Mitochondria Are Inherited From the *MAT*a Parent in Crosses of the Basidiomycete Fungus *Cryptococcus neoformans*

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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 206 single spores analyzed from six crosses, all but one inherited mtDNA from the *MAT***a** 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 *MAT***a** parent in *C. neoformans*. To distinguish two potential mechanisms, we obtained a pair of isogenic strains with different mating-type alleles, mtDNA types, and auxotrophic markers. Diploid cells from mating between these two strains were selected and 29 independent colonies were genotyped. These cells did not go through the hyphal stage or the meiotic process. All 29 colonies contained mtDNA from the *MAT***a** parent. Because no filamentation, meiosis, or spore formation was involved in generating these diploid cells, our results suggest a selective elimination of mtDNA from the *MAT*α parent soon after mating. To our knowledge, this is the first demonstration that mating type controls mtDNA inheritance in fungi.

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 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. Although 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, on the basis of evidence from amplified fragment length polymorphisms (AFLPs), Boekhout *et al.* (2001) proposed dividing *C. neoformans* into two separate species, with serotypes A, D, and AD included in the species *C. neoformans* (Sanfelice) Vuillemin, and strains of serotypes B and C comprising a new species, *C. 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 sero-

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type identifications, to avoid confusion only serotype identifications are 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 2002a).

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 is spontaneously generated asexual spores (called blastospores; 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 not the product of either karyogamy or 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 the 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 were 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 *MAT***a** 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 *MAT***a** parent. Additional experiments were performed to test for two alternative hypotheses on mating-type control of mtDNA inheritance.

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 (*MAT***a**) 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 2002a). Strain CDC46 was isolated from a patient in San Francisco, California, during an epidemiological survey conducted by the U.S. Centers for Disease Control and Prevention (CDC) in 1992. CDC46 is serotype AD and contains both *MAT***a** 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 2002a). Two other

strains, JEC34 and JEC50, were also used for testing mechanisms of mitochondrial inheritance. JEC34 is isogenic to JEC20 but contains the ura3− auxotrophic marker. JEC50 is isogenic to JEC21 and contains the ade2− auxotrophic marker.

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 CDC (kindly provided by Dr. Mary Brandt) and (ii) Nagasaki University in Japan (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 were confirmed by indirect immunofluorescence with a combination of polyclonal and monoclonal reagents. Serotypes for strains from Japan were determined by the Iatron (Tokyo) commercial kit. Mating-type alleles for strains from the CDC were recently reported by Yan *et al.* (2002). Mating-type alleles for strains from Japan were determined on the basis of serotype- and mating-type-specific PCR primers and followed the protocols described in Yan *et al.* (2002). Mitochondrial genotypes for strains from both the CDC and Japan were determined and reported by Xu (2002a).

Construction of isogenic strains with different mtDNA genotypes: Our initial screening demonstrated that CDC46 contained both *MAT***a** and *MAT*α (Yan *et al.* 2002). This strain was found to be 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 analyzed 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°), 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.

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 >400 strains (Lengeler *et al.* 2001; Yan *et al.* 2002). In the three initial strains, JEC20 had the serotype D-specific *MAT***a** (abbreviated as D**a**); JEC21 had the serotype D-specific *MAT*α (abbreviated as Dα); and CDC46 had both the serotype A-specific *MAT***a** allele (abbreviated as A**a**) and the serotype D-specific *MAT*α allele (abbreviated as Dα). Therefore, strain CDC46 had the mating-type A**a**Dα. Table 1 lists the sequences, annealing temperatures, and number of cycles for different primer pairs. Each PCR reaction contained 0.4 ng genomic DNA template, 0.5 μM of each primer, 2 mM magnesium chloride ($MgCl_2$), 0.1 mM deoxyribonucleotide triphosphate (dNTP), and 0.2 unit of Taq DNA polymerase in a total volume of 20 μl. Typical PCR reaction conditions were 3 min at 94°; followed by 40 cycles of 30 sec at 94°, 30 sec at the indicated annealing temperature, and 30 sec at 72°; and finally, 7 min of extension at 72°. 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.

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 2002a). Primer pairs and the number of cycles used are presented in Table 1. PCR conditions were similar to those described above. PCR products were digested by *Hin*dIII and *Alu*I for the URA5 and ND2 genes, respectively, according to manufacturer's recommendations. Restriction patterns were checked by agarose gel electrophoresis.

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′-GAGGGTGGCGGTTCT-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).

Electrophoretic karyotypes (contour-clamped homogeneous electric field): 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- to 200-sec switch at 4.5 V at 14° for 30 hr; block 2, 250- to 900-sec switch at 3.0 V at 14° for 46 hr. 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 1 and Table 2 (see RESULTS for details). The six strains listed in Table 2 were crossed to JEC20 or JEC21 to generate the following six crosses: JEC20-mtA-1 × JEC21; JEC20-mtA-2 × JEC21; JEC20-mtA-3 × JEC21; JEC21-mtA-1 × JEC20; JEC21-mtA-2 × JEC20; and JEC21-mtA-3 × 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 × JEC21 by micromanipulation using a Singer MSM 300 micromanipulator (Singer Instrument, Somerset, UK). 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 1 and Table 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*: Mat-

TABLE 1

PCR primers and their respective PCR conditions used in this study

Primer name	Primer sequence (5′–3′)	Annealing temp (°)	No. of cycles	Expected band size (bp)
STE20-Aα	Forward: ccaaaagctgatgctgtgga Reverse: aggacatctatagcagat	55–45	38	588
STE20-A**a**	Forward: tccactggcaaccctgcgag Reverse: atcagagacagaggagcaagac	55	35	865
STE20-D**a**	Forward: gatctgtctcagcagccac Reverse: aatatcagctgcccaggtga	60	32	~440
STE20-Dα	Forward: gatttatctcagcagccacg Reverse: aaatcggctacggcacgtc	61	28	443
URA5	Forward: acgcctgcctgttacttaa Reverse: ggacatgatgattggagt	50	40	744
ND2	Forward: caagctgcaccattccata Reverse: ccattagtggtggtactcc	50	40	533
M13 core	gagggtggcggttct	50	40	Variable
$(GACA)_4$	gacagacagacagaca	50	40	Variable
OPA17	gaccgcttgt	36	40	Variable
RP4	attgcgtcca	36	40	Variable

ing-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 × CDC46 and JEC21 × CDC46, we screened a total of 101 and 22 SSIs, respectively. 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**a**, Aα, D**a**, and Dα) that amplify the STE20 gene and two PCR-RFLP markers (URA5 and ND2).

Among the 101 SSIs from the cross between JEC20 and CDC46, 8 (~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 × 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 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 1 [gel pictures from RAPD primers M13, $(GACA)_4$, and OPA17 are not presented but results from these primers are summarized in Table 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. In all six crosses, mating-type alleles segregated at ratios not significantly different from 1:1 (Table 3), indicating Mendelian inheritance of nuclear genetic markers. The genotypes of 5 representative SSIs from two crosses are presented in Figures 2 and 3. In contrast to the balanced mating-

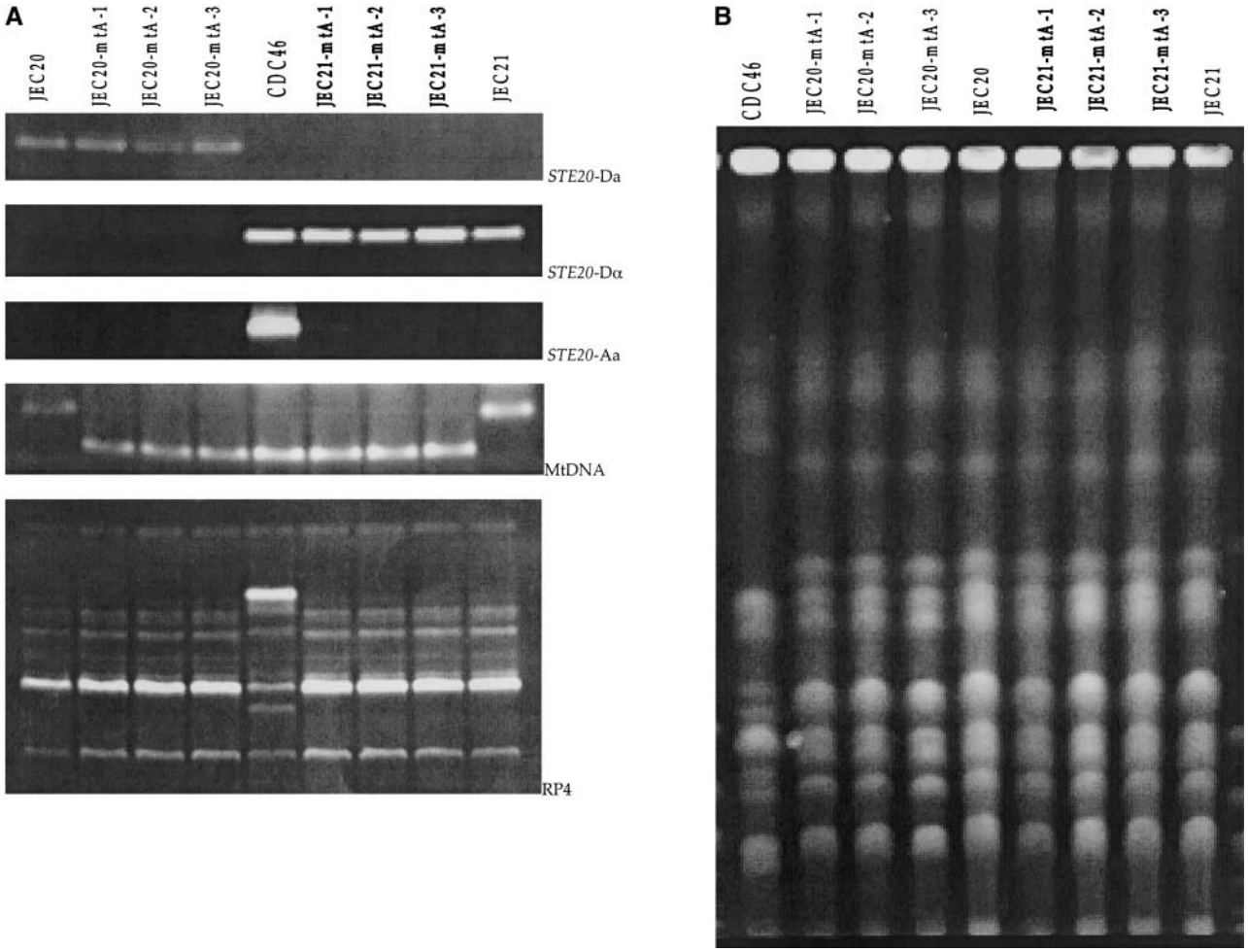

FIGURE 1.—Construction and genetic verification of strains with isogenic nuclear genotypes but different mitochondrial genotypes. (A) Top, names of strains corresponding to individual lanes; right side, primer pair and/or restriction digests used to generate individual genotypes. These primers and their PCR conditions are described in Table 1. (B) The chromosomal patterns of the same strains but in a different order.

type allele inheritance, mitochondrial inheritance was highly skewed. Among the 206 SSIs, only 1 inherited a mtDNA genotype from the *MAT*α parent; all other 205 SSIs inherited mtDNA from the *MAT***a** parent. No difference was observed between SSIs obtained by the two different protocols (*i.e.*, micromanipulation *vs.* picking of randomly germinated spores). Interestingly, 5 of the 206 SSIs contained both *MAT***a** 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 the *MAT***a** parent. Other crosses between JEC50 and JEC20-mtA-3 and between JEC34 and JEC21-mtA-1 also confirmed that progeny inherited mitochondrial DNA only from the *MAT***a** parent, regardless of mitochondrial genotype and other nuclear genetic markers (data not shown).

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 4. Data are arranged according to strain geographic origin and serotype identification. Genotypes of representative strains are presented in Figure 4. Among the 376 strains of serotype A, all had the serotype A-specific *MAT*α allele at the STE20 locus (Aα) 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 the STE20 locus (Dα) 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 4). In contrast, strains of serotype AD showed a mixed pattern of mating-type alleles and mitochondrial genotypes (Table 4).

TABLE 2

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

Strains	Specific alleles of STE20 gene			mtDNA: ND2 (*Alu*I digest)[b]	Nuclear genotype characterized by five different markers					
	Da	Dα	A**a**		URA5 (*Hin*dIII digest)	M13	$(GACA)_4$	OPA17	RP4	CHEF
JEC20	+[a]	−	−	JEC20[b]	JEC20	JEC20	JEC20	JEC20	JEC20	JEC20
JEC21	−	+	−	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21	JEC21
CDC46	−	+	+	CDC46[c]	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
JEC20-mtA-3	+	−	−	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

CHEF, contour-clamped homogeneous electric field.

[a] "+" indicates the presence of the allele identified by a specific PCR primer pair. "−" indicates the absence of such an allele.

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

[c] Genotype was identical to that of CDC46 at the indicated locus/genetic marker.

To facilitate comparisons of mating-type allele and mtDNA genotype among serotype AD strains, the 21 serotype AD strains were divided into five categories on the basis of our earlier findings of mating-type alleles (Yan *et al.* 2002; Table 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 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.

TABLE 3

Mitochondrial inheritance in six crosses

Cross	MtDNA genome of SSI from parent with *MAT***a** or *MAT*α		Mating-type allele distribution for SSIs[a]	
	*MAT***a**	*MAT*α	*MAT***a**	*MAT*α
JEC20 × JEC21-mtA-1	18	0	9	9
JEC20 × JEC21-mtA-2	25	0	12[b]	15[b]
JEC20 × JEC21-mtA-3	16	0	8[c]	9[c]
JEC21 × JEC20-mtA-1	25	1	16	10
JEC21 × JEC20-mtA-2	17	0	8	9
JEC21 × JEC20-mtA-3	33	0	20[d]	15[d]
JEC21 × JEC20-mtA-3[e]	72	0	32	41
Total	205	1	104	108

[a] 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 <2.0 (d.f. = 1, $P > 0.2$).

[b] Two of the 25 SSIs from this cross contained both *MAT***a** and *MAT*α.

[c] One of the 16 SSIs from this cross contained both *MAT***a** and *MAT*α.

[d] Two of the 33 SSIs from this cross contained both *MAT***a** and *MAT*α.

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

First, the 16 strains in AD-group 1 from both the United States and Japan had *MAT***a** from serotype A (A**a**). Their mtDNA types were identical to the serotype A-specific mtDNA haplotype (haplotype I). Second, the two strains in AD-group 2 had *MAT***a** from serotype D (D**a**), and their mtDNA types were identical to the 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***a** and *MAT*α (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 on the basis of the remaining mating-type allele information. Specifically, the strain in AD-group 3 should have initially contained (A**a**)Dα; the strain in AD-group 4 strain contained Aα(D**a**); and the strain in AD-group 5 contained (Aα)D**a** (note that 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*.

Testing two alternative hypotheses for mating-type control of mitochondrial inheritance: While the exact

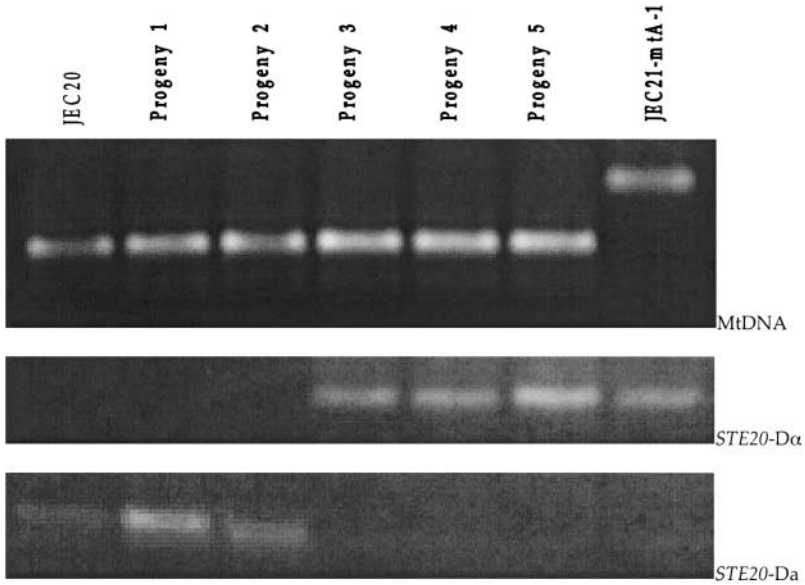

FIGURE 2.—Genotypes at the mitochondrial genome and the *STE20* locus for two parental strains and five representative progenies from a cross between JEC20 and JEC21-mtA-1. Top, strain names corresponding to individual lanes; right side, primers used to generate individual genotype profiles.

mechanisms are still unclear, abundant molecular and cellular evidence indicated that uniparental mitochondrial inheritance in the model unicellular alga *C. reinhardtii* was due to selective elimination of mtDNA from one mating partner. Similar to *C. neoformans*, two mating types are present in *C. reinhardtii*, designated mat^+ and mat^-. In sexual crosses, the progeny always inherit the mitochondrion of the mat^- parent (GILLHAM 1994). The mitochondrion from the mat^+ parent is selectively destroyed in the zygote by a presumed nuclear gene *mitd* (closely linked to mat^-) soon after the fusion of gametes (GILLHAM 1994).

Unlike *C. reinhardtii*, where no filamentous phase is involved in mating, hyphal filament formation is typical in mating in *C. neoformans*. The existence of a filamentous phase allows two alternative hypotheses to explain the potential mechanisms of mtDNA inheritance. The first is that during sexual mating, mitochondria and cytoplasm from the two mating partners were not completely mixed to form a homogeneous cytoplasm in the fused cell and that hyphal formation occurred preferentially on the *MAT***a** parent side, away from the *MAT*α parent cell. Therefore, mitochondria in the hyphae (and hence in sexual and asexual spores derived from these hyphae) would be from the *MAT***a** parent. The second hypothesis is that mitochondrial DNA from the *MAT*α parent is selectively destroyed by a *MAT***a**-linked gene after cell fusion, similar to the currently favored mechanism to explain uniparental organelle inheritance in *C. reinhardtii* (GILLHAM 1994).

Because these two hypotheses offer different predictions, we were able to design experiments to differentiate between them. In *C. neoformans*, stable diploid yeast cells can be synthesized directly from two mating partners without going through the filamentous stage (SIA *et al.* 2000; XU 2002b). Such cells can also be maintained

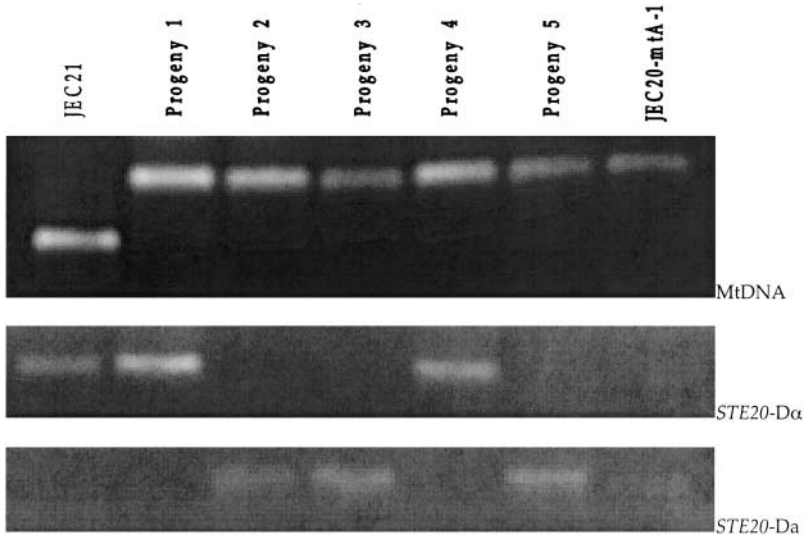

FIGURE 3.—Genotypes at the mitochondrial genome and the *STE20* locus for two parental strains and five representative progenies from a cross between JEC21 and JEC20-mtA-1. Top, strain names corresponding to individual lanes; right side, primers used to generate individual genotypes.

TABLE 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	Aα	Haplotype I
	D	12	Dα	Haplotype II
	AD-group 1	14	A**a**Dα	Haplotype I
	AD-group 2	2	AαD**a**	Haplotype II
	AD-group 3	1	Dα	Haplotype I
	AD-group 4	1	Aα	Haplotype II
	AD-group 5	1	D**a**	Haplotype II
	B	3	Bα	Haplotype III
Japan	A	52	Aα	Haplotype I
	AD-group 1	2	A**a**Dα	Haplotype I
	Not typable-1	1	Aα	Haplotype I
	Not typable-2	1	Dα	Haplotype II

[a] Mating types were determined on the basis of 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).

[b] Mitochondrial haplotypes were determined on the basis of restriction site polymorphisms at the mitochondrial ND2 locus. Mitochondrial haplotypes I and II are specific to serotypes A and D, respectively (XU 2002).

efficiently on nitrogen-rich medium (*e.g.*, YEPD). If diploid yeast cells derived through this process contained only mitochondria from the *MAT***a** parent, then the first hypothesis is refuted and the second hypothesis supported.

To test this prediction, we obtained strains with different mating types, mtDNA genotypes, and auxotrophic markers. Strain JEC50 (*MAT*α, ade−, mtD) was mated with JEC20-mtA-1 (*MAT***a**, mtA) and a SSI from this cross with the desired genotype (*MAT*α, ade−, mtA) was selected by a process similar to that described earlier. This strain was designated JEC50-mtA and was mated with strain JEC34 (*MAT***a**, ura−, mtD). After incubation at 25° on V8 juice agar for 11 hr (no hyphae were visible under microscope at this stage), the mating mixture was resuspended in sterile water and plated on the minimum medium SD. Both parental strains JEC34 and JEC50-mtA were plated as negative controls. While neither parental strain grew, prototrophic colonies appeared from the mating mixture. Further analyses of 29 such colonies confirmed that each was prototrophic for adenine and uracil and all contained both *MAT***a** and *MAT*α. All 29 colonies had the mitochondrial genotype from the *MAT***a** parent JEC34; no mtDNA from the *MAT*α parent (JEC50-mtA) was found. Because no filamentous stage was involved in this mating, this result therefore suggests a selective elimination mechanism for mitochondrial inheritance in *C. neoformans*, a process potentially similar to that postulated for *C. reinhardtii*.

DISCUSSION

In this study, we examined the effect of the 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. In addition, we demonstrated that uniparental mtDNA inheritance in *C. neoformans* was likely due to a selective elimination mechanism similar to that postulated for *C. reinhardtii*. To our knowledge, this is the first unambiguous evidence demonstrating that the 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 × CDC46 and JEC21 × CDC46) were analogous to the widely described diploid × haploid (or dikaryon × monokaryon, heterokaryon × homokaryon) matings examined in many filamentous basidiomycete species (*e.g.*, RAPER 1966; CARVALHO *et al.* 1995; 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 reassortment process is also highly efficient in generating new nuclear-mitochondrial combinations in the basidiomycete yeast species *C. neoformans*.

While nuclear and mitochondrial genome reassort-

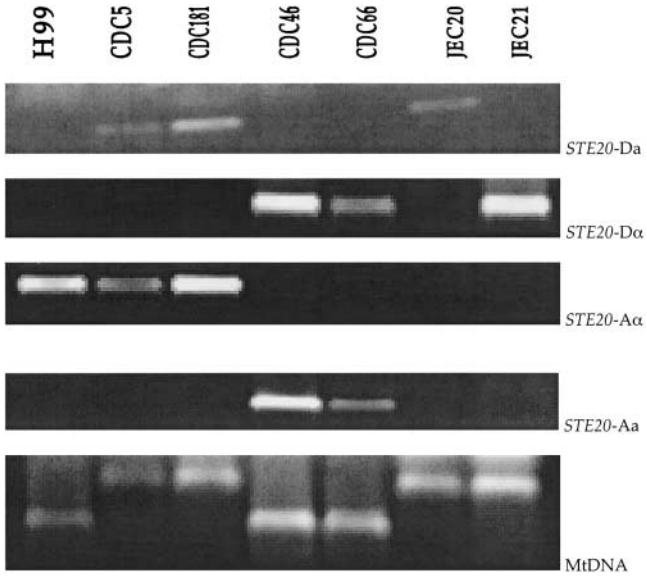

FIGURE 4.—Genotypes at the mating-type locus (four primer pairs) and mitochondrial genome in representative strains of *C. neoformans*. Strain H99 is the model laboratory strain for serotype A. It has the serotype A-specific mtDNA haplotype and Aα allele at the STE20 locus. Strains JEC20 and JEC21 have the serotype D-specific mtDNA haplotypes and the D**a** and Dα alleles, respectively, at the STE20 locus. Strains CDC5 and CDC181 are serotype AD. They have *MAT***a** from a serotype D parent (D**a**) plus a *MAT*α from a serotype A parent (Aα). Their mtDNA genotypes were identical to those of serotype D strains. Strains CDC46 and CDC66 are also serotype AD. However, they have *MAT***a** from a serotype A parent (A**a**) and *MAT*α from a serotype D parent (Dα). Their mtDNA genotypes were identical to those of serotype A strains. Therefore, as shown in Table 4, mitochondria genotypes in serotype AD strains are always consistent with those of the assumed *MAT***a** parent.

ments 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 the 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 does not 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. As our results showed, mitochondria from the *MAT*α parent were selectively eliminated at a very early stage during mating, before the formation of hyphae. On the basis of these findings, basidiospores, blastospores, and hyphae can all be used equally to assess mitochondrial inheritance in *C. neoformans*. Results in Table 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). 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***a** (*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 the mating-type locus in *C. neoformans*. Spontaneous leaky mtDNA inheritance was also reported for uniparental organelle inheritance in the model alga *C. reinhardtii* (BOYTON *et al.* 1987; GILLHAM 1994).

Interestingly, the cross JEC20 × 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 *MAT***a**; therefore, mtDNA from a *MAT***a**/*MAT*α mating partner could also be transmitted to progeny. When CDC46 (the *MAT***a**/α parent) was mated to the *MAT*α parent JEC21, all 22 SSIs inherited mtDNA from CDC46, the strain that contained *MAT***a**. In contrast, the contribution was relatively limited when CDC46 was mated to the *MAT***a** parent JEC20. Most SSIs (~92%) from this cross inherited mtDNA from the parent JEC20 that contained only *MAT***a**.

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 *C. 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 a_2 mating type inherited mitochondria almost exclusively from the a_2 parent. In contrast, progeny with the a_1 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 cellullar 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, 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 control of *C. neoformans* infections await further investigation.

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