphisms for these two genes were presented elsewhere (Currie et al.,1994; Xu, 2002). Primer pairs and the number of cycles used are presented in Table 3.1. PCR conditions were similar to those described above. PCR products were digested by *Hind* III and *Alu* I for the *URA5* and *ND2* genes, respectively, according to manufacturer's recommendations. Restriction patterns were checked by agarose gel electrophoresis.

(III) Randomly amplified polymorphic DNA: Four individual PCR primers were used to examine genotypes at nonspecific locations in the genome. The two long primers, M13 phage core sequence (5'-GAGGGTGGCGTTCT-3') and (GACA)₄ (5'-GACA GACA GACA GACA-3'), have been used extensively for DNA fingerprinting of human pathogenic fungi. Primers OPA17 (5'-GACCGCTTGT-3') and RP4 (5'-ATTGCGTCCA-3') were both 10-mers found to be diagnostic for distinguishing CDC46

from JEC20 and JEC21. Conditions of PCR are presented in Table 1 and followed those described by Xu et al. (1999; 2000b).

(IV) Electrophoretic karyotypes (CHEF): Pulsed field gel electrophoresis was performed following the protocols described in Forche et al. (2001). The chromosomes were separated under the following running conditions: Block 1, 100-200 seconds switch at 4.5V at 14⁰C for 30hrs; Block 2, 250-900 seconds switch at 3.0V at 14⁰C for 46 hours. The gel was then stained with ethidium bromide and visualized under ultraviolet light.

Except for the mating type locus, JEC20 and JEC21 showed no differences at other genetic markers, consistent with previous reports (Kwon-Chung et al., 1992; Heitman et al., 1999). In contrast, CDC46 showed different patterns from those of JEC20 and JEC21 for all genotyping methods.

Genetic crosses to determine the effects of mating type locus on mitochondrial inheritance in *C. neoformans*. Six single-spore isolates with identical nuclear genotypes to either JEC20 or JEC21 but with mtDNA from CDC46 were obtained from the above two crosses. Their genotypes are presented in Figure 3.1 and Table 3.2 (see Results for details). The six strains listed in Table 3.2 were crossed to JEC20 or JEC21 to generate the following six crosses: JEC20-mtA-1 X JEC21; JEC20-mtA-2 X JEC21; JEC20-mtA-3 X JEC21; JEC21-mtA-1 X JEC20; JEC21-mtA-2 X JEC20; and JEC21-mtA-3 X JEC20. A total of 206 SSIs were isolated and analyzed. Among these SSIs, 134 SSIs were isolated by spore suspension, dilution, plating on YEPD agar medium (1% yeast extract, 2% Bacto-peptone, 2% dextrose, and 2% Bacto-agar in distilled water), and picking of randomly germinated spores following the protocols described in Xu et al. (2000a). An additional 72

SSIs were obtained from cross JEC20-mtA-3 x JEC21 by micromanipulation using a Singer MSM 300 micromanipulator (Singer Instrument Co. Ltd., Somerset, U.K.). Each SSI was individually characterized for mating type and mitochondrial genotype. Genotypes at other gene loci and genetic markers were expected to be identical for all parents in these crosses (see Figure 3.1 and Table 3.2). Therefore, other genotyping markers were not used to examine SSIs from these six crosses.

Comparative analyses of mating type alleles and mtDNA genotypes for natural and clinical populations of *C. neoformans*. Mating type alleles for the 358 strains of *C. neoformans* from the CDC were recently reported by Yan et al. (2002). Similarly, mtDNA genotypes were determined for all 358 strains from the CDC (Xu 2002), and in addition, for 56 strains from Japan (Xu 2002). To allow complete comparison of the two genomic regions, we further determined mating type alleles for the 56 strains from Japan, following the protocol described above and by Yan et al. (2002)

RESULTS

Construction of strains with identical nuclear genomes but different mitochondrial genomes to JEC20 and JEC21. From two crosses, JEC20 X CDC46 and JEC21 X CDC46, we respectively screened a total of 101 and 22 SSIs. These SSIs included both basidiospores and blastospores, as these two groups of spores were not easily distinguishable during spore isolation either by micromanipulations or by spore suspension and plating. All 123 SSIs were first genotyped using six gene-specific PCR

primer pairs, including four serotype- and mating type- specific PCR primer pairs (A α , A α , Da, and D α) that amplify the *STE20* gene, and two PCR-RFLP markers (*URA5* and *ND2*).

Among the 101 SSIs from the cross between JEC20 and CDC46, eight (~8%) had identical mating type alleles and *URA5* genes as JEC20 but the mitochondrial genotype at the *ND2* locus from CDC46. In contrast, a higher percentage (17 of the 22, 77%) of the SSIs from cross JEC21 X CDC46 had the identical mating type alleles and *URA5* genes as JEC21 but the mitochondrial genotype at the *ND2* locus from CDC46. Genotypes at these loci for three selected SSIs from each of the two crosses are presented in Figure 3.1. No SSI was found to contain all alleles at the mating type locus and the *URA5* locus from both mating partners. None had mixed mitochondrial genotypes.

Further tests were conducted using RAPDs amplified by four individual primers and by pulsed-field gel electrophoresis. To increase the efficiency of identifying strains with the desired combinations, six SSIs (three from each cross) from the candidate pools above were randomly picked for these further tests. All six showed identical genotype profiles to JEC20 or JEC21 for all genotyping tests. The results of the tests are presented in Figure 3.1 (gel pictures from RAPD primers M13, (GACA)₄, and OPA17 are not presented but results from these primers are summarized in Table 3.2). Because of the obvious genetic differences between mating partners CDC46 vs. JEC20 and JEC21, the lack of any genetic change between these six SSIs and JEC20 or JEC21 as determined by our genotyping methods suggested that these six SSIs were blastospores, derived from mitotic budding along hyphae without going through karyogamy and meiosis. For ease of communication, these six SSIs are designated JEC20-mtA-1, JEC20-mtA-2, JEC20-mtA-3,

JEC21-mtA-1, JEC21-mtA-2, and JEC21-mtA-3. These designations imply that they have nuclear genotype from strains JEC20 or JEC21, but with the serotype A- specific mtDNA genotype. The last digit refers to the isolation number.

Mitochondrial inheritance in crosses between strains that differ only at the mating type locus and the mitochondrial genome. Six crosses were constructed and results are presented in Table 3.3. In all six crosses, mating type alleles segregated at ratios not significantly different from 1:1 (Table 3.3), indicating Mendelian inheritance of nuclear genetic markers. The genotypes of five representative SSIs from two crosses are presented in Figures 3.2 and 3.3. In contrast to the balanced mating type allele inheritance, mitochondrial inheritance was highly skewed. Among the 206 SSIs, only one inherited mtDNA genotype from the MAT α parent; all other 205 SSIs inherited mtDNA from the MAT α parent. No difference was observed between SSIs obtained by the two different protocols (i.e. micromanipulation vs. picking of randomly germinated spores).

Interestingly, five of the 206 SSIs contained both MAT α and MAT α , suggesting potential abnormal nuclear disjunction or other processes causing incomplete meiosis. However, in all five cases, their mitochondrial genotype was identical to that of MAT α parent.

Comparative analyses of mating type and mitochondrial genotype in environmental and clinical samples of *C. neoformans*. Summary results of mating types and mitochondrial genotypes from natural and clinical sources are presented in Table 3.4. Data are arranged according to strain geographic origin and serotype identification. Genotypes of representative strains are presented in Figure 3.4. Among the 376 strains of

serotype A, all had the serotype A-specific MAT α allele at the *STE20* locus (A α) and all had the same, serotype A-specific restriction site pattern for the mitochondrial gene *ND2* (haplotype I). Similarly, all 12 serotype D strains had the serotype D-specific MAT α allele at *STE20* locus (D α) and the serotype D-specific mtDNA haplotype (haplotype II). For the three strains of serotype B, all had the MAT α mating type and a mtDNA haplotype different from strains of serotypes A and D. For the two strains with undefined serotypes from Japan, one had the serotype A-specific mtDNA type while the other had the serotype D-specific mtDNA type (Table 3.4). In contrast, strains of serotype AD showed a mixed pattern of mating type alleles and mitochondrial genotypes (Table 3.4).

To facilitate comparisons of mating type allele and mtDNA genotype among serotype AD strains, the 21 serotype AD strains were divided into five categories based on our earlier findings of mating type alleles (Yan et al., 2002; Table 3.4). Earlier studies confirmed that strains of serotype AD were often diploid or aneuploid (e.g. Lengeler et al., 2001) and the results of recent hybridization between strains of serotypes A and D (Xu et al., 2002). As shown in Table 3.4 and explained below, the hypothesis that mating type controls mtDNA inheritance is consistent with the observed patterns of mtDNA distribution in this group of serotype AD strains.

First, the 16 strains in AD-group 1 from both the US and Japan had MATa from serotype A (Aa). Their mtDNA types were identical to the serotype A-specific mtDNA haplotype (haplotype I). Second, the two strains in AD-group 2 had MATa from serotype D (Da), and their mtDNA types were identical to serotype D-specific mtDNA haplotype (haplotype II). Third, even though only one mating type allele is present in each of the

other three serotype AD strains and therefore inferences about relationships between mating types and mtDNA haplotypes are less obvious, data from these three strains are still consistent with our hypothesis. Assuming these three serotype AD strains were of hybrid origins from strains of serotypes A and D, similar to other serotype AD strains, these strains must have initially contained both MAT α and MAT a (Lengeler et al., 2001; Xu et al., 2002). Subsequently, one of the two mating type alleles was lost during mitotic or meiotic divisions. If so, the missing mating type allele could be inferred based on the remaining mating type allele information. Specifically, the strain in AD-group 3 should have initially contained (Aa)D α ; the strain in AD-group 4 strain contained A α (Da); and the strain in AD-group 5 contained (A α)Da (Note: the putative missing alleles were indicated in parentheses). Again, a simple comparison revealed that their mtDNA haplotypes were inherited from the MAT a parent in sexual crosses in *C. neoformans*.

DISCUSSION

In this study, we examined the effect of mating type locus on mitochondrial inheritance in the human pathogenic yeast *C. neoformans*. To achieve this goal, we constructed isogenic strains that differed only in mitochondrial DNA. We showed in six independent crosses that, with one exception, all SSIs inherited mitochondria from the MAT a parent. Mating type control of mitochondrial inheritance was also suggested among natural hybrids through the joint analyses of mating types and mitochondrial DNA haplotypes. To our knowledge, this is the first unambiguous evidence demonstrating that mating type locus controls mitochondrial inheritance in sexual crosses in a fungus.

The initial observation of uniparental mtDNA inheritance in *C. neoformans* was based on six crosses between strains of serotypes A and D (Xu et al., 2000a). While such a result suggested a genetic mechanism, it was not known where such genetic loci might be residing. This was because serotypes A and D were highly divergent from each other (Franzot et al., 1999; Xu et al., 2000c).

The two initial crosses described in this study (JEC20 X CDC46 and JEC21 X CDC46) were analogous to the widely described diploid X haploid (or dikaryon X monokaryon, heterokaryon X homokaryon) matings examined in many filamentous basidiomycete species (e.g. Carvalho et al., 1995; Raper, 1966; Xu et al., 1996). For example, in both the plant pathogen *Armillaria gallica* (Carvalho et al., 1995) and the commercial mushroom *Agaricus bisporus* (Xu et al., 1996), independent assortment/segregation of mitochondrial genomes and nuclear genomes was observed from matings between vegetative hyphae. Our results here indicated that such a re-assortment process is also highly efficient in generating new nuclear-mitochondrial combinations in the basidiomycete yeast species *C. neoformans*.

While nuclear and mitochondrial genome re-assortments can happen in these “illegitimate” matings, there are differences in mating and reproduction between the basidiomycete yeast *C. neoformans* and filamentous basidiomycete species. For example, in most filamentous basidiomycete species, asexual reproduction occurs only through extension of hyphal mycelia while sexual reproduction occurs only in mature, macroscopic mushroom fruiting bodies (Raper 1966). In *C. neoformans*, there is no macroscopic fruiting body and both sexual and asexual spores are present along filamentous mycelium

(e.g. Erke, 1976). Therefore, our collection of SSIs included both sexual and asexual spores. However, our inability to accurately dissect sexual from asexual spores doesn't in any way affect interpretations of our results and conclusions. All basidiospores and blastospores were isolated far from mating mixtures that contained parental yeast cells. Both types of spores were generated from hyphae and therefore must obtain their mitochondrial genotypes from hyphae. Based on these findings, basidiospores, blastospores and hyphae can all be equally used to assess mitochondrial inheritance in *C. neoformans*. Results in Table 3.3 further indicated that SSIs derived from micromanipulation and from picking of randomly germinated colonies showed similar patterns of inheritance for mitochondrial DNA and for the mating type alleles.

Using isogenic strains with different mtDNA genotypes, we constructed six independent crosses, three each with JEC20 and JEC21. The mtDNA inheritance patterns strongly indicated that the mating type locus or a gene closely linked to the mating type locus controlled mitochondrial inheritance. However, there was one exception among the 206 SSIs analyzed (Table 3.3). Because MAT α strains could undergo haploid fruiting under typical mating conditions (Wickes, et al. 1996), we tested whether haploid fruiting by JEC21 was responsible for this SSI. Our test identified that this progeny contained MAT α (i.e. that from parent JEC20-mtA-1) but a mitochondrial genotype from the other parent (JEC21), thus indicating that this SSI was not the result of haploid fruiting. This exception in mtDNA inheritance suggests that mitochondrial inheritance is not under absolute control of mating type locus in *C. neoformans*. Spontaneous leaky mtDNA

inheritance was also reported for uniparental organelle inheritance in the model alga *Chlamydomonas reinhardtii* (Boyton et al., 1987; Gillham, 1994).

Interestingly, the cross JEC20 X CDC46 showed evidence for biparental transmission of mitochondrial DNA. Among the 101 SSIs examined, 8 had mitochondrial genotypes from parent CDC46 and the other 93 had mtDNA inherited from JEC20. This result is not in conflict with our hypothesis that mating type controls mitochondrial inheritance. Strain CDC46 had both MAT α and MATa; therefore, mtDNA from a MATa/MAT α mating partner could also be transmitted to progeny. When CDC46 (the MATa/MAT α parent) was mated to the MAT α parent JEC21, all 22 SSIs inherited mtDNA from CDC46, the strain that contained MATa. In contrast, the contribution was relatively limited when CDC46 was mated to the MATa parent JEC20. Most SSIs (~92%) from this cross inherited mtDNA from the parent JEC20 that contained only MATa.

Uniparental inheritance of mtDNA has been observed in three other unicellular, isogamous species, the slime mold *Polysphondylium pallidum* (Mirfakhrai et al., 1990), and the model unicellular algae *Chlamydomonas reinhardtii* and *Chlamydomonas smithii* (Gillham, 1994). In these species, genes at or close to the mating type locus were found to be responsible for the observed uniparental inheritance. Doubly uniparental inheritance was observed in an isogamous fungus, *Ustilago violacea* (Wilch et al., 1992). In *U. violacea*, progeny expressing the a₂ mating type inherited mitochondria almost exclusively from the a₂ parent. In contrast, progeny with the a₁ mating type inherited mitochondria DNA equally frequently from either parent (Wilch et al., 1992). At present, the detailed

molecular mechanisms of mating type control of mtDNA inheritance are not known in any of these species.

Because mitochondria play a vital role in cellular metabolism, mutations and accessory genetic elements in mitochondria could have significant phenotypic effects on host cells. For example, amplification of segments of mitochondrial DNA, mitochondrial plasmids, and mtDNA genomic mutations have been found associated with senescence in the filamentous ascomycetes *Podospora anserina* and *Neurospora crassa* (for detailed descriptions, please see a recent review by Bertrand 2000). In the Chestnut Blight fungus *Cryphonectria parasitica*, double-stranded RNA viruses and circular plasmids in the mitochondria could reduce virulence of this pathogen (Bertrand, 2000; Monteiro-Vitorello, et al., 2000). Indeed, these viruses and genetic elements are currently being exploited as a strategy for biological control of *C. parasitica*. If similar genetic elements are found in mitochondrial genomes in *C. neoformans*, such elements could be introduced into natural strains of *C. neoformans* to reduce their virulence or increase their rates of senescence. Whether such elements exist and how they could be exploited for controls of *C. neoformans* infections await further investigation.

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REFERENCES

- Aulakh, H. S., S. E. Straus and K. J. Kwon-Chung. 1981. Genetic relatedness of *Filobasidiella neoformans* (*Cryptococcus neoformans*) and *Filobasidiella bacillispora* (*Cryptococcus bacillispora*) as determined by DNA base composition and sequence homology studies. *International Journal Systematic Bacteriology*. 31: 97-103.
- Bertrand, H. 2000. Role of mitochondrial DNA in the senescence and hypovirulence of fungi for plant disease control. *Annual Review of Phytopathology*. 38: 397-422.
- Birky, C. W. Jr. 1996. Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc. Natl. Acad. Sci. USA*. 92:11331-11338.
- Boekhout, T., B. Theelen, M. Diaz, J. W. Fell, W. C. J. Hop, E. C. A. Abeln, F. Dromer and W. Meyer. 2001. Hybrid genotypes in the pathogenic yeast *Cryptococcus neoformans*. *Microbiology*. 147:891-907.
- Boyton, J. E., E. H. Harris, B. D. Burkhart, P. M. Lamerson and N. W. Gillham. 1987. Transmission of mitochondrial and chloroplast genomes in crosses of *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA*. 84: 2391-2395.
- Carvalho, D. B., M. I. Smith and J. B. Anderson. 1995. Genetic exchange between diploid and haploid mycelia of *Armillaria gallica*. *Mycological Research*. 99: 641-647.
- Casadevall, A. and J.R. Perfect. 1998. *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology Press
- Currie, B. P., L. F. Freundlich and A. Casadevall. 1994. Restriction fragment length polymorphism analysis of *Cryptococcus neoformans* isolates from environmental

- (pigeon excreta) and clinical sources in New York City. *Journal of Clinical Microbiology*. 32:1188-1192.
- Erke, K. H. 1976. Light microscopy of basidia, basidiospores, and nuclei in spores and hyphae of *Filobasidiella neoformans* (*Cryptococcus neoformans*). *Journal of Bacteriology*. 128: 445-455.
- Evans, E. E. 1950. The antigenic composition of *Cryptococcus neoformans*. I. A serologic classification by means of the capsular and agglutination reactions. *Journal of Immunology*. 64: 423-430.
- Forche, A., J. Xu, R. Vilgalys and T. G. Mitchell. 2001. Development and characterization of a genetic linkage map of *Cryptococcus neoformans* var. *neoformans* using amplified fragment length polymorphisms (AFLP) and other markers. *Fungal Genetics and Biology*. 31: 180-203.
- Franzot, S. P., I. F. Salkin and A. Casadevall. 1999. *Cryptococcus neoformans* var. *grubii*: separate varietal status for *Cryptococcus neoformans* serotype A isolates. *Journal of Clinical Microbiology*. 37: 838-840.
- Gillham, N. W. 1994. *Organelle Genes and Genomes*. Oxford University Press. N. Y.
- Heitman, J., B. Allen, J. A. Alspaugh and K. J. Kwon-Chung. 1999. On the origins of congenic MAT α and MAT α strains of the pathogenic yeast *Cryptococcus neoformans*. *Fungal Genetics and Biology*. 28:1-5.
- Ikeda, R., T. Shinoda, Y. Fukazawa, and L. Kaufman. 1982. Antigenic characterization of *Cryptococcus neoformans* serotypes and its application to serotyping of clinical isolates. *Journal of Clinical Microbiology*. 16: 22-29.

- Kabasawa, K., H. Itagaki, R. Ikeda, T. Shinoda, K. Kagaya and Y. Fukazawa. 1991. Evaluation of a new method for identification of *Cryptococcus neoformans* which uses serologic tests aided by selected biological tests. *Journal of Clinical Microbiology*. 29:2873-2876.
- Kwon-Chung, K. J. 1976. Morphogenesis of *Filobasidiella neoformans*, the sexual state of *Cryptococcus neoformans*. *Mycologia*. 68:821-833.
- Kwon-Chung, K. J., J. E. Bennett and J. C. Rhodes. 1982. Taxonomic studies on *Filobasidiella* species and their anamorphs. *Antonie Van Leeuwenhoek*. 48:25-38.
- Kwon-Chung, K. J., J. C. Edman and B. L. Wickes. 1992. Genetic association of mating type and virulence in *Cryptococcus neoformans*. *Infection and Immunity*. 60:602-605.
- Lengeler, K. B., G. M. Cox and J. Heitman. 2001. Serotype AD strains of *Cryptococcus neoformans* are diploid or aneuploid and are heterozygous at the mating-type locus. *Infection and Immunity*. 69:115-122.
- May, G. and J. W. Taylor. 1988. Patterns of mating and mitochondrial DNA inheritance in the agaric basidiomycete *Coprinus cinereus*. *Genetics*. 118:213-220.
- Mirfakhrai, M., Y. Tanaka and K. Yanagisawa. 1990. Evidence for mitochondrial DNA polymorphism and uniparental inheritance in the cellular slime mold *Polysphondylium pallidum*: effect of intraspecies mating on mitochondrial DNA transmission. *Genetics*. 124: 607-613.

- Monteiro-Vitorello, C. B., D. Baidyaroy, J. A. Bell, G. Hausner, D. W. Fulbright, H. Bertrand. 2000. A circular mitochondrial plasmid incites hypovirulence in some strains of *Cryphonectria parasitica*. *Current Genetics*. 37: 242-256.
- Raper, J. R. 1966. *Genetics of sexuality in higher fungi*. New York: The Ronald Press Company.
- Sia, R. A., K. B. Lengeler and J. Heitman. 2000. Diploid strains of the pathogenic basidiomycete *Cryptococcus neoformans* are thermally dimorphic. *Fungal Genetics and Biology*. 29:153-163.
- Wickes, B. L., M. E. Mayorga, U. Edman, and J. C. Edman. 1996. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the alpha-mating type. *Proc. Natl. Acad. Sci. USA*. 93: 7327-7331.
- Wilch, G., S. Ward and A. Castle. 1992. Transmission of mitochondrial DNA in *Ustilago violacea*. *Current Genetics*. 22: 135-140.
- Wilson, D. E., J. W. Bennett, and J. W. Bailey. 1968. Serological grouping of *Cryptococcus neoformans*. *Proc. Soc. Exp. Biol. Med.* 127: 820-823.
- Xu, J. 2002. Mitochondrial DNA polymorphisms in the human pathogenic fungus *Cryptococcus neoformans*. *Current Genetics*. 41: 43-47
- Xu, J., R. Ali, D. Gregory, D. Amick, S. Lambert, H. Yoell, R. J. Vilgalys and T. G. Mitchell. 2000a. Uniparental mitochondrial transmission in sexual crosses in *Cryptococcus neoformans*. *Current Microbiology*. 40:269-273.
- Xu, J., P. A. Horgen and J. B. Anderson. 1996. Somatic recombination in the cultivated mushroom *Agaricus bisporus*. *Mycological Research*. 100: 188-192.

- Xu, J., A. R. Ramos, R. J. Vilgalys and T.G. Mitchell. 2000b. Clonal and spontaneous origins of fluconazole resistance in *Candida albicans*. *Journal of Clinical Microbiology*. 38:1214-1220.
- Xu, J., G. Luo, R. Vilgalys, M. E. Brandt and T. G. Mitchell. 2002. Multiple origins of hybrid strains of *Cryptococcus neoformans* with serotype AD. *Microbiology*. 148: 203-212.
- Xu, J., R. J. Vilgalys and T.G. Mitchell. 2000c. Multiple gene genealogies reveal recent dispersion and hybridization in the human pathogenic fungus *Cryptococcus neoformans*. *Molecular Ecology*. 9:1471-1481.
- Xu, J. Estimating the spontaneous mutation rate of loss of sex in the human pathogenic fungus *Cryptococcus neoformans*. *Genetics* (in press)
- Yan, Z., X. Li and J. Xu. 2002. Geographic distribution of mating type alleles of *Cryptococcus neoformans* in four areas of the United States. *Journal of Clinical Microbiology*. 40: 965-972.

Table 3.1 PCR primers and their respective PCR conditions used in this study.

Primer name	Primer sequence (5'-3')	Annealing temp ($^{\circ}$ C)	Number of cycles	Expected band size (bp)
STE20-A α	Forward: ccaaaagctgatgctgtgga Reverse: aggacatctatagcagat	55-45	38	588
STE20-Aa	Forward: tccactggcaaccctgcgag Reverse: atcagagacagaggagcaagac	55	35	865
STE20-Da	Forward: gatctgtctcagcagccac Reverse: aatatcagctgccaggtga	60	32	~440
STE20-D α	Forward: gatttatctcagcagccag Reverse: aaatcggctacggcagctc	61	28	443
URA5	Forward: acgcctgcctgttacttaa Reverse: ggacatgatgattggagt	50	40	744
ND2	Forward: caagctgcaccattccata Reverse: ccattagtgggtgactcc	50	40	533
M13 core	gagggtggcggttct	50	40	Variable
(GAGA) $_4$	gacagacagacagaca	50	40	Variable
OPA17	gaccgcttgt	36	40	Variable
RP4	attgcgtcca	36	40	Variable

Table 3.2 Summary of genotypic tests for six isogenic strains constructed in this study.

Strains	Specific alleles of <i>STE20</i> gene			Nuclear genotype characterized by five different markers						
	Da	D α	Aa	mtDNA <i>ND2</i> (<i>AluI</i> digest)	<i>URA5</i> (<i>HindIII</i> digest)	M13	(GACA) ⁴	OPA17	RP4	CHEF
JEC20	+ ¹	-	-	JEC20 ²	JEC20	JEC20	JEC20	JEC20	JEC20	JEC20
JEC21	-	+	-	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21
CDC46	-	+	+	CDC46 ³	CDC46	CDC46	CDC46	CDC46	CDC46	CDC46
JEC20- mtA-1	+	-	-	CDC46	JEC20	JEC20	JEC20	JEC20	JEC20	JEC20
JEC20- mtA-2	+	-	-	CDC46	JEC20	JEC20	JEC20	JEC20	JEC20	JEC20
JEC21- mtA-1	-	+	-	CDC46	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21
JEC21- mtA-2	-	+	-	CDC46	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21
JEC21- mtA-3	-	+	-	CDC46	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21

¹, Sign “+” indicates the presence of the allele identified by specific PCR primer pair.

Sign “-“ indicates the absence of such an allele.

², Genotype at the indicated locus/loci was identical to JEC20. Except at the mating type locus, JEC20 and JEC21 have identical genotype as shown before (Kwon-Chung et al. 1992) and by all our current genotyping methods.

³, Genotype was identical to that of CDC46 at the indicated locus/genetic marker.

Table 3.3 Mitochondrial inheritance in six crosses.

Cross	MtDNA genome of SSI from parent with MATa or MAT α		Mating type allele distribution for SSIs ¹	
	MATa	MAT α	MATa	MAT α
JEC20 X JEC21-mtA-1	18	0	9	9
JEC20 X JEC21-mtA-2	25	0	12 ²	15 ²
JEC20 X JEC21-mtA-3	16	0	8 ³	9 ³
JEC21 X JEC20-mtA-1	25	1	16	10
JEC21 X JEC20-mtA-2	17	0	8	9
JEC21 X JEC20-mtA-3	33	0	20 ⁴	15 ⁴
JEC21 X JEC20-mtA-3 ⁵	73	0	32	41
Total	206	1	104	108

¹, None of the progeny mating type allele ratios in these crosses was significantly different from an expected 1:1 Mendelian segregation ratio. In all crosses, Chi-square values were less than 2.0 (df=1, P > 0.2).

², Two of the 25 SSIs from this cross contained both MATa and MAT α .

³, One of the 16 SSIs from this cross contained both MATa and MAT α .

⁴, Two of the 33 SSIs from this cross contained both MATa and MAT α .

⁵, Progeny from this cross was obtained by micromanipulation, unlike the other six SSIs samples that were obtained by picking randomly germinated spores. Strain designations are described in text.

Table 3.4. Mating type and mitochondrial genotype distributions among natural and clinical populations of *C. neoformans*.

Country of origin	Serotype	No. of strains	Mating type ^a	Mitochondrial type ^b
USA	A	324	A α	Haplotype I
	D	12	D α	Haplotype II
	AD-group 1	14	AaD α	Haplotype I
	AD-group 2	2	A α Da	Haplotype II
	AD-group 3	1	D α	Haplotype I
	AD-group 4	1	A α	Haplotype II
	AD-group 5	1	Da	Haplotype II
	B	3	B α	Haplotype III
Japan	A	52	A α	Haplotype I
	AD-group 1	2	AaD α	Haplotype I
	Not Typable-1	1	A α	Haplotype I
	Not Typable-2	1	D α	Haplotype II

¹, Mating types were determined based on the presence of serotype and mating type-specific alleles at the *STE20* locus (See Materials and Methods for details; see also Yan et al., 2002).

², Mitochondrial haplotypes were determined based on the restriction site polymorphisms at the mitochondrial *ND2* locus. Mitochondrial haplotypes I and II are specific to serotypes A and D respectively (Xu, 2002).

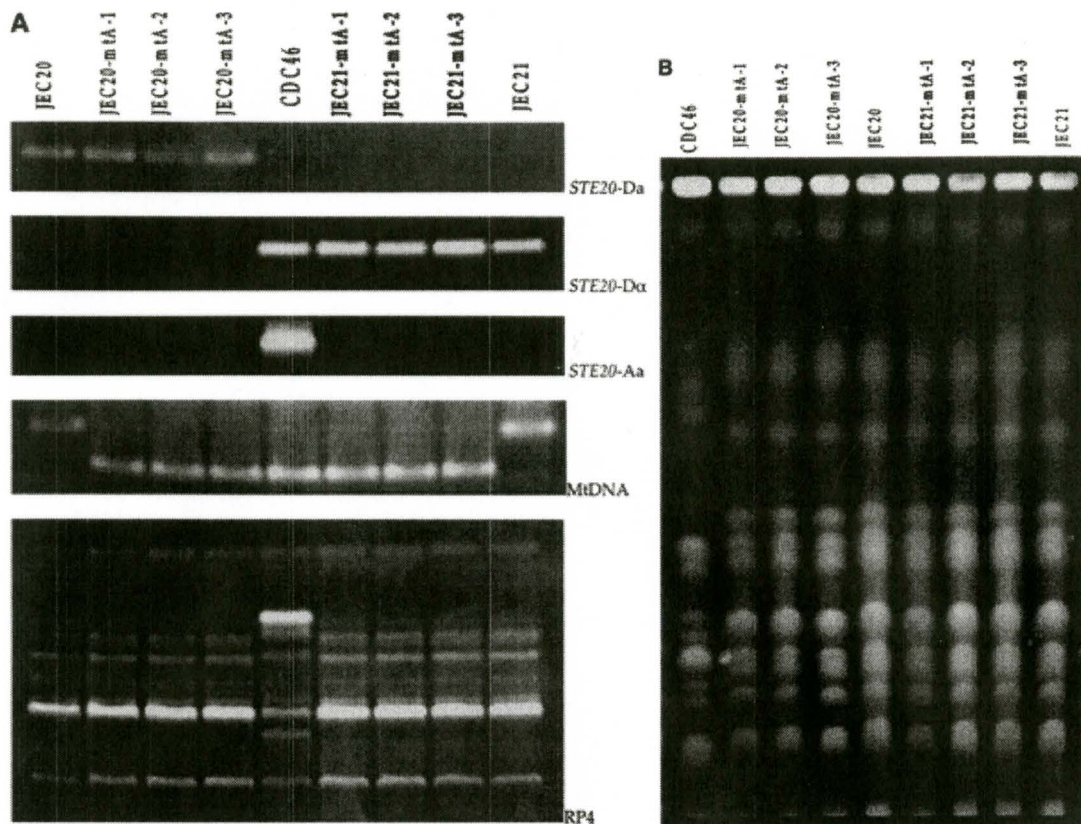


Figure 3.1 Construction and genetic verification of strains with isogenic nuclear genotypes but different mitochondrial genotypes. (A) Top, names of strains corresponding to individual lanes; right side, primer pair and/or restriction digests used generate individual genotypes. These primers and their PCR conditions are described in Table 3.1. (B) The chromosomal patterns of the same strains but in a different order.

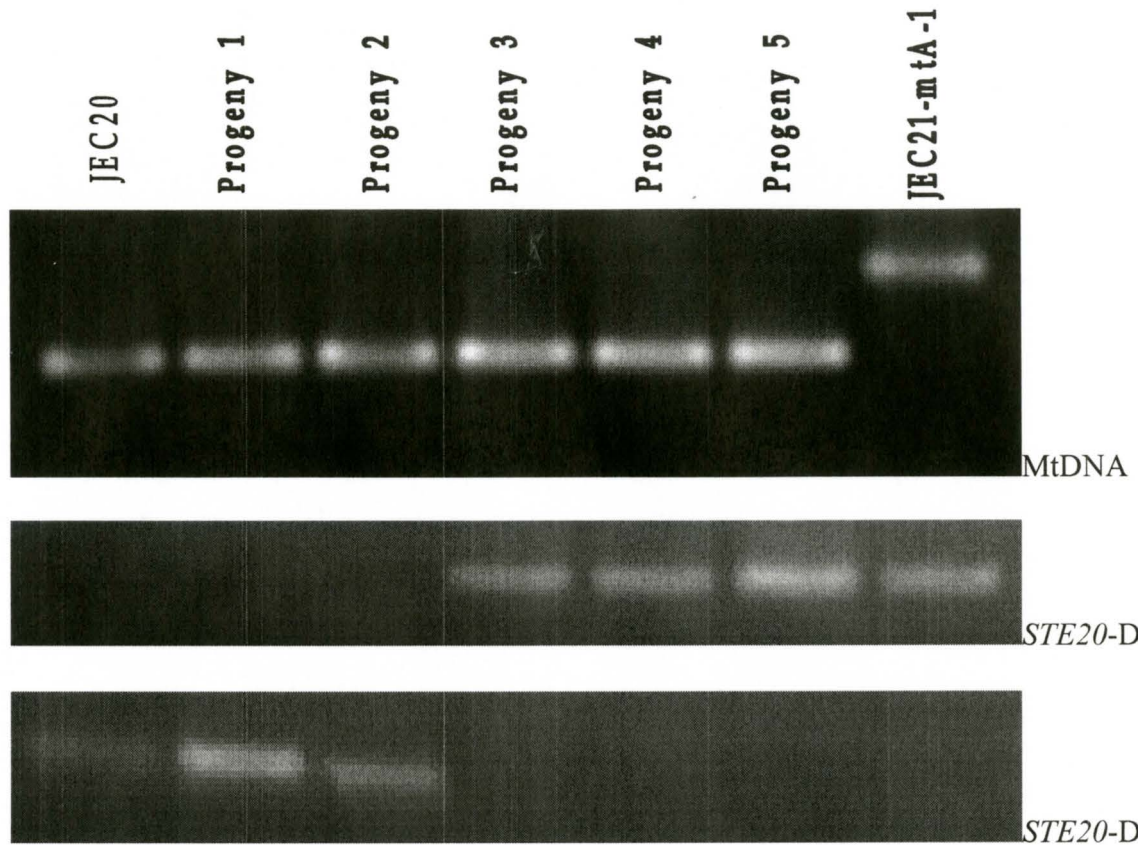


Figure 3.2 Genotypes at the mitochondrial genome and the *STE20* locus for two parental strains and five representative progenies from cross between JEC20 and JEC21-mtA-1. Labels on the top indicate strain names corresponding to individual lanes. Labels to the right of panels correspond to primers used to generate individual genotype profiles.

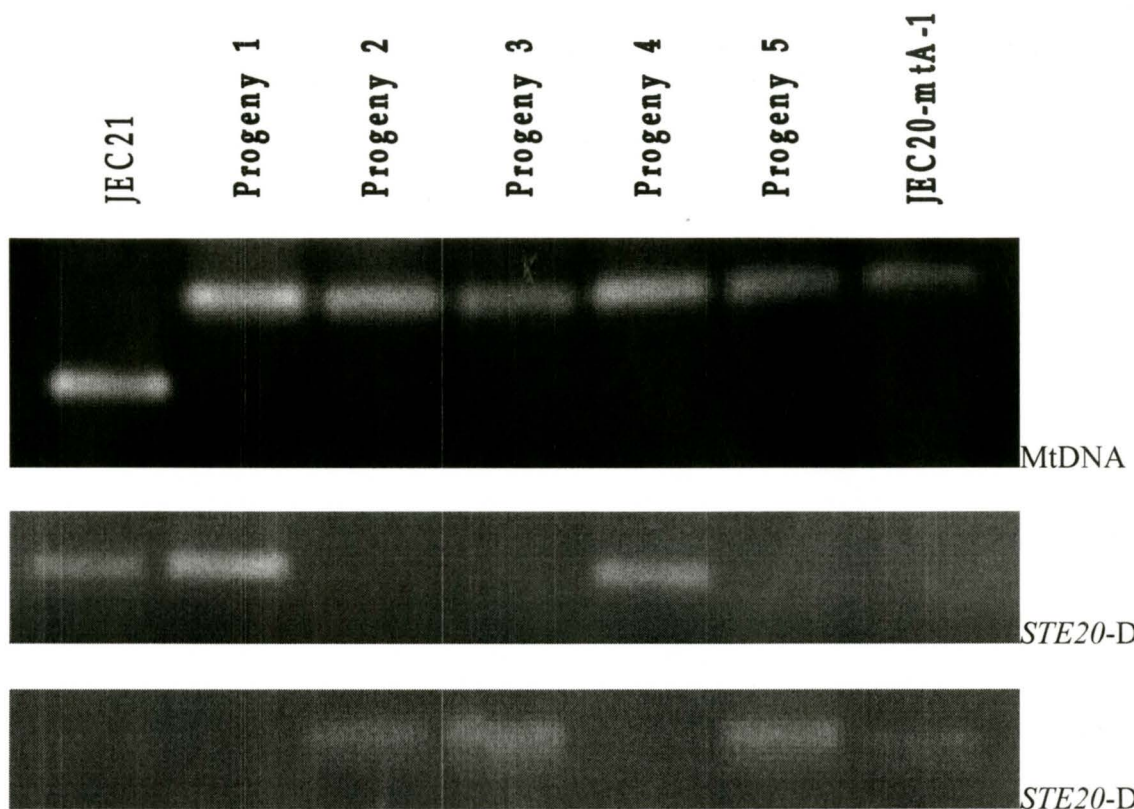


Figure 3.3 Genotypes at the mitochondrial genome and the *STE20* locus for two parental strains and five representative progenies from cross between JEC21 and JEC20-mtA-1. Labels on the top indicated strain names corresponding to individual lanes. Labels to the right of panels correspond to primers used to generate individual genotypes.

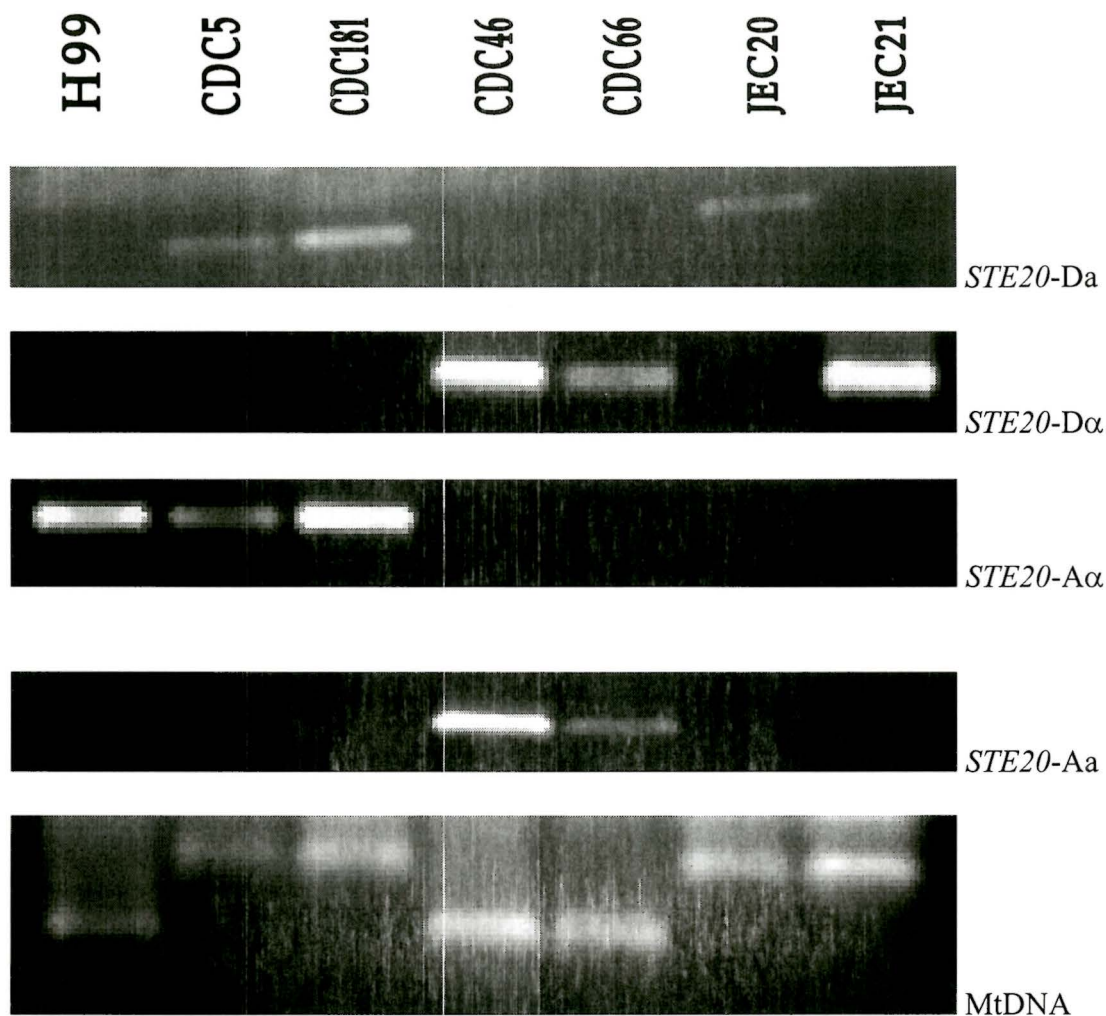


Figure 3.4 Genotypes at the mating type locus (four primer pairs) and mitochondrial genome in representative strains of *C. neoformans*. Strain H99 is the model laboratory strain for serotype A. It has the serotype A-specific mtDNA haplotype and A α allele at the *STE20* locus. Strains JEC20 and JEC21 have the serotype D-specific mtDNA haplotypes and the Da and D α alleles respectively at the *STE20* locus. Strains CDC5 and CDC181 are serotype AD. They have MATa from a serotype D parent (Da) plus a

MAT α from a serotype A parent (A α). Their mtDNA genotypes were identical to those of serotype D strains. Strains CDC46 and CDC66 are also serotype AD.

However, they have MATa from a serotype A parent (Aa) and MAT α from a serotype D parent (D α). Their mtDNA genotypes were identical to those of serotype A strains.

Therefore, as shown in the Table 3.4, mitochondria genotypes in serotype AD strains are always consistent with those of the assumed MATa parent.

Chapter 4: Mating type α -specific gene *SX11 α* controls uniparental mitochondrial inheritance in *Cryptococcus neoformans*

Preface:

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Mating type α -specific gene *SXII α* controls uniparental mitochondrial inheritance in *Cryptococcus neoformans*

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Running title: *SXII α* controls mtDNA inheritance

ABSTRACT:

In the great majority of sexual eukaryotes, mitochondrial genomes are inherited almost exclusively from a single parent (Gillham, 1994; Birky, 2001). However, the genetic mechanisms for uniparental mitochondrial inheritance remain largely unknown. Here, we report the identification of a sex-specific gene that controls mitochondrial inheritance in the fungus *Cryptococcus neoformans*. In typical crosses between strains of mating type a (MATa) and MAT α in *C. neoformans*, the progeny inherit mitochondrial DNA (mtDNA) from the MATa parent. Disruption of the MAT α -specific gene *SXI α* results in biparental mtDNA inheritance, significant heteroplasmy (zygote with organelle genomes from both parents), and the recombination of mitochondrial genomes. Thus, *SXI α* is the first identified sex-determining gene controlling mitochondrial inheritance.

RESULTS AND DISCUSSION

Strains of the basidiomycete yeast *Cryptococcus neoformans* are predominantly haploid and exist in one of two sexes (mating types), MATa and MAT α (Kwon-Chung, 1976). We recently identified that mtDNA was transmitted from the MATa parent in sexual crosses of *C. neoformans* (Yan and Xu, 2003). Genomic sequences were recently obtained from two strains each for both the MATa and the MAT α loci (Lengeler et al., 2002). Sequence comparisons indicated that the homeodomain sexual identity-determining gene *SXI α* was the only difference between the MATa and the MAT α loci, existing only in MAT α strains (Hull et al., 2002).

To test the role of *SXI1 α* in mtDNA inheritance in *C. neoformans*, we constructed a series of strains (Table 4.1) and crosses (Table 4.2). For each cross, cells from two genetically marked haploid strains of opposite mating types were mixed on V8-juice agar medium (Kwon-Chung, 1976). After 16-20 hr incubation, cell mixtures were transferred to selective media that allowed growth of only diploid zygotes. Randomly picked zygote colonies from selective media were then genotyped for their mtDNA types at two loci NADH dehydrogenase subunit 2 (*ND2*) and subunit 4 (*ND4*), using PCR and restriction enzyme digests of the PCR products (Figure 4.1).

Similar to results obtained in previous studies (Yan and Xu, 2003; Xu et al., 2000), crosses involving strains with wild-type MATa and MAT α loci produced zygotes containing mtDNA almost exclusively from the MATa parent (crosses #1 and 2, Table 4.2). Neither the mitochondrial genomes nor the auxotrophic markers used for selection influenced mitochondrial inheritance. In contrast, zygotes from mating involving the *sxi1 α* mutant CHY618 showed a biparental mitochondrial inheritance, significant heteroplasmy, and the recovery of a recombinant mtDNA genotype (Table 4.2, cross #3; Figure 4.1). Heteroplasmic zygotes included the mitochondrial genes *ND2* and *ND4* from both parental strains. As shown in cross #4, the change in mtDNA inheritance was not due to the addition of the transforming vector DNA into the genome nor due to the introduction of the nourseothricin (*NAT*)-resistance gene used to disrupt the *SXI1 α* gene (Tables 4.1 and 4.2). Re-introduction of the wild type *SXI1 α* gene into strain CHY618 re-established uniparental mtDNA inheritance, with almost all progeny inheriting mtDNA from the MATa parent (Table 4.2, cross #5). Again, this change was not due to the introduced transforming

vector DNA (Table 4.2, cross #6). Results from these crosses clearly show that the *SXI1 α* gene controls mtDNA transmission during sexual crosses in *C. neoformans*.

SXI1 α is identified as a putative homeodomain transcription factor (Hull et al., 2002). Therefore, its control of mtDNA inheritance is likely through regulating the expression of other genes. At present, the downstream targets of *SXI1 α* are unknown. However, there are two non-mutually exclusive hypotheses. The first is that *SXI1 α* controls mtDNA transmission through active degradation of mtDNA in the MAT α parent. Deletion of the *SXI1 α* gene would abolish the degradation activity of its own mtDNA. Selective degradation of organelle genomes has been recently observed in the model green alga *Chlamydomonas reinhardtii* (Nishimura et al., 2002) and the multi-sexual protist *Physarum polycephalum* (Moriyama and Kawano, 2003). In *C. reinhardtii*, the chloroplast DNA from the *mt-* parent was rapidly degraded by a putative nuclease soon after zygote formation. As a result, all progeny inherits chloroplast DNA from the *mt+* parent (Nishimura et al., 2002). Similarly, in *P. polycephalum*, a rapid, selective digestion of mtDNA was observed soon after sexual mating, resulting in uniparental mtDNA inheritance. The selectivity of mtDNA digestion in *P. polycephalum* had a hierarchical pattern based on mating type alleles (Moriyama and Kawano, 2003). In these two organisms, the nature of the nuclease enzymes, their relationships to the mating type loci, and the mechanisms of control are unknown at present. Whether this process operates in *C. neoformans* is unknown at present.

The second possibility is that uniparental mtDNA inheritance in *C. neoformans* is the result of unidirectional migration of the MAT α nuclei into the MAT α cells. In this process, the mitochondria from the MAT α parent are left behind and the diploid zygote

would contain mitochondria from only the MAT α parent. Unidirectional migration of the MAT α nuclei into MAT α cells was recently observed in *C. neoformans* (McClelland et al., 2004). In the basidiomycete species *Schizophyllum commune* and *Coprinus cinereus*, nuclear migration instead of mitochondria during sexual mating resulted uniparental inheritance (see introduction, fertilization mechanism). Based on this hypothesis, deletion of the *SXI1 α* gene in *C. neoformans* would be predicted to perturb or impair nuclear migration of the MAT α nucleus, thus contributing to greater cytoplasmic mixing, biparental mitochondrial inheritance, heteroplasmy, and the recombination of mitochondrial genomes. Indeed, strains with *sxi1 α* deletion have impaired filamentation, consistent with the lack of nuclear migration in the developing zygote (Hull et al., 2002).

The control of mtDNA inheritance by *SXI1 α* in *C. neoformans* may be part of an evolutionary conserved pathway. *SXI1 α* has a similar structure and function as other sex-determining genes such as the *SRY* gene in mammals (Haqq and Donahoe, 1998) and the gamete-specific gene *GSP1* in *C. reinhardtii* (Kurvari et al., 1998). *SRY* is male-specific, located on mammalian Y-chromosomes. In contrast, *GSP1* is present in both “+” and “-” mating types but is expressed only in the “+” gamete. At present, experimental evidence for *SRY* and *GSP1* in mitochondrial inheritance is not available. However, it is worth noting that in these evolutionary divergent systems, parental gametes with these homeodomain genes function as males and contribute nuclei but not mitochondria to their offspring zygote. Therefore, it is possible that the control of uniparental mitochondrial inheritance by homeodomain genes may have evolved early in the eukaryotic lineage.

MATERIALS AND METHODS:

Strains: All strains used in this study along with their genotypes are presented in Table 4.1. Strains JEC34 and JEC43 were originally obtained from Dr. Jeff Edman. Strains CHY618 and CHY648 were from Hull et al (2002). Other strains were constructed in this study according to the protocols described in before (Yan and Xu, 2003; Hull et al., 2002).

Mating, Zygote Screening and Genotyping: In a typical mating, about 10^8 cells from each mating partner were thoroughly mixed on V8-juice agar medium (Yan and Xu, 2003). At least three independent matings were performed for each cross. Individual parental cells were plated as negative controls. After 16-20 hr incubation at 25°C, cell mixtures were scraped off the agar surface, washed in 1ml sterile distilled water, and spread-plated on minimum medium (SD) supplemented without (crosses #1, 2, 3, and 4) or with (crosses # 5 and 6) the antibiotic G418. Plates were then incubated at 37°C for 4 days. Individual single colonies were directly subjected to DNA isolation according to a published protocol (Xu et al., 2000), without further sub-culturing. No colony was recovered on the selective media from negative controls for any of the 8 individual strains in Table 4.1. Mitochondrial genotype for each colony was determined using polymorphic restriction sites at two loci: the mitochondrial *ND2* and *ND4*. These two genes are separated by 16972 bp in one direction and 15179 bp in the other direction on the circular mitochondrial chromosome as determined by genomic sequencing of the laboratory strain JEC21 at the Stanford University Genome Technology Center. Primer sequences for the *ND2* gene fragment are (5'→3'), forward: caagctgcaccattccata; reverse: ccattagtgggtgactcc. This primer pair amplifies a 533 bp DNA fragment. Primer sequences for the *ND4* gene fragment are,

forward: gggagaatttgattcaagtgaac; reverse: catacatggaaaggtactag. This primer pair amplifies a 515bp DNA fragment. Restriction enzymes AluI and XhoI were used to digest PCR products amplified with *ND2* and *ND4* primer pairs respectively to distinguish the mtDNA genotypes. The polymorphic patterns for mtA type and mtD type of *ND2* and *ND4* genes are presented in Figure 4.1.

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REFERENCES:

- Birky Jr., C. W. (2001). The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Ann. Rev. Genet.* 35, 125-148.
- Brown, A. J., and Casselton, L. A. (2001). Mating in mushrooms: increasing the chances but prolonging the affair. *Trends in Genetics.* 17, 393-400.
- Gillham, N. W. (1994). *Organelle Genes and Genomes* (New York: Oxford University Press).
- Haqq, C. M., and Dorahoe, P. K. (1998). Regulation of sexual dimorphism in mammals. *Physiol. Rev.* 78, 1-33
- Hull, C. M., Davidson, R. C., and Heitman, J. (2002). Cell identity and sexual development in *Cryptococcus neoformans* are controlled by the mating-type-specific homeodomain protein *SXII α*. *Genes Dev.* 16, 3046-3060.

Figure 4.1 DNA restriction site polymorphisms for the mitochondrial *ND2* and *ND4* genes.

Lanes M: DNA ladder; Lanes 1: mtA type; Lanes 2: mtD type; Lanes 3: heteroplasmy where both parental mtDNA genomes are present; Lanes 4 and 5: mtDNA recombinants.

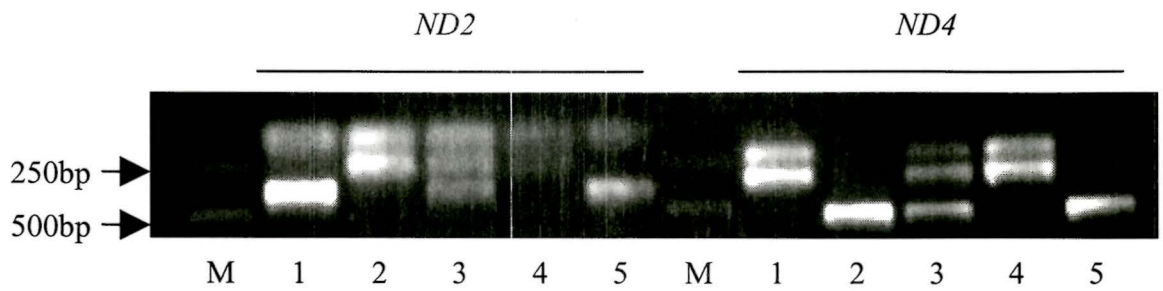


Table 4.1 Strains and their genotypes used in this study.

Strain	Genotype
JEC43	MAT α <i>ura5 mtD</i>
YZX1	MAT α <i>ade2 G418 mtA</i>
CHY618	MAT α <i>ura5 mtD sxi1α::NAT</i>
CHY620	MAT α <i>ura5 mtD SXIIα NAT</i>
CHY647	MAT α <i>ura5 mtD sxi1α::NAT URA5 ectopic pPM8 vector</i>
CHY648	MAT α <i>ura5 mtD sxi1α::NAT URA5 ectopic pPM8-SXIIα</i>
JEC34	MATa <i>ura5 mtD</i>
YZX2	MATa <i>ade2 G418 mtA</i>

All strains above are isogenic except at the indicated loci. MATa: mating type allele a; MAT α : mating type allele α . Strains with *ura5* and *ade2* auxotrophic markers require uracil and adenine respectively for growth on minimum medium SD. *mtA* and *mtD* are two mitochondrial genotypes distinguished by restriction site polymorphisms at two loci *ND2* and *ND4*.

Table 4.2 *SXI1* α controls mtDNA inheritance in *C. neoformans*.

Cross	mtDNA from MATa parent	mtDNA from MAT α parent	Recombinant mtDNA	Zygote with mtDNA from both parents (heteroplasmic)	Total
1. JEC43 x YZX2	48	3	0	0	51
2. JEC34 x YZX1	29	0	0	0	29
3. CHY618 x YZX2	37	161	1	43	242
4. CHY620 x YZX2	156	6	0	2	164
5. CHY648 x YZX2	121	6	0	2	129
6. CHY647 x YZX2	30	40	3	18	91

Chapter 5: The mating type-specific homeodomain genes *SXI1 α* and *SXI2a* coordinately control uniparental mitochondrial inheritance in *Cryptococcus neoformans*

Preface:

This chapter is being prepared for submission.

This study was conducted under the guidance of my supervisor, Dr. Jianping Xu. I performed all the experiments, prepared the figures and tables and contributed to the writing of the paper. Dr. Hull and Dr. Heitman supplied us mutant strains (CHY618, CHY620, CHY795, CHY766 and CHY771) and considerable advice. Dr. Xu helped the design of the experiment, interpretation of the data and writing of the manuscript.

The mating type-specific homeodomain genes *SXI1α* and *SXI2a* coordinately control uniparental mitochondrial inheritance in *Cryptococcus neoformans*

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Running title: *SXI2a* controls mtDNA inheritance

ABSTRACT

In the great majority of sexual eukaryotes, mitochondrial genomes are inherited almost exclusively from a single parent. While many mechanisms have been proposed to explain this phenomenon, very little is known about the genetic elements controlling uniparental mitochondria inheritance. In the bipolar, isogamous basidiomycete yeast *Cryptococcus neoformans*, progeny from crosses between strains of mating type a (MATa) and mating type α (MAT α) typically inherit mitochondrial DNA (mtDNA) from the MATa parent. We recently demonstrated that a mating type α (MAT α) -specific gene, *SXI1 α* , controls mitochondrial inheritance in *C. neoformans*. Here we show that a MATa specific homeodomain gene *SXI2a* is also involved in controlling mitochondria inheritance in this fungus. Similar to results from the *sxi1 α* Δ mutant, disruption of *SXI2a* results in biparental mtDNA inheritance and heteroplasmy (zygotes with mitochondrial genomes from both parents). Together with results from previous studies, the data here suggest that *SXI1 α* and *SXI2a* coordinately control mitochondria inheritance in *C. neoformans*.

INTRODUCTION

One of the major differences between prokaryotic and eukaryotic cells is that eukaryotic cells contain a diverse array of subcellular organelles. Among these organelles, the ubiquitously distributed mitochondria in almost all eukaryotes are distinctive in that they contain their own genetic material. In contrast to the Mendelian patterns of inheritance for nuclear genes, in the majority of sexual eukaryotes, mitochondrial genes and genomes do not follow Mendelian laws. Early studies of mitochondria inheritance focused on sexual plants and animals. In these species, mitochondrial genes and genomes were inherited almost exclusively from the maternal parent (Birky, 2001; Gillham, 1994; Xu, 2005).

Because sexual plants and animals have morphologically differentiated gametes of different sizes (anisogamy) and with very different numbers of mitochondria, the observed uniparental mitochondrial DNA (mtDNA) inheritance in these species have been traditionally regarded as a byproduct of two processes during gamete fusion (Birky, 2001; Gillham, 1994; Xu, 2005). In the first, mitochondria from the smaller paternal gamete (sperm or pollen) may fail to enter the larger maternal gamete (egg or ovule). Second, even if mitochondrial genomes from the paternal gamete enter the maternal gamete during fertilization, we may not be able to detect the paternal mtDNA because of the large difference in the number of mitochondrial genomes in favor of maternal gametes.

Consistent with these hypotheses, biparental mtDNA inheritance was observed in several isogamous species (i.e. species with undifferentiated and morphologically similar gametes). For example, in the model isogamous yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, mtDNA inheritance was biparental, with significant

proportions of progeny inheriting mtDNA from the MAT α parent, the MAT α parent, both the MAT α and MAT α parents, and recombinant types (Berger and Yaffe, 2001; Birky, 2001). Biparental mtDNA inheritance has also been observed in the human pathogenic protist *Trypanosoma brucei* that causes African sleeping sickness (Gibson, 2001).

While uniparental and maternal mtDNA inheritance is the predominant mode of mtDNA inheritance in sexual plants and animals, there are exceptions. Using highly sensitive techniques such as immunofluorescent microscopy, Southern hybridization and the polymerase chain reaction (PCR) to analyze mitochondrial inheritance in anisogamous plants and animals, both paternal inheritance and biparental inheritance have been reported (Xu, 2005). At present, the genetic determinants for paternal and biparental mtDNA inheritance in sexual plants and animals remain unknown.

Interestingly, uniparental mtDNA inheritance has been observed in several isogamous sexual eukaryotes, including the green alga *Chlamydomonas reinhardtii* (Boynton et al., 1987), the slime molds *Physarum polycephalum* (Moriyama and Kawano, 2003) and *Polysphondylium pallidum* (Mirfakhrai et al., 1990), and the fungus *Cryptococcus neoformans* (Xu et al. 2000). In these species, the mating type locus has been found to play a key role in controlling mtDNA inheritance. For example, sexual progeny in *C. reinhardtii* inherit mtDNA almost exclusively from the mt- parent (Boynton et al., 1987). Unlike *C. reinhardtii*, which has only two mating types (mt+ and mt-), there are at least 13 functionally different alleles at the sex-determining matA locus in *P. polycephalum*. MtDNA in this protist is inherited largely uniparentally according to the relative sexuality determined by the mating type alleles. In fact, the 13 matA alleles can be ranked in a linear

hierarchy of dominance. The mtDNA donor is typically the strain that possesses the dominant *matA* allele in the linear hierarchy (Moriyama and Kawano, 2003). A similar mating type-dependent uniparental mtDNA inheritance was observed in another slime mold, *P. pallidum*. *P. pallidum* has two mating types, *mat1* and *mat2*. Progeny from crosses between *mat1* and *mat2* strains typically inherit mtDNA from the *mat2* parent (Mirfakhrai et al., 1990). In *C. reinhardtii*, *P. polycephalum* and *P. pallidum*, there is evidence for selective degradation of mitochondrial genome from one of the two parents. Interestingly, ubiquitin tagging and active degradation of sperm mtDNA has also been observed during fertilization of mammalian eggs (Sutovsky et al., 2000). However, the gene(s) involved in controlling the degradation has not been identified.

Similar to the observations in *C. reinhardtii*, *P. polycephalum* and *P. pallidum*, in typical crosses, the mitochondrial genome is inherited uniparentally in *Cryptococcus neoformans*. *C. neoformans* has two mating types, *MATa* and *MAT α* . In laboratory crosses, progeny inherit mtDNA almost exclusively from the *MATa* parent (Yan and Xu, 2003). Molecular genetic analyses further identified that a homeodomain gene *SXI1 α* located within the *MAT α* locus controls mtDNA inheritance in *C. neoformans* (Yan et al., 2004). Disruption of the *SXI1 α* gene results in biparental inheritance (Yan et al., 2004).

In a recent study, a *MATa*-specific homeodomain gene called *SXI2a* was reported and the *Sxi2a* protein was found to interact directly with the *Sxi1 α* protein in a yeast two-hybrid screen (Hull et al., 2005). Since *SXI1 α* was found to control mtDNA inheritance, we hypothesize that *SXI2a* will similarly influence mtDNA inheritance. To test this hypothesis, we analyzed mitochondrial genotypes from several crosses involving strains with defined

genotypes. Our results support the hypothesis that *SXI1a* and *SXI2a* coordinately control mtDNA inheritance in *C. neoformans*.

MATERIALS AND METHODS

Strains: All strains used in this study along with their genotypes are presented in Table 5.1. Strain JEC34 and JEC43 were originally obtained from Dr. Jeff Edman. The *sxi1a*Δ strain CHY618 and its positive control CHY620 were from Hull et al (2002) and crosses involving these two strains were described in Yan et al. (2004). The *sxi2a*Δ mutant CHY766 and its positive control CHY771 were from Hull et al. (2005). Strain CHY795 was from Hull et al. (2002). Strains YZX1 and YZX2 were from Yan et al. (2004). Strain YZX24 was constructed for the present study according the protocols described in Yan and Xu (2003).

Mating, Zygote Screening and Genotyping: In a typical mating, about 10⁸ cells from each mating partner were thoroughly mixed on V8-juice agar medium. At least three independent matings were performed for each cross. Individual parental cells were plated as negative controls. After 16-20 hr incubation at 25°C, cell mixtures were scraped off the agar surface, washed in 1ml sterile distilled water, and spread-plated on SD minimum medium (table 5.2, crosses #1, 2, 6 and 7) or SD supplemented with antibiotic G418 (NEO, table 5.2, crosses #3, 4 and 8) or nourseothricin (NAT, table 5.2, crosses #5). Plates were then incubated at 37°C for 4 days. Individual single colonies (i.e. diploid zygotes) were directly subjected to DNA isolation according to a published protocol, without further sub-

culturing (Xu et al., 2000). No colony was recovered on the selective media from negative controls for any of the parental strains in Table 5.1. The mitochondrial genotype for each colony was determined using the length difference polymorphisms of PCR products from the *ND5* gene locus (Toffaletti et al. 2004). The length difference between the mtA and mtD mitochondria genotypes was due to the presence of an intron of 815bp in the *ND5* gene in the mtD genome. The primer sequences were (5'→3'), forward: ctattggtgttacaggagctcac; reverse: gagccttcatacctgccttattgc. The expected PCR products were 435bp and 1250bp respectively for the mtA and mtD genotypes. The mtA and mtD mitochondria genotypes were originally defined based on restriction site polymorphisms (Xu, 2002). The conditions for PCR, agarose gel electrophoresis, and scoring follow those of Yan and Xu (2003) and Toffaletti et al. (2004). In each crosses, the two parental strains have different mitochondrial genotypes (Tables 5.1 and 5.2). Therefore, the progeny mtDNA genotype can be easily determined and assigned to the parental type. The presence of both fragments in one colony indicates that the initial zygote contained mitochondrial genomes from both parents.

RESULTS AND DISCUSSIONS

The results of our experimental crosses are presented in Table 5.2. Similar to results obtained in previous studies (Xu et al. 2000; Yan and Xu 2003; Yan et al. 2004), crosses involving wild type MAT α and MAT α strains produced zygotes containing mtDNA almost exclusively from the MAT α parent (crosses #1 and 2, Table 5.2). Neither the mitochondrial genomes themselves nor the auxotrophic and drug resistance markers used for selection influenced mitochondrial inheritance in the crosses here. In contrast, zygotes from matings

between a wild type MAT α strain YZX1 and a MAT α *sxi2a* Δ strain CHY766 showed biparental mtDNA inheritance and significant heteroplasmy (cross #3, Table 5.2). As shown in cross #4, the change in mtDNA inheritance was not due to the addition of the transforming vector DNA into the genome nor due to the introduction of the *URA5* gene used to disrupt the *SXI2a* gene (Tables 5.1 and 5.2). Results from these crosses clearly support our hypothesis that the *SXI2a* gene is involved in controlling mtDNA transmission during sexual crosses in *C. neoformans*.

To better compare the effects of *SXI1 α* and *SXI2a* on mitochondria inheritance, we included the mitochondrial inheritance pattern of two crosses originally presented in Yan et al (2004). Cross #6 involved the *sxi1 α* Δ mutant CHY618 and cross #7 involved its positive control CHY620. As can be seen from Table 5.2, the overall patterns are highly similar between crosses #3 and #6 and between crosses #4 and #7. Disruptions of both genes either individually or together result in biparental inheritance and heteroplasmy (crosses #3, #5 and #6, Table 5.2).

The coordinated control of mtDNA inheritance in *C. neoformans* by *SXI1 α* and *SXI2a* is further supported by cross #8 (Table 5.2). In this cross, the MAT α strain CHY795 containing an ectopic copy of the *SXI1 α* gene was crossed to the MAT α strain YZX1. Zygotes were selected on SD minimal medium containing G418. If *SXI1 α* were the only gene controlling mtDNA inheritance independent of *SXI2a*, progeny from this cross should show a biparental mtDNA inheritance. However, we observed a uniparental mtDNA inheritance, similar to those between wild type MAT α and MAT α strains (Table 5.2).

How do *SXI1 α* and *SXI2a* control mitochondrial inheritance in *C. neoformans*? At

present, the exact molecular processes are unknown. However, results from this and previous studies suggest two hypotheses. To help illustrate these hypotheses, we will first briefly review the function and expression patterns of *SXI1 α* and *SXI2a* genes obtained in previous studies. *SXI1 α* and *SXI2a* encode homeodomain proteins belonging to the HD1 and HD2 groups respectively and they directly interact to regulate sexual development in *C. neoformans* (Hull et al., 2002, 2005). Proteins homologous to HD1 and HD2 are widespread in fungi including the ascomycete yeast *S. cerevisiae*, the basidiomycete mushroom *Coprinus cinereus*, and the basidiomycete plant pathogen *Ustilago maydis* (Brown and Casselton 2001). In these divergent species, HD1 and HD2 proteins from compatible mating partners are needed to fully initiate sexual development such as meiosis and sexual spore formation (Brown and Casselton 2001; Hull et al. 2005).

Because *Sxi1 α* and *Sxi2a* are interacting homeodomain transcription factors (Hull et al., 2005), their control of mtDNA inheritance is likely through regulating the expression of other genes. In haploid MAT α and MATa cells, both *SXI1 α* and *SXI2a* genes are expressed at very low levels and mutations at both loci had little effect on mating ability of these strains (Hull et al., 2005). However, their expressions increased dramatically soon after the fusion of the MATa and MAT α cells. The increased expression was associated with decreased expression of pheromone genes and potentially other genes involved in mating in both parents. In contrast, the increased expression of *SXI1 α* and *SXI2a* are positively correlated to downstream zygote developments such as filamentation, meiosis and sexual spore formation in *C. neoformans* (Hull et al., 2005). Indeed, ectopic expression of the *SXI1 α* gene in a MATa strain or the *SXI2a* gene in a MAT α strain are sufficient to drive the

complete sexual developmental processes in the recipient MAT α and MAT α strains respectively (Hull et al. 2002; 2005). At present, the detailed downstream targets of *SXI1 α* and *SXI2 α* are unknown.

Two mutually non-exclusive hypotheses have been proposed to explain the observed uniparental mtDNA inheritance in *C. neoformans* (Yan and Xu 2003; Yan et al. 2004). Here we discuss the relevance of both hypotheses to new observations. In the first hypothesis, the uniparental mtDNA inheritance in *C. neoformans* may be the byproduct of unidirectional migration of the MAT α nuclei into the MAT α cells. In this process, the mitochondria from the MAT α cells are left behind and the diploid zygote would contain mitochondria from only the MAT α parent. Furthermore, it was suggested that new diploid cells would bud from the MAT α parent side, away from the site where initial conjugation occurred (Yan and Xu, 2003). Unidirectional migration of the MAT α nuclei into MAT α cells has been observed in *C. neoformans* (McClelland et al., 2004). If this hypothesis were true, deletion of the *SXI1 α* and *SXI2 α* genes in *C. neoformans* would perturb or impair nuclear migration of the MAT α nucleus, thus contributing to greater cytoplasmic mixing and biparental mitochondrial inheritance.

Current observations indicated that while *sxi1 α Δ* and *sxi2 α Δ* mutants have similar capacities for normal conjugation tube formation and mating as the wild type strains, they have impaired abilities for hyphal formation and hyphal extension, consistent with their potential contribution in cytoplasmic mixing and mitochondria inheritance (Hull et al. 2005). In other basidiomycete species *Schizophyllum commune* and *Coprinus cinereus*, genes homologous to *SXI1 α* and *SXI2 α* have shown to control clamp cell formation, synchronized

nuclear migration, and septation while the pheromone genes control reciprocal nuclear migration – all essential steps for the formation of sexually fertile dikaryotic hyphae during sexual mating (for a review see Brown and Casselton 2001). However, different from *C. neoformans* where mating involves the fusion of yeast cells, mating in *S. commune* and *C. cinereus* involve reciprocal migration of nuclei into the resident hyphae of compatible mating partners. Because mitochondria do not move along with the nuclei, mating products (i.e. fertile dikaryotic hyphae) in *S. commune* and *C. cinereus* have uniform nuclear genotype but mosaic mitochondrial types (for a review see Yan and Xu 2005). In addition, while the homeodomain proteins in *C. neoformans* influence the expressions of pheromone genes, they do not seem to in *S. commune* and *C. cinereus* (Brown and Casselton 2001; Hull et al. 2005). These differences suggest that the molecular mechanisms controlling mitochondrial inheritance may be different between *C. neoformans* and other filamentous basidiomycetes. At present, the detailed cellular effects of *sxi1 α* Δ and *sxi2a* Δ on nuclear migration, budding site formation, and their relationship to mitochondria inheritance in *C. neoformans* remain to be investigated.

Our second hypothesis states that *SXI1 α* and *SXI2a* control mtDNA transmission through active degradation of the mitochondrial genome from the MAT α parent. Under this hypothesis, *SXI1 α* and *SXI2a* control uniparental mtDNA inheritance through regulating other genes. This control may first involve differential tagging of their respective mitochondria right before cell fusion. After mating and cell fusion, the protein complex of *SXI1 α* and *SXI2a* would target the differentially tagged mitochondria for either destruction (the MAT α parent mitochondria) or future inheritance (the MAT α parent mitochondria).

Deletion of the *SXI1 α* and/or *SXI2a* genes would abolish the tagging and/or degradation processes and preserve the mtDNA from both mating partners. Selective degradation of organelle genomes has been observed recently in the green alga *C. reinhardtii* (Nishimura et al., 2002) and the multi-sexual protist *P. polycephalum* (Moriyama and Kawano 2003). In *C. reinhardtii*, the chloroplast DNA from the *mt-* parent was rapidly degraded by a putative nuclease soon after zygote formation. As a result, all progeny inherits chloroplast DNA from the *mt+* parent (Nishimura et al., 2002). Similarly, in *P. polycephalum*, a rapid, selective digestion of mtDNA was observed soon after sexual mating, resulting in uniparental mtDNA inheritance. In these two organisms, the nature of the nuclease enzymes, their relationships to the mating type locus, and the mechanisms of control remain to be elucidated.

In conclusion, we identified that both homeodomain genes *SXI1 α* and *SXI2a* from compatible mating partners are essential to regulate uniparental mitochondria inheritance in *C. neoformans*. Our results suggested two specific, mutually non-exclusive hypotheses govern uniparental mtDNA inheritance in *C. neoformans*. A detailed understanding of the molecular processes underlying mtDNA inheritance in *C. neoformans* may help reveal the potential mechanisms of uniparental mtDNA inheritance in other species. Structurally, *Sxi1 α* and *Sxi2a* are highly similar to a group of homeodomain transcription factors that function to determine sexual identity in divergent groups of organisms. These genes include the *SRY* gene in mammals (Haqq and Donahoe 1998) and the gamete-specific gene *GSP1* in *C. reinhardtii* (Kurvari et al. 1998). At present, the potential roles of *SRY* and *GSP1* in mitochondrial inheritance in mammals and algae have not been examined.

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REFERENCES:

- Birky Jr., C. W. 2001. The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Ann. Rev. Genet.* 35:125-148.
- Boynton, J.E., Harris, E.H., Burkhart, B.D., Lamerson, P.M., and Gillham, N.W. 1987. Transmission of mitochondrial and chloroplast genomes in crosses of *Chlamydomonas*. *Proc. Natl. Acad. Sci. USA.* 84:2391-2395.
- Brown, A, J. and Casselton, L. A. 2001. Mating in mushrooms: increases the chances but prolonging the affair. *Trends in Genetics.* 17:393-400.
- Gibson, W. 2001. Sex and evolution in trypanosomes. *International Journal for Parasitology.* 31:643-647.
- Gillham, N. W. 1994. *Organelle Genes and Genomes.* Oxford University Press. New York.
- Haqq, C. M., and Donahoe, P. K. 1998. Regulation of sexual dimorphism in mammals. *Physiol. Rev.* 78:1-33.
- Hull, C. M., Davidson, R. C., and Heitman, J. 2002. Cell identity and sexual development in *Cryptococcus neoformans* are controlled by the mating-type-specific homeodomain protein *SXI1 α* . *Genes Dev.* 16:3046-3060.
- Hull, C. M., Boily, M. J., and Heitman J. 2005. Sex-specific homeodomain proteins *Sxi1 α* and *Sxi2 α* coordinately regulate sexual development in *Cryptococcus neoformans*. *Eukaryot Cell.* 4:526-535.
- Kurvari, V., Grishin, N. V., and Snell, W. J. 1998. A gamete-specific, sex-limited homeodomain protein in *Chlamydomonas*. *J. Cell Biol.* 143:1971-1980.

- McClelland, C. M., Chang, Y. C., Varma, A., and Kwon-Chung, K. J. 2004. Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends in Microbiology*. 12:208-212.
- Mirfakhrai, M., Tanaka, Y., and Yanagisawa, K. 1990. Evidence for mitochondrial DNA polymorphism and uniparental inheritance in the cellular slime mold *Polysphondylium pallidum*: effect of intraspecies mating on mitochondrial transmission. *Genetics*. 124:607-613.
- Moriyama, Y., and Kawano, S. 2003. Rapid, selective digestion of mitochondrial DNA in accordance with the *matA* hierarchy of multiallelic mating types in the mitochondrial inheritance of *Physarum polycephalum*. *Genetics*. 164:963-975.
- Nishimura, Y., Misumi, O., Kato, K., Inada, N., Higashiyama, T., Momoyama, Y., and Kuroiwa, T. 2002. An mt(+) gamete-specific nuclease that targets mt(-) chloroplasts during sexual reproduction in *C. reinhardtii*. *Genes Dev*. 16:1116-1128.
- Sutovsky, P., Moreno, R. D., Ramalho-Santos, J., Dominko, T., Simerly, C., and Schatten, G. 2000. Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biol. Reprod*. 63:582-590.
- Toffaletti, D. L., Nielsen, K., Dietrich, F., Heitman, J. and Perfect, J. R. 2004. *Cryptococcus neoformans* mitochondrial genomes from serotype A and D strains do not influence virulence. *Curr Genet*. 46:193-204.
- Xu, J. 2002. Mitochondrial DNA polymorphisms in the human pathogenic fungus *Cryptococcus neoformans*. *Curr. Genet*. 41:43-47.
- Xu, J. 2005. The inheritance of organelle genes and genomes: patterns and mechanisms. *Genome*. 48:951-958.

- Xu, J., Ali, R.Y., Gregory, D. A., Amick, D., Lambert, S. E., Yoell, H. J., Vilgalys, R. J., and Mitchell, T. G. 2000. Uniparental mitochondrial transmission in sexual crosses in *Cryptococcus neoformans*. *Curr. Microbiol.* 40:269-273.
- Xu, J., Ramos, A., Vilgalys, R., and Mitchell, T. G. 2000. Clonal and spontaneous origins of fluconazole resistance in *Candida albicans*. *J. Clin. Microbiol.* 38:1214-1220.
- Yan, Z, Hull, C. M., Heitman, J., Sun, S., and Xu, J. 2004. *SXII* α controls uniparental mitochondrial inheritance in *Cryptococcus neoformans*. *Current Biology.* 14:R743-R744.
- Yan, Z., and Xu, J. 2003. Mitochondria are inherited from the MATa parent in crosses of the basidiomycete fungus *Cryptococcus neoformans*. *Genetics.* 163:1315-1325.
- Yan, Z. and Xu, J. 2005. Fungal mitochondrial inheritance and evolution. In *Evolutionary Genetics of Fungi* (J. Xu editor). Horizon Scientific Press, England. Pp. 221-252.

Table 5.1 Strains and their genotypes used in this study.

Strain	Genotype
JEC43	MAT α <i>ura5</i> mtD
YZX1	MAT α <i>ade2</i> mtA <i>NEO</i>
YZX24	MAT α <i>ade2</i> mtA <i>sxi1α::NAT</i>
CHY618	MAT α <i>ura5</i> mtD <i>sxi1α::NAT</i>
CHY620	MAT α <i>ura5</i> mtD <i>SXI1α NAT</i>
JEC34	MATa <i>ura5</i> mtD
CHY766	MATa <i>ura5</i> mtD <i>sxi2a::URA5</i>
CHY771	MATa <i>ura5</i> mtD <i>SXI2a URA5</i>
CHY795	MATa <i>ura5</i> mtD <i>ectopic pRCD85 -SXI1α</i>
YZX2	MATa <i>ade2</i> mtA <i>NEO</i>

All strains above are isogenic except at the indicated loci. MATa: mating type allele a; MAT α : mating type allele α . Strains with *ura5* and *ade2* auxotrophic markers require uracil and adenine respectively for growth on the minimal medium SD. mtA and mtD are two mitochondrial genotypes for serotypes A and D respectively as originally defined based on restriction site polymorphisms by Xu (2002). In this study, these two mtDNA types are distinguished based on length polymorphisms at the *ND5* locus due to the presence of an intron in the serotype D mitochondrial genome (Toffaletti et al. 2004). *NAT* and *NEO* encode genes resistant to antibiotics nourseothricin and G418 respectively.

Table 5.2 *SXI2a* controls mtDNA inheritance in *Cryptococcus neoformans*.

Cross	mtDNA from the MAT α parent	mtDNA from the MAT α parent	mtDNA from both parents (Heteroplasmy)	Total
1. JEC34 x YZX1 ¹	29	0	0	29
2. JEC43 x YZX2 ¹	48	3	0	51
3. CHY766 x YZX1	54	92	8	154
4. CHY771 x YZX1	125	1	0	126
5. CHY766 x YZX24	19	40	6	65
6. CHY618 x YZX2	37	161	43	241
7. CHY620 x YZX2 ¹	156	6	2	164
8. CHY795 x YZX1	21	2	0	23

¹, These four crosses were from Yan et al. (2004).

Chapter 6. Intron mobility and the control of selfish cytoplasmic element in *Cryptococcus neoformans*

Preface:

This chapter is prepared for submission

This study was conducted under the guidance of my supervisor, Dr. Jianping Xu. I performed all the experiments, prepared the figures, tables and wrote the manuscript. Dr. Xu helped the design of the experiment, interpretation of the data and writing of the manuscript.

**Intron mobility and the control of selfish cytoplasmic
element in *Cryptococcus neoformans***

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Running title: intron and mtDNA inheritance

Abstract

Introns are found in the genomes of all major groups of organisms. Some of these introns contain endonuclease genes (HEGs) that can initiate the mobility of their host introns. These HEGs are thought to be selfish or parasitic genes that can spread rapidly in populations because their catalytic activity results in self-propagation. However, the genetic control for these selfish genes remains little known. Here we report that the mating type (MAT) α -specific homeodomain gene *SXII α* influences the transmission of selfish HEGs and their associated introns in the mitochondrial genome of *Cryptococcus neoformans*. *C. neoformans* has two mating types, MAT α and MAT β . In typical crosses, the majority of progeny (>93%) inherit the entire mitochondrial genome of the MAT α parent. Deletion of the *SXII α* gene results in biparental mitochondrial inheritance and significant heteroplasmy. Interestingly, in the sexual cross involving *sxII α* deletion, the majority (>95%) of the progeny inherit the selfish HEGs and associated introns from the MAT β parent. Our results suggest that the nuclear encoded, sex-specific *SXII α* gene in *C. neoformans* controls not only mitochondrial transmission but also the spread of selfish HEGs in the mitochondrial genome. Our results provide direct experimental support for the hypothesis that uniparental mitochondrial inheritance might have evolved to limit the spread of selfish cytoplasmic DNA.

Introduction

Homing endonuclease genes (HEGs) are enzymes that catalyze DNA sequence specific double-strand breaks and can significantly stimulate homologous recombination at these breaks. They widely exist in fungi, protists, bacteria, and viruses (Belfort and Roberts, 1997). These HEGs have no known host function. Rather, they are capable of enhancing their own transmission relative to other elements in an individual's genome. Consequently, these HEGs have traditionally been considered as selfish or parasitic genetic elements (Hurst and Werren, 2001; Goddard and Burt, 1999). At eukaryotes, they are also often found in organelles associated with group I or group II self-splicing introns and played a important role in the mobility and spread of their associated introns.

The mobility of HEG associated introns is achieved by a process termed "homing". For example, in a heteroplasmic individuals, in which there are both HEG⁺ and HEG⁻ mitochondrial genomes, the endonuclease encoded by the HEG can recognize and cut the HEG⁻ genomes, that creates a double-strand break (Chevalier and Stoddard . 2001).; the HEG⁺ genomes are protected because the presence of the gene interrupts the recognition sequence. The broken mitochondrial genome is then repaired through recombinational repair system, which uses the homologous HEG⁺ mitochondrial genome as template. After repair, the HEG together with its associated intron are found on both mitochondrial genomes. Consequently, these genes show strong transmission ratio distortion; they are often inherited by more than 95% of progeny, rather than the Mendelian 50% during sexual crosses (Jacquier and Dujon, 1985; Gimble and Thorner, 1992). This process of "homing" also explains how such a gene can increase in frequency and spread within a

population. For example, with a transmission rate of 95%, a gene would increase its frequency from 0.001 to 0.999 in about 15 outcrossed generations (Goddard and Burt, 1999). However, the genetic control for these selfish genes remains little known. One of the hypothesis explains that the uniparental cytoplasmic inheritance may evolved to control the spread of selfish deleterious cytoplasmic elements (Grun, 1976; Coleman, 1982; Law and Huston; Birky, 1995). However, experimental evidence for this hypothesis is lacking.

Introns are widespread in fungal mitochondrial genomes. Of the four mitochondrial genomes recently sequenced for the model pathogenic yeast *Cryptococcus neoformans*, all four contained introns (Litter et al. 2005; Tofallentii et al. 2004). However, these strains differ in their numbers of introns in their mitochondrial genomes. The two mitochondrial genomes from the serotype D strains (*C. neoformans* var. *neoformans*) JEC21 and IFM5844 had an identical intron distribution pattern with a total of 10 introns dispersed in four genes, two each in *COB1* and *LsrRNA*, five in *COX1*, and one in *ND5*. In contrast, the mitochondrial genomes from the two serotype A strains (*C. neoformans* var. *grubii*) H99 and IFO410 had only one intron each at the same location in *COB1* gene. Based on sequence analysis, the two introns in the *COB1* gene and four of the five introns in the *COX1* gene in the mtD genomes contained the LAGLIDADG motif, characteristic of HEGs (Litter et al. 2005). The mobility of these introns is, however, not known.

C. neoformans has a bipolar mating system with two alleles, mating types α (MAT α) and α (MAT α). In typical crosses between strains of MAT α and MAT α , the progeny

inherit mitochondrial genome almost exclusively from the MAT α parent (Yan and Xu, 2003). A recent study identified that a MAT α specific gene, *SXII α* , plays an important role in controlling mitochondrial inheritance in *C. neoformans*. Deletion of this gene resulted in biparental mitochondrial inheritance, significant heteroplasmy and the recovery of recombinant mitochondrial genomes. Re-introducing the wild-type gene into the mutant strain restored uniparental mitochondrial inheritance (Yan et al. 2004).

The discoveries of naturally existing HEG associated intron distribution polymorphisms in mitochondrial genomes and a gene that controls uniparental mitochondrial inheritance afford us an unique opportunity to investigate the mobility of these HEG associated introns and determine how the spread of selfish genetic element, such as HEGs, might be influenced by the gene *SXII α* . To assay intron mobility, we compared the inheritance patterns of the introns with those of other genes in the mitochondrial genomes. We identified that while the introns in *ND5* gene were immobile, the HEG associated introns within the *COXI* gene were mobile. Furthermore, in the crosses involving *sxii α* mutant, the spread of HEG associated introns within the *COXI* gene was significantly enhanced. Our results suggest that the homeodomain gene *SXII α* can control the spread of selfish cytoplasmic elements in *C. neoformans*.

Materials and Methods

Strains

The strains used in this study are listed in Table 6.1. All strains are serotype D and are isogenic except at the indicated loci. YZX2 is a mating type α (MAT α) strain

with a characteristic mitochondrial genotype of serotype A strains based on genotyping at *ND2*, *ND4* and the small subunit of the ribosomal RNA genes (*SsrRNA*, Xu 2002; Yan et al. 2004). However, though no intron was found in *ND5* and *COX1* gene in strain YZX2 (similar to the two sequenced serotype A strains H99 and IFO410), two introns were found in both the *COB1* gene and *LsrRNA* genes, similar to those in the two sequenced strains of serotype D, JEC21 and IFM5844. The intron distribution in *COB1* and *LsrRNA* in strain YZX2 is thus different from the two sequenced serotype A strains H99 and IFO410 where only one intron was found in *COB1* gene and none was found in the *LsrRNA* genes (See below in Results).

Strains CHY618 and CHY620 are mating type α (MAT α) and they have identical mitochondrial genotypes characteristic of serotype D strains that contain introns within *COB1*, *COX1*, *ND5* and *LsrRNA* genes (Litter et al. 2005; Toffaletti et al. 2004). In strain CHY618, the gene *SXII α* was deleted through homologous recombination and replaced by the gene coding for *NAT* resistance while strain CHY620 has the wild-type *SXII α* gene but with an ectopic copy of the *NAT* resistance gene.

As shown in Table 6.1, the three strains contain genetic markers that can be used to select for mating zygotes on selective medium. The origins of strain YZX2 and of strains CHY618 and CHY620 were described in Yan et al. (2004) and in Hull et al. (2002) respectively.

Mating and zygote selection

To prepare cells for mating, all three strains were first retrieved from the -70°C freezer, spread onto YEPD (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% dextrose, 2% agar) plates, and allowed to grow at room temperature for 2-4 days. Two pairs of strains (CHY618 x YZX2 and CHY620 x YZX2) were then mated on V8 medium [5% V8-vegetable juice (Campbell Soup Co.), 0.5 g/L KH₂PO₄, 4% agar and pH 7.2], following our previously described method (Yan et al., 2004). After 16–20 hour incubation, the mating mixtures were transferred to the minimum medium SD [1.7g Yeast Nitrogen Base without Amino Acids (Difco), 20g Dextrose, 5g (NH₄)₂SO₄, 20g Agar, per liter] that allowed the growth of only diploid zygotes. After 4 days of growth on SD medium at 37°C, zygotes were randomly picked for genotyping at various loci within their mitochondrial genomes.

Identification of mitochondrial genotype

The mitochondrial genotype was determined by PCR or PCR-restriction fragment length polymorphisms (PCR-RFLP) at the following marker gene loci: *ND2*, *ND4*, *ND5*, *COB1*, *LsrRNA* and *COX1* (Table 6.2). The mitochondrial genotypes at *ND2* and *ND4* loci were determined based on PCR-RFLP as described by Xu (2002) and Yan et al. (2004). The genotypes for *ND5*, *COX1*, *COB1* and *LsrRNA* were determined by PCR using primers located in intron-flanking regions as described by Toffaletti et al. (2004) and Litter et al. (2005). As the parental strains only differ at gene *ND2*, *ND4*, *ND5* and *COX1* (see results), the mitochondrial genotype of individual zygotes was determined only at these four loci.

Results

The distribution of introns in the study strains

Based on the sequenced mitochondrial genomes, we synthesized primers to first test the existence or absence of introns in the study strains (Table 6.2). Our analyses indicated that CHY618 and CHY620 had all the 10 introns described for the sequenced strains JEC21 and IFM5844 with five in the *COX1* gene, two each in the *COB1* and *LsrRNA* genes, and one in the *ND5* gene. This result is consistent with our expectation because JEC21 was the progenitor strain of CHY618 and CHY620. However, while no intron was found within *ND5* and *COX1* gene (similar to sequenced serotype A strains H99 and IFO410), the MAT α parental strain YZX2 was found to contain four introns two each within *COB1* and *LsrRNA* genes, identical to the two serotype D strains but different from the two sequenced serotype A strains. The distributions of intron in each of *COB1*, *ND5* and *COX1* genes in four strains are shown in Figure 6.1.

The differences in intron distribution between the MAT α and MAT α strains in Table 6.1 thus offered us an opportunity to examine the potential mobility of introns in *ND5* and *COX1* genes during sexual crosses.

The HEG associated introns in the *COX1* gene are mobile

To examine intron mobility, we first analyzed the cross between strains CHY620 (MAT α *ura5* mtD *SXI1 α* *NAT*) and YZX2 (MAT α *ade2* mtA G418) and directly analyzed the diploid zygotes for their mitochondrial genotypes. The genetic markers

include PCR-RFLP markers in *ND2* and *ND4* genes and PCR fragment size differences due to the presence and absence of introns in genes *ND5* and *COX1*. Similar to results from a previous study (Yan et al. 2004), cross between the wild type *MAT α* strain CHY620 and *MAT α* strain YZX2 produced zygotes predominantly (254/273; 93%) containing mitochondrial alleles only from the *MAT α* parent (Table 6.4). Of the remaining 19 zygotes, 12 had a mitochondrial alleles from only the *MAT α* parent CHY620; 4 had alleles from both parents at all loci; 2 had most of the mitochondrial alleles from the *MAT α* parent but contained *COX1* introns from the *MAT α* parent; and 1 had most of the alleles from the *MAT α* parent but contained no intron within the *COX1* gene. DNA sequence analysis of portions of the *COX1* gene from the three recombinants indicated that the *COX1* gene of the first two recombinants were from the *MAT α* parent while the last one was from the *MAT α* parent.

The three recombinants analyzed above might have been generated by one of two processes. In the first, normal homologous recombination between the two parental mitochondrial genomes could have generated all these recombinants. In the second, intron mobility initiated by HEGs could have generated two of the three recombinants with the 3rd one generated by normal homologous recombination. Because of the low number of recombinants, neither possibility could be rejected. As was shown earlier (Yan et al. 2004), the low recombination frequency was due to the stringent control of uniparental mitochondrial inheritance by the sex-determining gene *SXI α* . Therefore, to further test for intron mobility, we decided to examine the intron inheritance pattern in a cross using the *sxi1c Δ* mutant CHY618 as a parent.

Deletion of *SXI1 α* gene enhances the spread of HEGs associated introns in *COX1*

To test the effect of *SXI1 α* on intron mobility, we crossed strains CHY618 (MAT α *ura5* mtD *sxi1 α ::NAT*) and YZX2 (MAT α *ade2* mtA). Strains CHY618 and CHY620 are isogenic except that the *sxi1 α* gene was deleted in strain CHY618 but present and functional in strain CHY620 (Hull et al. 2002; Yan et al. 2004). Different from the cross between strains CHY620 and YZX2, zygotes from cross between strains CHY618 and YZX2 showed a biparental mitochondrial inheritance when examined with the majority of the mitochondrial markers (except *COX1*); a greater percentage of heteroplasmy (6.9% in this cross vs 1.4% in the cross between CHY620 and YZX2); and the recovery of a large number of recombinant mtDNA genotypes (Table 6.4). Most significantly, excluding the 8 heteroplasmic zygotes, while 65% (70/108) of the zygotes have mitochondrial genomes from the intron-containing MAT α parent CHY618, over 95% (103/108) contained the HEG associated introns in the *COX1* gene from this parent. That is, intron inheritance independent of the majority of mitochondrial genome increased from 0.7% (2/273) in the cross between CHY620 and YZX2 to 28% (30/108) in the cross between CHY618 and YZX2. In addition, no evidence of reciprocal recombination was observed between *COX1* gene and other genetic markers (Table 6.4). The results clearly indicate that the HEG associated introns in *COX1* gene are mobile and that the deletion of the *SXI1 α* gene significantly enhanced the spread of HEG associated introns in *COX1* from the intron-containing allele to the intronless allele in *C. neoformans*.

In contrast to the evidence for mobility of the HEG associated introns in *COX1* gene, we observed no evidence for the mobility of introns in *ND5* gene. The intron in *ND5* gene of strain CHY618 contains no HEG. The transmission of *ND5* intron followed the same inheritance pattern as the intronless genetic markers of *ND2* and *ND4* genes.

The spread of the HEG associated introns are confined to the *COX1* gene region.

To further examine the extent of the transferred regions from the *COX1* intron-containing mitochondrial genome to the *COX1* intronless mitochondrial genome, we sequenced the *COX1* gene and its flanking regions for 10 random zygotes (out of 30) with recombinant mitochondrial genotypes, using the 8 primer pairs listed in Table 6.3. These sequences are then compared to the sequences from the two parental strains. The results show that the recombination borders for all 10 progeny lie close to the borders of *COX1* gene, either right within the *COX1* gene exon or in the adjacent flanking regions just within 48 base pairs from the borders of *COX1* gene (Table 6.5, Figure 6.2). This result further confirmed that the HEG associated introns in the *COX1* gene are mobile elements capable of transferring to intronless *COX1* gene.

Discussions

In this study, we examined the mobility of mitochondrial introns in the human pathogenic yeast *C. neoformans*. We found that while the intron in *ND5* gene was not mobile in these crosses, the HEG associated introns in the *COX1* gene were mobile.

Furthermore, we demonstrated that the mobility of the *COXI* introns was controlled by the MAT α -specific homeodomain gene *SXI/* α . Deletion of this gene significantly enhanced the mobility of *COXI* introns.

In our crosses, we observed both homologous recombination and unidirectional intron transfer in the mitochondrial genomes. These two processes have been observed in other species. For example, homologous mitochondrial genome recombination has been reported in *Coprinus cinereus* (Baptista-Ferreira et al., 1983), *Agrocybe aegerita* (Barroso and Labarère, 1997), and *Pleurotus ostreatus* (Matsumoto and Fukumasa-Nakai, 1996). Similarly, independent transfers of HEG associated introns and mitochondrial plasmids have also been reported in several species. A classical example of HEG associated intron mobility was observed in the baker's yeast *Saccharomyces cerevisiae*. In this species, there was a unidirectional gene conversion of the mitochondrial large ribosomal subunit RNA (*LsrRNA*) gene in a ω^- strain that contained no intron in *LsrRNA* by the allele in a ω^+ strain that contained a HEG associated group I intron in *LsrRNA* (Dujon et. al. 1989). Direct observations of HEG associated group I intron mobility was also reported in the mitochondrial genome of the green alga *Chlamydomonas smithii*, and in the chloroplast genomes of two other green algae *Chlamydomonas eugametos* and *Chlamydomonas reinhardtii* (Gillham 1994). In species of the model filamentous fungal genus *Neurospora*, mitochondrial plasmids with sequences very similar to group I introns have been found to independently transfer to strains without such plasmids (Gillham 1994). However, in none of the above cases

was a gene identified that controls both mitochondrial inheritance and HEG associated intron mobility as shown in the study by Yan et al. (2004) and in this study, respectively.

At present, because our sequence analyses of recombinant mitochondrial genomes all showed similar recombination boundaries very close to the borders of *COXI* gene, we can not determine the individual mobility of each of the four introns within the *COXI* gene. Sequencing more recombinants might allow us to pin-point the recognition sequences and cut site(s) for intron integration. In addition, the detailed mechanism of intron mobility in *COXI* gene in *C. neoformans* is not known. Based on sequence analyses Four of the five introns in the *COXI* gene of strain CHY618 contain HEGs Therefore, any one of them could have initiated the intron homing process.

Relevance to the evolution of uniparental mtDNA inheritance

In the great majority of sexual eukaryotes, mitochondria are inherited almost exclusively from a single parent (Gillham, 1994; Birky, 2001; Xu 2005). Because uniparentally inherited genomes are prone to mutation accumulation (Muller, 1964; Xu, 2004), the predominance of uniparental mitochondrial inheritance has posed a serious challenge for biologists. Indeed, many hypotheses have been proposed to explain the prevalence of uniparental mitochondrial inheritance (reviewed by Birky, 1995). One of the commonly discussed stated that uniparental organelle inheritance was evolved to prevent the spread of selfish cytoplasmic DNA (Grun, 1976; Coleman, 1982; Law and Huston; Birky, 1995). Possible selfish cytoplasmic elements include intracellular parasites as well as defective organelle genes and genomes. These selfish elements may

have a replication and/or transmission advantage relative to other genes in the host cell, resulting in over-representation in subsequent generations. Such an over-representation may cause deleterious effect for the hosts. One example of the selfish elements is the petite mutants of yeast (Williamson, 2002). Petite yeast mutants are typically characterized by large deletions in the mitochondrial genomes and by their inability to respire. In heteroplasmic cells, due to its smaller genome size and relatively more origins of replication, a mitochondrial genome from petite mutants may exhibit a 2-fold transmission advantage compared to the wild type genome (MacAlpine et al., 2002). In an organism with biparental mitochondrial inheritance, a deleterious cytoplasmic element present in only one of the two parents could be transmitted to all of the progeny and spread rapidly in the population. To prevent the spread of such deleterious cytoplasmic elements in the population, several scientists (Hastings, 1992; Hurst and Hamilton 1992) proposed that a nuclear gene would evolve to control the transmission of the deleterious cytoplasmic elements. The consequence of a complete control would be a uniparental cytoplasmic inheritance. In the organism with a uniparental cytoplasmic inheritance, the spread of selfish detrimental cytoplasmic elements will be limited to the cytoplasmic descendants of the cell it originally invaded. Our earlier results (Yan et al. 2004) and the results here thus provide direct empirical evidence for the existence of a nuclear gene (i.e. *SXII α*) that controls both uniparental mitochondrial inheritance and the spread of selfish cytoplasmic genes, such as HEGs and their associated introns. .

References

- Baptista-Ferreira, J.L.C., Economou, A., and Casselton, L.A. (1983). Mitochondrial genetics of *Coprinus*: recombination of mitochondrial genomes. *Curr Genet* 7: 405-407.
- Barroso, G., and Labarere, J. (1997). Genetic evidence for nonrandom sorting of mitochondria in the basidiomycete *Agrocybe aegerita*. *Appl Environ Microbiol* 63:4686-4691.
- Belfort, M., Perlman, P.S. (1995). Mechanisms of intron mobility. *J Biol Chem.* 270:30237–30240.
- Belfort, M., and Roberts, R.J. (1997). Homing endonucleases: keeping the house in order. *Nucleic Acids Res.* 25(17):3379-88.
- Birky, C.W. Jr. (1995). Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc Natl Acad Sci U S A.* 92(25):11331-8.
- Birky, C.W. Jr. (2001). The inheritance of genes in mitochondria and chloroplasts: laws, mechanisms, and models. *Annu. Rev. Genet.* 35. 125–148.
- Chevalier, B.S., and Stoddard, B.L. (2001). Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucleic Acids Res.* 29(18):3757-74.
- Coleman, A.W. (1982). Sex is dangerous in a world of potential symbionts or the basis of selection for uniparental inheritance. *J. Theor. Biol.* 97: 367-369.
- Dujon, B. (1989). Group I introns as mobile genetic elements: facts and mechanistic speculations-a review. *Gene.* 82:91–114.

- Dujon, B., Belfort, M., Butow, R.A., Jacq, C., Lemieux, C., Perlman, P.S., and Vogt, V.M. (1989). Mobile introns: definition of terms and recommended nomenclature. *Gene*. 82(1):115-8.
- Gillham, N.W. (1994). *Organelle Genes and Genomes*. Oxford University Press, New York.
- Goddard, M.R., and Burt, A. (1999). Recurrent invasion and extinction of a selfish gene. *Proc Natl Acad Sci U S A*. 96(24):13880-5.
- Grun, P. (1976). *Cytoplasmic genetics and evolution*. Columbia University Press. New York.
- Hastings, I.M. (1992). Population genetic aspects of deleterious cytoplasmic genomes and their effect on the evolution of sexual reproduction. *Genet Res*. 59(3): 215-25.
- Haugen, P., Simon, D.M., and Bhattacharya, D. (2005). The natural history of group I introns. *Trends Genet*. 21(2):111-9.
- Hull, C.M., Davidson, R.C., and Heitman, J. (2002). Cell identity and sexual development in *Cryptococcus neoformans* are controlled by the mating-type-specific homeodomain protein Sx1 α . *Genes Dev*. 16(23):3046-60.
- Hurst, G.D., and Werren, J.H. (2001). The role of selfish genetic elements in eukaryotic evolution. *Nat Rev Genet*. 2(8):597-606.
- Hurst, L.D. and Hamilton, W.D. (1992) Cytoplasmic fusion and the nature of sexes. *Proc. R. Soc. Lond. Biol*. 247: 189-194.

- Law, R., and Hutson, V. (1992). Intracellular symbionts and the evolution of uniparental cytoplasmic inheritance. *Proc. R. Soc. Lond. Biol.* 248:69-77.
- Litter, J., Keszthelyi, A., Hamari, Z., Pfeiffer, I., and Kucsera, J. (2005). Differences in mitochondrial genome organization of *Cryptococcus neoformans* strains. *Antonie Van Leeuwenhoek.* 88(3-4):249-55.
- MacAlpine, D.M., Kolesar, J., Okamoto, K., Butow, R.A., and Perlman, P.S. (2001). Replication and preferential inheritance of hypersuppressive petite mitochondrial DNA. *EMBO J.* 20(7):1807-17.
- Matsumoto, T., and Fukumasa-Nakai, Y. (1996) Mitochondrial DNA inheritance in sexual crosses of *Pleurotus ostreatus*. *Curr Genet* 30: 549-552.
- Muller, H.J. (1964). The relation of recombination to mutational advance. *Mutat Res* 106: 2-9.
- Toffaletti, D.L., Nielsen, K., Dietrich, F., Heitman, J., and Perfect, J.R. (2004). *Cryptococcus neoformans* mitochondrial genomes from serotype A and D strains do not influence virulence. *Curr Genet.* 46(4):193-204.
- Williamson, D. (2002). The curious history of yeast mitochondrial DNA. *Nature Reviews Genetics* 3: 475-81.
- Xu, J. (2002). Mitochondrial DNA polymorphisms in the human pathogenic fungus *Cryptococcus neoformans*. *Curr Genet.* 41(1):43-7.

- Xu, J. (2004). Genotype-environment interactions of spontaneous mutations affecting vegetative fitness in the human pathogenic fungus *Cryptococcus neoformans*. *Genetics*. 168:1177-1188.
- Xu, J. (2005). The inheritance of organelle genes and genomes: patterns and mechanisms. *Genome*. 48(6):951-8.
- Yan, Z., and Xu, J. (2003). Mitochondria are inherited from the MATa parent in crosses of the basidiomycete fungus *Cryptococcus neoformans*. *Genetics*. 163(4):1315-25.
- Yan, Z., Hull, C.M., Heitman, J., Sun, S., and Xu, J. (2004). *SXI1alpha* controls uniparental mitochondrial inheritance in *Cryptococcus neoformans*. *Curr Biol*. 14(18): R743-R744.

Table 6.1 Strains and their genotypes

Strain	Genotype	Origin
CHY618	MAT α <i>ura5</i> mtD <i>sxi1</i> α :: <i>NAT</i>	Hull et al. 2002
CHY620	MAT α <i>ura5</i> mtD <i>SXII</i> α <i>NAT</i>	Hull et al. 2002
YZX2	MATa <i>ade2</i> mtA G418	Yan et al. 2004

¹, The above strains are isogenic except at the indicated loci. MATa: mating type allele a; MAT α : mating type allele α . Strains with *ura5* and *ade2* auxotrophic markers require uracil and adenine respectively for growth on minimum medium. mtA and mtD are two mitochondrial genotypes distinguished by polymorphisms at four loci *ND2*, *ND4*, *ND5* and *COX1*.

Table 6.2 Primers used for the identification of mitochondrial genotype

Primer pair	Sequence (5'→3')	Location
ND2F	CAAGCTGCACCATTCCATA	<i>ND2</i>
ND2R	CCATTAGTGGTGGTACTCC	<i>ND2</i>
ND4F	GGGAGAATTTGATTCAAGTGCAAC	<i>ND4</i>
ND4R	CATACATGGAAAGGTACTAG	<i>ND4</i>
Da20	GACACTACACAAGATGCCTC	<i>COX1</i> exon 1
Da3	GCAATAGCATATAACCATCCCG	<i>COX1</i> exon 3
Da22	CTCGAGCTTACTTTACAGCAG	<i>COX1</i> exon 3
Da19	GTACTACTCCTGTTAGTCCTC	<i>COX1</i> exon 4
Da26	CAACGGCATAACGGTGGATCTATCC	<i>COX1</i> exon 4
Da15	CTGTTAGATATGATGGTGTGC	<i>COX1</i> exon 6
COB1F	CCACAACCTATTAACATTAGCTACGC	<i>COB1</i> exon 1
COB1R	CGTCTCCATCTACAAAGCCAGCAAAC	<i>COB1</i> Intron 2
ND5F	CTATTGGTGTTACAGGAGCTCAC	<i>ND5</i>
ND5R	GAGCCTTCATACCTGCCTTATTTGC	<i>ND5</i>
LsrRNAF	CAGCAGAACCCTTCCCAGC	Upstream <i>LsrRNA</i>
LsrRNAR	CCTCCACTGTCTCATGCGG	<i>LsrRNA</i> exon 3

Table 6.3 Primers used for the identification of the recombination border

Primer pairs	Sequence (5'→3')	Location
Cox1F	TGTCTGGAGCTGGTAACCAAT	<i>COX1</i>
Cox1R	CAAAAGAGGTGTTTCATATAGAACTGG	<i>COX1</i>
ATP6F	GACACACTTTATTACATCTCCAC	<i>ATP6</i>
ATP6R	GAAGTTCAATGGCATCCTTG	<i>ATP6</i>
N2A8F	AACTCCCCACATAGTTATGG	<i>ND2-ATP8 ig</i> ^a
N2A8R	CATCCCTGTTATTAATTCACT	<i>ND2-ATP8 ig</i>
ATP8F	TTC AATGGGTGCTGTGTTC	<i>ATP8-COX1 ig</i> ^b
ATP8R	CCGAATGTAATTTGGTTTACCC	<i>ATP8</i>
ATP9F	CGGACTATCAGGAGCTGGAG	<i>ATP9</i>
ATP9R	TGGTGGTCACCGTTTAGAA	<i>ATP9-COX1 ig</i> ^c
ND6F	ACTTGATCTTCTTGCATTTGG	<i>ND6</i>
ND6R	TTATGTTTCGTGGTCGTAGACA	<i>ND6</i>
Da26	CAACGGCATAACGGTGGATCTATCC	<i>COX1</i> exon 4
Da15	CTGTTAGATATGATGGTGTGC	<i>COX1</i> exon 6
Da20	GACACTACACAAGATGCCTC	<i>COX1</i> exon 1
Da3	GCAATAGCATATACCATCCCG	<i>COX1</i> exon 3

^a*ND2-ATP8 ig* represents the primer located at the intergenic region between *ATP8* and

COX1.

^b *ATP8-COX1 ig* represents the primer located at the intergenic region between *ATP8* and *COX1*.

^c *ATP9-COX1 ig* represents the primer located at the intergenic region between *ATP8* and *COX1*.

Table 6.4 Intron mobility and mitochondrial inheritance in crosses with and without the *SX11 α* gene

Origins of mtDNA gene		Crosses	
<i>ND2, ND4</i> and <i>ND5</i> ¹	<i>COX1</i> gene ³	CHY618 (MAT α <i>sxi1α</i> mtD) x YZX2 (MATa mtA)	CHY620 (MAT α <i>SX11α</i> mtD) x YZX2 (MATa mtA)
MATa alleles	MATa allele	5	254
MAT α allele	MAT α allele	70	12
MATa alleles	MAT α allele	30	2
MAT α alleles	MATa allele	0	1
Recombinant ²	MAT α allele	3	0
MATa and MAT α alleles	MATa and MAT α alleles	8	4
Total		116	273
% zygote with introns in the <i>COX1</i> gene		95.6	6.59

¹, MATa alleles refer to those from the MATa parent; MAT α alleles refer to those from the MAT α parent, CHY618 or CHY620.

², Recombinant here refers to the genotype with a combination of alleles from MATa and MAT α parents for loci *ND2, ND4, ND5* and *COX1*. However, it does not have alleles for any of the four loci from both parents.

³, MATa allele (YZX2) has no intron in the *COX1* gene; MAT α allele has 5 introns in the *COX1* gene.

Table 6.5 Location of recombination borders in ten zygotesstrains.

Strains	Left border within this region	Location of left border	Right border within this region	Location of right border
OYZ1	13127~13237 ^a	- <i>COXI</i> ^b	14440~14786	<i>COXI</i> - ^c
OYZ2	13639~13840	<i>COXI</i> ^d	14440~14786	<i>COXI</i> -
YZ150	13456~13588	<i>COXI</i>	14440~14786	<i>COXI</i> -
YZ151	13127~13237	- <i>COXI</i>	14440~14786	<i>COXI</i> -
YZ152	13855~13887	<i>COXI</i>	14440~14786	<i>COXI</i> -
YZ153	13456~13588	<i>COXI</i>	14440~14786	<i>COXI</i> -
YZ155	13855~13887	<i>COXI</i>	14440~14786	<i>COXI</i> -
YZ158	13855~13887	<i>COXI</i>	14374~14407	<i>COXI</i>
YZ159	13639~13840	<i>COXI</i>	14297~14374	<i>COXI</i>
YZ160	13855~13887	<i>COXI</i>	14297~14374	<i>COXI</i>

^a The number shows the location of recombination borders using the published serotype A mitochondrial genome (NC_004336) as a reference. ^b -*COXI* represents that the left border of recombination is located either within the *COXI* gene or in the intergenic region between *ATP9* and *COXI*. ^c *COXI*- represents that the right border of recombination is located either within the *COXI* gene or in the intergenic region between *COXI* and *ATP8*. ^d *COXI* represents that the border of recombination is located within the *COXI* gene.

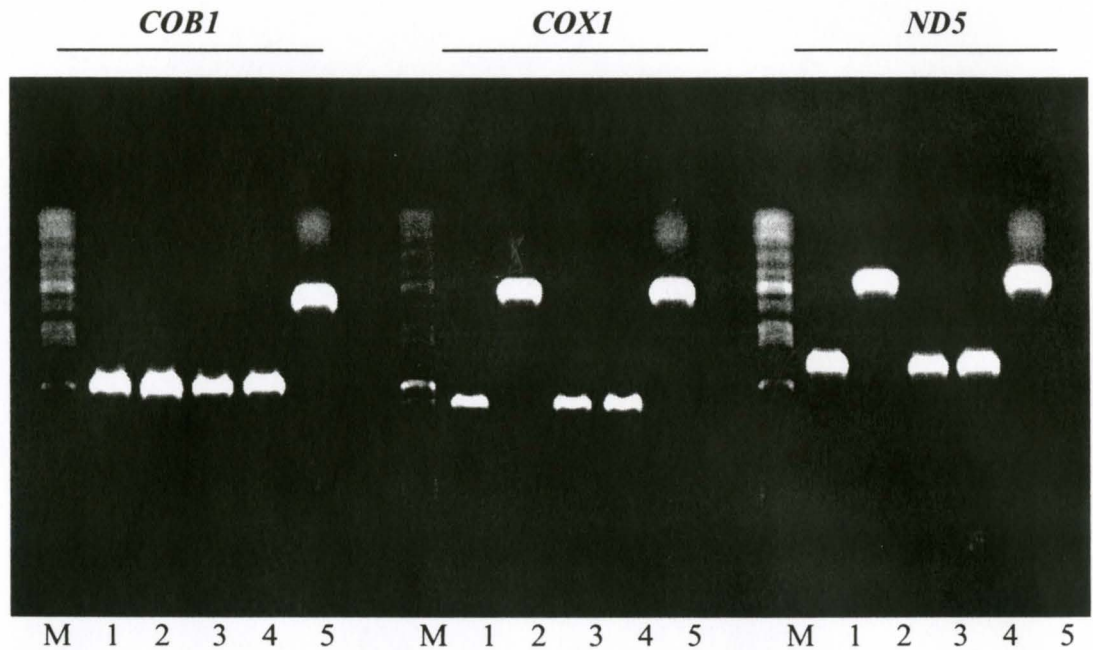


Figure 6.1 Comparisons of intron distributions among five strains of *C.*

neoformans for four introns, one intron within the *COB1* gene (intron 1 in Litter et al. 2005), one intron in *ND5* gene (intron in Toffaletti et al. 2004) and two introns within the *COX1* gene (introns 4 and 5 together in Toffaletti et al. 2004). Lanes 1, 2, 3, 4 and 5 correspond to strains JEC21, YZX2, CHY618, CHY620, and H99 respectively. H99 is the sequenced serotype A strain. JEC21 is the sequenced serotype D strain. Lane M: 1kb ladder.

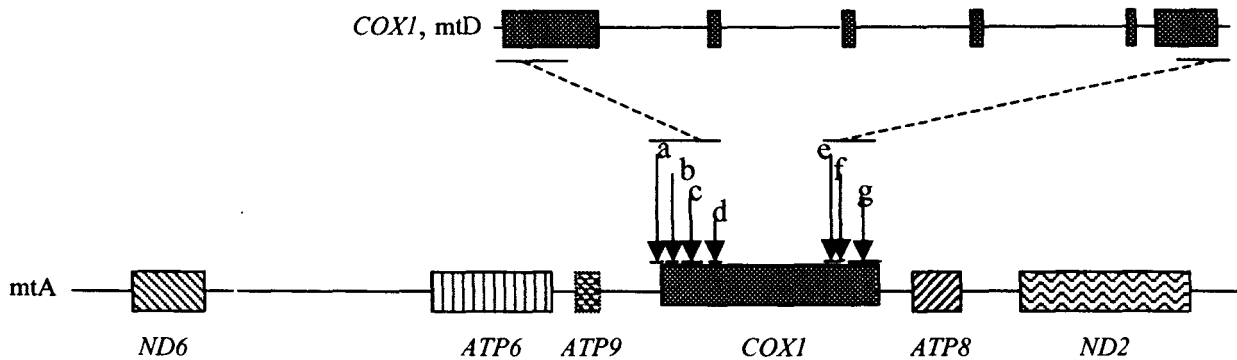


Figure 6.2 This figure shows the borders of recombination in the ten random zygotes with recombinant mitochondrial genotypes. The arrows show the approximate regions of recombination borders. All the borders are located either within the *COX1* gene or within the intergenic region between *COX1* and two other genes (between *ATP9* and *COX1* for the left border and between *COX1* and *ATP8* for the right border). Arrow “a” shows the left borders of 2 strains (OYZ1 and YZ151). Arrow “b” points to the left borders of 2 strains (YZ150 and YZ153). Arrow “c” points to the left borders of 2 strains (OYZ2 and YZ159). Arrow “d” points to the left borders of 4 strains (YZ152, YZ155, YZ158 and YZ160). Arrow “e” points to the right borders of 2 strains (YZ159 and YZ160). Arrow “f” points to the right border of YZ158. Arrow “g” points to the right border of 7 strains (OYZ1, OYZ2, YZ150, YZ151, YZ152, YZ153 and YZ155).

CHAPTER 7

GENERAL CONCLUSIONS AND PERSPECTIVES

1. The composition of MAT alleles in natural populations of *C. neoformans*

In order to better understand the population structure of *C. neoformans*, the second chapter of this thesis examined the composition of MAT alleles in 385 natural strains collected in four areas of the US. The results showed that the A α (serotype A and mating type α) allele was the most prevalent. One of the interesting discoveries in this chapter is that some serotype AD strains, especially those collected in San Francisco area, contained the Aa allele. This finding suggests that the fertile Aa (serotype A and mating type a) strains which thought to have been extinct (Lengeler et al., 2000) still exist in some specific areas. This prediction was supported recently by the discoveries of fertile Aa strains in Italy and Africa (Viviani et al., 2001; Keller et al., 2003; Nielsen et al., 2003; Litvintseva et al., 2003).

Another interesting finding in this chapter was the composition of MAT alleles in serotype AD strains. Serotype AD strains are the hybrids between serotype A and serotype D strains. It could be produced by two processes. One is crossing between Aa and D α (serotype D and mating type α) strains. The other is crossing between A α and Da (serotype D and mating type ϵ) strains. To distinguish the serotype AD strains produced by these two different processes, I used AaD α to represent serotype AD strains produced by crossing between Aa and D α strains; A α Da to represent serotype AD strains produced by crossing between A α and Da strains. Because Da strain is more prevalent than Aa strain and A α strain is more common than D α strain in nature, we predicted that most of the serotype AD strains should belong to the A α Da type. However, in our survey, I found that most of the AD strains were the AaD α type. This result contradicted our expectation. One possible explanation is that the mating type allele combination in AaD α strain is more virulent than

that in A α Da strain. This hypothesis can be tested experimentally by comparing in vivo virulence between AaD α and A α Da congenic serotype AD strains.

2. The functional differences of mitochondrial genomes in *C. neoformans*

In chapter 3 of this thesis, we found that the mitochondrial genome could be very easily replaced through a parasexual process in *C. neoformans*. This finding may offer us an opportunity to identify the functional differences of mitochondrial genomes and the interaction between nuclear and mitochondrial genomes. Despite the small size, mitochondrial genomes in fungi have been found to influence many characters of its host cells, such as virulence, mycelial growth, and senescence (Esser and Tudzynski, 1977; de la Bastide *et al.*, 1997; Olson and Stenlid, 2001). Systematic comparison among mitochondrial genomes in *C. neoformans* has not been performed. In a recent study, Toffaletti and her colleagues (2004) compared the virulence between two serotype AD strains created either by mating Aa with D α strains or A α with Da strains. They concluded that the mitochondrial genomes from serotype A and D strains do not influence virulence. Unfortunately, this conclusion ignored the influence by MAT locus since the two serotype AD strains used in their experiment differ not only at the mitochondrial genome but also at the MAT locus. A further experiment is thus necessary to clarify the influence of mitochondrial genomes on virulence. The parasexual process found in this thesis can create strains differing only at the mitochondrial genome, therefore, offering us a chance to address this issue.

3. The unique mitochondrial inheritance pattern in *C. neoformans*

In this thesis, we found that progeny always inherited mitochondrial from the MAT α parent in sexual crosses between serotype D strains. Similar mitochondrial inheritance pattern has also found in crosses between serotype A strains of *C. neoformans* (a pilot experiment, data not shown). This type of inheritance pattern is commonly found in animals but is unique in fungi. This result raises a question with regard to the origin of this unique mitochondrial inheritance pattern in fungi. A potential way to address this question is to identify the mitochondrial inheritance pattern in species closely related to *C. neoformans*. Using the phylogenic information, we may be able to estimate when this phenomenon emerged and/or lost.

4. The mechanisms for the uniparental inheritance in *C. neoformans*

In this thesis, we identified two genes at the MAT locus that control mitochondrial inheritance. However, the detailed molecular mechanism for the uniparental inheritance is still unclear. We proposed two hypotheses to explain how these two genes, *Sxi1 α* and *Sxi2 α* , control mitochondrial inheritance. First, these two genes may control unilateral nuclear migration during the formation of the zygote. Specifically, in crosses with wild-type genotypes, after fusion of MAT α and MAT α cells, only the MAT α nucleus migrates towards the MAT α parent cell, while the mitochondrial DNA didn't mix immediately. Subsequently, the budding might have occurred preferentially at the MAT α parent side, which results in most of the progeny inheriting mitochondria from the MAT α parent. In contrast, in crosses with *Sxi1 α* or *Sxi2 α* deletions, the unidirectional nuclear migration may be prevented, resulting in biparental inheritance. To further test this hypothesis, differential labelling of nucleus and mitochondria with different colours of dyes for both mating

partners are needed and the mating processes need to be recorded using fluorescent microscopy. There are many mitochondrial specific dyes available including MitoTracker Green and MitoTracker Red etc. These dyes are essentially nonfluorescent in aqueous solution and become fluorescent when their chloromethyl moieties form covalent bonds with protein thiols in the mitochondrion (Haugland, 1996). These dyes have been successfully used to observe the mitochondrial behavior in the large fertilized eggs (Nishimura et al., 2006; Cao et al., 2004).

The second hypothesis explains that the mitochondria from the two parents are recognized or tagged and those from the MAT α parent are selectively destroyed (active model). This mechanism has been reported for organelle inheritance in other organisms where methylation and ubiquitination were found involved in the organelle recognition process (Sutovsky et al., 1999; 2000; Feng and Chiang 1984; Umen and Goodenough, 2001). It is possible that a similar process is involved in the uniparental mitochondrial inheritance in *C. neoformans*. Several environmental factors (Eg. UV irradiation, drugs) have been found capable of influencing the process of methylation and ubiquitination. Thus, the above hypothesis can be tested by identifying the influence of environmental factors on mitochondrial inheritance. In addition, finding the downstream genes of *Sxi1a* and *Sxi2a* may provide clues for the underlying mechanism responsible for uniparental mitochondrial inheritance in *C. neoformans*. The downstream genes may be identified through subtractive cloning or micro array analysis.

5. Why is mitochondrial inheritance uniparental in most sexual eukaryotes?

Many hypotheses have been proposed to explain the prevalence of uniparental mitochondrial inheritance in sexual eukaryotes (see General Introduction). One of the hypotheses explains that it is evolved to prevent the spread of selfish deleterious cytoplasmic elements (Grun, 1976; Coleman, 1982). However, evidence supporting this hypothesis is still sparse. In Chapter 6 of this thesis, we found a mobile intron in the mitochondrial genome of *C. neoformans* and the spread of this selfish element can be prevented by the nuclear genes *SXI1a*. This result therefore is consistent with the above hypothesis. Selfish cytoplasmic elements exist widely in nature. Except genes in the mitochondrial genome, selfish cytoplasmic elements could also be intracellular viruses or bacteria. To further test the selfish cytoplasmic element hypothesis, experiments can be conducted to examine whether *SXI1a* and *SXI2a* genes could prevent the spread of intracellular virus or bacteria in *C. neoformans*. At present, no virus or bacteria is known to exist in *C. neoformans*. In addition, it would be interesting to test the fitness consequences of the selfish intron in *C. neoformans* and how widely this intron has spread in natural populations of *C. neoformans*.

6. Genomic conflict between mitochondrial and nuclear genomes

The differential transmission between the nuclear and the mitochondrial genomes may create genomic conflicts. The nuclear genome usually transmits one copy of itself to the next generation via strict replication and segregation mechanisms, thus each nuclear gene generally has an equal chance to be transmitted to the next generation. In contrast, the replication and segregation of mitochondrial genomes are less stringent. Differential transmission of mitochondrial genes can occur either by differential replication of

mitochondrial genomes in heteroplasmic cells or by differential segregation of mitochondrial genomes during mitosis and meiosis. In the absence of strict segregation mechanisms that ensure a fair cytoplasmic segregation in the daughter cells of a heteroplasmic cell, selfish cytoplasmic elements may evolve that give an intra-cytoplasmic advantage, but lower the fitness of the host cell. One example of the selfish mitochondrial genes is the petite mutants of yeast (Williamson, 2002). Petite mitochondrial genomes are characterized by large deletions and their inability to respire. The smaller petite mitochondrial genomes therefore can replicate faster than wild-type genomes resulting in a nearly 2-fold transmission advantage when the two coexist within a cell (MacAlpine *et al.*, 2001). The fitness of the petite mutant, however, is lower than wild type due to the deficiency in respiration. The differential transmission rules between nuclear and mitochondrial genomes are therefore expected to lead to genomic conflict: natural selection may favour a gene at the level of the mitochondrial genome but dis-favour it at the level of the nuclear genome (organism level).

To avoid such potential genomic conflicts created during mating, a nuclear gene would evolve to control mitochondrial inheritance and suppress the spread of selfish mitochondria genomes. Indeed, in this thesis, we found a sex-determining genes, *SXII α* , in the *C. neoformans* nuclear genome that can suppress the spread of selfish mitochondrial genes by controlling mitochondrial inheritance. The uniparental inheritance is, however, a dead end for the mitochondria of the MAT α parent. Selection on the mitochondria should therefore favor variants capable of influencing fertility of its host. This phenomenon was found in many organisms. For example, in flowering plants, mitochondrial mutants can

convert hermaphrodites into females by undermining their ability to produce pollen (Saumitou-Laprade et al., 1994). Feminization of hermaphrodites is advantageous to mitochondria because relocation of plant resources from male to female would increase female survival and thereby enhancing mitochondria transmission. Similar phenomenon was also reported in insects where the intracellular bacteria, *Wolbachia*, evolved the ability to manipulate sex ratio of its host by either killing the male embryos, feminizing the genetic male into female or inducing parthenogenesis (Charlat et al., 2003).

The relationship between sex and mitochondria may therefore be a two-way street: in one direction, the sex-determining genes control mitochondrial inheritance. In the other direction, the mitochondria can also influence the sex of its host. In our experiment, we have confirmed one direction of the two way street that sex-determining genes control mitochondria inheritance in *C. neoformans*. However, we did not test the possible effect of the other direction of the two way street. It would be interesting to test if the mitochondria or the selfish genes in the mitochondrial genome have any effect on the fertility of its host.

REFERENCES

- Cao, L., Kenchington, E., and Zouros, E. (2004). Differential segregation patterns of sperm mitochondria in embryos of the blue mussel (*Mytilus edulis*). *Genetics*. 166(2):883-94.
- Charlat, S., Hurst, G.D., and Mercot, H. (2003). Evolutionary consequences of *Wolbachia* infections. *Trends Genet.* 19(4):217-23.

- de la Bastide, P.Y., Sonnenberg, A.S.M., Van Griensven, L.J.L.D., Anderson, J.B., and Horgen, P.A. (1997). Mitochondrial haplotype influences mycelial growth of *Agaricus bisporus* heterokaryons. *Appl Environ Microbiol* 63: 3426-3431.
- Esser, K., and Tudzynski, P. (1977). Prevention of senescence in the ascomycete *Podospora anserine* by antibiotic tiamulin. *Nature* 265: 454-456.
- Haugland, R. P. (1996). *Handbook of Fluorescent Probes and Research Chemicals*, Ed. 6. Molecular Probes, Eugene, OR.
- Keller, S.M., Viviani, M.A., Esposto, M.C., Cogliati, M., and Wickes, B.L. (2003). Molecular and genetic characterization of a serotype A MATa *Cryptococcus neoformans* isolate. *Microbiology*. 149:131-42.
- Lengeler, K.B., Wang, P., Cox, G.M., Perfect, J.R., and Heitman, J. (2000). Identification of the MATa mating-type locus of *Cryptococcus neoformans* reveals a serotype A MATa strain thought to have been extinct. *Proc Natl Acad Sci U S A*. 97(26):14455-60.
- Litvintseva, A.P., Marra, R.E., Nielsen, K., Heitman, J., Vilgalys, R., and Mitchell, T.G. (2003). Evidence of sexual recombination among *Cryptococcus neoformans* serotype A isolates in sub-Saharan Africa. *Eukaryot Cell*. 2(6):1162-8.
- Nielsen, K., Cox, G.M., Wang, P., Toffaletti, D.L., Perfect, J.R., and Heitman, J. (2003). Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic alpha and alpha isolates. *Infect Immun*. 71(9):4831-41.

- Nishimura, Y., Yoshinari, T., Naruse, K., Yamada, T., Sumi, K., Mitani, H., Higashiyama, T., and Kuroiwa, T. (2006). Active digestion of sperm mitochondrial DNA in single living sperm revealed by optical tweezers. *Proc Natl Acad Sci U S A*. 103(5):1382-7.
- Olson, A., and Stenlid, J. (2001) Mitochondrial control of fungal hybrid virulence. *Nature* 411: 438.
- Saumitou-Laprade, P., Cuguen, J., and Vernet, P. (1994) Cytoplasmic male sterility in plants: molecular evidence and the nucleocytoplasmic conflict. *Trends Ecol Evol* 9: 431–435.
- Toffaletti, D.L., Nielsen, K., Dietrich, F., Heitman, J., and Perfect, J.R. (2004). *Cryptococcus neoformans* mitochondrial genomes from serotype A and D strains do not influence virulence. *Curr Genet*. 46(4):193-204.
- Viviani, M.A., Esposto, M.C., Cogliati, M., Montagna, M.T., and Wickes, B.L. (2001). Isolation of a *Cryptococcus neoformans* serotype A MATa strain from the Italian environment. *Med Mycol*. 39(5):383-6.