

Environment factors can influence mitochondrial inheritance in the fungus *Cryptococcus neoformans*

Zhun Yan, Sheng Sun, Mori Shahid, Jianping Xu *

Department of Biology, McMaster University, 1280 Main Street W, Hamilton, Ont., Canada L8S 4K1

Received 4 May 2006; accepted 4 October 2006

Available online 7 November 2006

Abstract

Cryptococcus neoformans is a model basidiomycete yeast. Strains of this species belong to one of two mating types: mating type a (MATa) or mating type alpha (MAT α). In typical crosses between MATa and MAT α strains, the progeny inherit mitochondria from the MATa parent. However, the underlying mechanisms remain largely unknown. To help elucidate the molecular mechanisms, we examined the effects of four environmental factors on the patterns of mtDNA inheritance. These factors are temperature, UV irradiation, and the addition of either the methylation inhibitor 5-aza-2'-deoxycytidine (5-adc) or the ubiquitination inhibitor ammonium chloride. Except temperature, the other three factors have been shown to influence organelle inheritance during sexual mating in other eukaryotes. Our results indicate that while the application of 5-adc or ammonium chloride did not influence mtDNA inheritance in *C. neoformans*, both UV irradiation and high temperature treatments did. Progeny from a cross involving a high temperature-sensitive mutant with the calcineurin subunit A gene deleted showed biparental mtDNA inheritance in all examined temperatures, consistent with a role of calcineurin and temperature in mtDNA inheritance. Furthermore, the zygote progeny population from a cross performed at a high-temperature environment had a greater variability in their vegetative fitness than that from the same cross conducted at a low temperature. Our results indicate a potentially adaptive role of biparental mtDNA inheritance and mtDNA recombination in certain environments in *C. neoformans*.

© 2006 Elsevier Inc. All rights reserved.

Keywords: *Cryptococcus neoformans*; Environment factors; Mitochondrial inheritance; mtDNA recombination, Biparental mtDNA inheritance; Adaptation

1. Introduction

In the majority of sexual eukaryotes, the mitochondrial DNA (mtDNA) is inherited from only one parent (Gillham, 1994). However, the uniparental inheritance of mtDNA does have exceptions. In some organisms, the transmission of mtDNA is biparental or primarily uniparental with some leakage from the minority parent (Xu, 2005; Yan and Xu, 2005). While the detailed genetic mechanisms for mtDNA inheritance remain poorly understood, preliminary analyses indicated that certain physical (ultra-

violet irradiation) or chemical factors (e.g. methylation inhibitor) could influence the inheritance of chloroplast DNA in the model green alga *Chlamydomonas reinhardtii* (Sager and Ramanis, 1967; Umen and Goodenough, 2001). Whether environmental factors could influence mtDNA inheritance in fungi remains unknown.

To investigate the effects of environmental factors on mtDNA inheritance, a study system with the following features would be ideal: (i) a uniparental mtDNA inheritance; (ii) readily identifiable and neutral genetic markers that distinguish mtDNA genotypes; (iii) strains with isogenic genetic backgrounds but with different mtDNA genotypes; and (iv) the lack of confounding factors such as gamete size differences (Gamete size dimorphism (anisogamy) predominates in higher plants and animals). The basidiomycete

* Corresponding author.

E-mail address: jpxu@mcmaster.ca (J. Xu).

yeast *Cryptococcus neoformans* satisfies all these requirements. Previous studies have established that: (i) mtDNA in *C. neoformans* is inherited from a single parent in typical crosses (Xu et al., 2000), with progeny inheriting mtDNA predominantly from the MAT α parent (Yan and Xu, 2003); (ii) there are abundant mtDNA polymorphisms in this species (Litter et al., 2005; Xu, 2004a; Yan and Xu, 2003; Toffaletti et al., 2004); (iii) there is a series of isogenic strains with auxotrophic markers for both mating types containing different mtDNA genotypes (Yan and Xu, 2003; Yan et al., 2004); and (iv) *C. neoformans* is an isogamous yeast without morphologically differentiated gametes of different sizes, such as those in higher plants and animals.

In anisogamous species such as higher plants and animals, mtDNA is inherited predominantly from the maternal parent. As a result, the mechanism for uniparental mitochondrial inheritance in these species has been traditionally attributed to (i) the failure of mitochondria from the small gamete (e.g. sperm) to enter the large gamete (e.g. egg), or (ii) the relatively smaller number of organelles in the small gamete than those in the larger gamete, even if sperm mitochondria enter the egg during fertilization. However, these two mechanisms are not applicable for explaining uniparental mtDNA inheritance in *C. neoformans* since the gametes from MAT α and MAT α cell are very similar in size.

In a previous paper, we proposed that uniparental mtDNA inheritance in *C. neoformans* might be the result of the MAT α mtDNA being selectively destroyed after cell fusion (Yan and Xu, 2003), similar to the currently favored mechanism to explain uniparental chloroplast inheritance in *C. reinhardtii* (Gillham, 1994). A prerequisite for the selective degradation model is that the mitochondria or mitochondrial DNA from the two gametes must be differentially tagged or recognized. Methylation and ubiquitination are two common mechanisms involved in the recognition process with the methylated DNA or ubiquitinated organelles from one gamete destroyed while those not methylated or ubiquitinated maintained in the progeny. This mechanism was supported by the findings that treatments influencing methylation [such as UV irradiation or treatment with methylation inhibitor 5-aza-2'-deoxycytidine (5-*adc*)] or ubiquitination (such as treatment with ammonium chloride) could influence chloroplast inheritance in *C. reinhardtii* (Sager and Ramanis, 1967; Umen and Goodenough, 2001) and mitochondrial behavior in mammals during mating (Sutovsky et al., 2000), respectively.

To examine the potential role of selective degradation for uniparental mtDNA inheritance in *C. neoformans*, we tested the effects of UV irradiation, 5-*adc*, and ammonium chloride on mitochondrial inheritance in *C. neoformans*. In addition, we also examined the effect of temperature. *C. neoformans* has a global distribution, from the tropics to temperate regions and these areas differ significantly in seasonal temperature dynamics. Therefore, previous laboratory studies using temperatures between 22–25 °C for

mating and mtDNA inheritance investigations might not be representative of those in nature. Our results demonstrated that UV irradiation and high temperature treatments both influenced mitochondrial inheritance. However, treatments with 5-*adc* or ammonium chloride during mating did not. We further examined a high temperature-sensitive mutant and found that crosses involving this strain showed biparental mtDNA inheritance, consistent with the hypothesis that high temperature induces biparental mtDNA inheritance in *C. neoformans*. Our quantitative analysis of progeny fitness suggested that biparental mtDNA inheritance might play an adaptive role in certain environments.

2. Materials and methods

2.1. Strains

Four strains of *C. neoformans* were used in this study. These four strains are all serotype D and their genotypes are presented in (Table 1). Strain YZX1 has the MAT α mating type and requires adenine for growth on minimum SD media. Strain YZX2 is the MAT α counterpart of YZX1 (Yan et al., 2004). Both strains YZX1 and YZX2 have the mitochondrial genotype typical of serotype A strains (mtA) as defined previously by Xu (2002). In contrast, strains JEC43 and MCC3 have the mitochondrial genotype typical of serotype D strains (mtD) and both require uracil for growth (Cruz et al., 2001; Yan et al., 2004). JEC43 has the MAT α mating type while MCC3 has the MAT α genotype. In addition, strain MCC3 has a deletion in the gene coding for subunit A of the calcineurin protein. Calcineurin is required for growth at high temperature (37 °C) as well as for hyphal elongation during mating and haploid fruiting (Odom et al., 1997; Cruz et al., 2001). These four strains are all isogenic except at the loci indicated above (Table 1).

2.2. Crosses between strains JEC43 and YZX2 to determine the effect of environmental factors on mtDNA inheritance in *C. neoformans*

Prior to the mating experiments, two parental strains JEC43 and YZX2 were first inoculated on YPD agar (1% Bacto Yeast Extract, 2% Bacto Peptone, 2% Dextrose; 1.8% agar) and incubated at 22 °C for 3 days. Cells were then scraped off the plates and resuspended in 500 μ l of sterile distilled water. Cell densities were adjusted to OD₆₀₀ = 0.5. Equal volumes of suspension (400 μ l) from the two parental strains in each cross were mixed thoroughly in a new tube

Table 1
Strains used in this study and their genotypes

Strain	Genotype	Source
JEC43	MAT α <i>ura5</i> mtD	Jeff Edman
MCC3	MAT α <i>ura5</i> <i>cna1</i> mtD	Cruz et al. (2001)
YZX1	MAT α <i>ade2</i> mtA NEO	Yan et al. (2004)
YZX2	MAT α <i>ade2</i> mtA NEO	Yan et al. (2004)

through vortex. Small aliquots (20 μ l) of the cell mixture were then plated on selected mating media and incubated at various conditions (see below for details). At least four repeats were done for each individual treatment. Single parental strains were plated as negative controls for screening and mtDNA genotyping.

2.2.1. UV irradiation

To determine the effects of UV irradiation on mtDNA inheritance, cell suspensions were plated on V8-juice agar [5% V8 vegetable juice (Campbell Soup Co.), 0.5 g/l KH_2PO_4 , 4% agar and pH 7.2], a common medium for mating in *C. neoformans*. These plates were then irradiated at fluorescent rate of 2.1 J/m²/s using a germicidal bulb emitting at 254 nm in a dark room. Four exposures were included in our analysis: 0, 11, 22, and 45 s. These dosages included the upper and lower limits of UV exposure that were effective at influencing the inheritance pattern of chloroplast DNA in the green alga *C. reinhardtii* (Sager and Ramanis, 1967). After UV exposure, the V8-juice agar plates were covered by aluminum foil and incubated 22°C for 16–24 h before mating products were screened on selection medium.

2.2.2. 5-*adc* treatment

To test the effect of 5-aza-2'-deoxycytidine (5-*adc*) on mtDNA inheritance in *C. neoformans*, cells of YZX2 and JEC43 were prepared differently from those for UV irradiation. The parental cells were first grown separately on YPD medium supplemented with the selected concentrations of 5-*adc* for 3 days at 22°C. Four concentrations were analyzed in our study: 0, 50, 250, and 500 μ M. The concentrations around 200 μ M were previously found to influence the inheritance pattern of chloroplast DNA in the green alga *C. reinhardtii* (Umen and Goodenough, 2001). Cell suspensions and mating mixtures were then prepared as described above. The mating mixtures were then plated on V8-juice agar medium supplemented with the corresponding concentrations of 5-*adc*, i.e. to V8-juice agar plates with 0, 50, 250, and 500 μ M of 5-*adc*, respectively. Plates were incubated at 22°C for 16–24 h before mating products were screened on selection medium.

2.2.3. Ammonium chloride treatment

Similar to cell preparations during the treatment of 5-*adc*, strains YZX2 and JEC43 were first plated on YPD medium supplemented with selected concentrations of ammonium chloride (0, 10, and 100 mM) and allowed to grow for 3 days at 22°C. Sutovsky and his colleagues (2000) reported that the use of 10 mM of ammonium chloride to treat fertilized mammal eggs could prevent the degradation of sperm mitochondria. The treated cells were resuspended and the mating mixtures were prepared as described above. The mating mixtures were then plated on V8-juice agar medium supplemented with the corresponding concentrations of ammonium chloride. Plates were

incubated at 22°C for 16–24 h before mating products were screened on selection medium.

2.2.4. Temperature treatment

To determine the effects of temperature on mtDNA inheritance, cell mixtures were prepared as described above and plated on V8-juice agar medium, the same as those for UV irradiation treatments. These agar plates were then incubated at temperatures 13, 22, 28, and 33°C, respectively, for 16–24 h. In a pilot experiment, we found no mating occurred at temperatures of 34°C and above.

2.2.5. Selecting for diploid mating products

After mating, individual cell mixtures were scraped off the agar plates, re-suspended in sterile distilled water, and spread-plated on SD minimal medium without amino acids (Difco) to select for diploid mating products. Single colonies of diploids could usually be seen after 2–3 days of incubation at 37°C. Single parental strains were used as negative controls in all mating experiments. Neither parental strain grew by themselves on the minimum selection medium in any of the above treatments.

2.2.6. Determining progeny mtDNA genotype

The diploid mating products were randomly picked and their mitochondrial genotypes were directly determined by PCR, without first extracting their DNA, using primers flanking the *ND5* gene [Forward (5' \rightarrow 3'): CTATTGG TGTTACAGGAGCTCAC; Reverse (5 \rightarrow 3'): GAGCCT TCATACCTGCCTTATTTGC] (Toffaletti et al., 2004). In the intron-less *ND5* gene of the mtA-type mitochondrial genome (strains YZX1 and YZX2), this pair of primers amplifies a fragment of 435 bp. In contrast, in the intron-containing *ND5* gene of the mtD-type mitochondrial genome (strains MCC3 and JEC43), this primer pair amplifies a fragment of 1250 bp. In this assay, progeny containing both fragments are scored as heteroplasmic. To determine whether heteroplasmy results in mtDNA recombination, multiple single-celled colonies from one heteroplasmic clone were analyzed using two additional polymorphic markers at *ND2* and *ND4* genes. These markers have shown previously capable of discriminating the mtA and mtD mitochondrial genotypes used in this study (Yan et al., 2004). The procedures of mtDNA genotyping at *ND2* and *ND4* loci followed those described in Yan et al. (2004).

Each PCR reaction contains 2 mM MgCl_2 , 0.05 mM of each dNTP, 0.8 μ M each primer, and 0.05 U of Taq polymerase (Invitrogen) in a total of 15 μ l of volume. A small number of cells ($\sim 10^4$) were directly added to the PCR reaction tube. The PCR reaction conditions were 4 min at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2.5 min at 72°C; and finally, 7 min of extension at 72°C. PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide for visualization and genotype scoring.

Table 2
Effects of environmental factors on mitochondrial DNA inheritance in *C. neoformans*

Treatment	Dosage or concentration	No. progeny with mtDNA from the MATa parent	No. progeny with mtDNA from the MAT α parent	No. progeny with mtDNA from both parents	Percent leakage (% progeny with mtDNA from MAT α parent)
UV irradiation	(In seconds)				
	0	206	13	5	8.0
	11	70	20	2	23.9
	22	89	66	8	45.4
	45	91	78	6	48
5-adc	(In μ M)				
	0	69	2	1	4.2
	125	59	1	0	1.7
	250	57	3	0	5.0
	500	70	1	1	2.8
(NH ₄)Cl	(In mM)				
	0	59	1	0	1.7
	10	58	1	1	3.3
	100	59	0	1	1.7
Temperature	(In $^{\circ}$ C)				
	13	151	7	3	6.8
	22	103	4	0	3.9
	28	98	11	8	16.2
	33	97	73	14	47.3

2.3. The effect of a high temperature-sensitive mutation on mtDNA inheritance in *C. neoformans*

In the above experiments, a high temperature environment was found to induce mtDNA leakage and biparental mtDNA inheritance in *C. neoformans* (see detailed results below). Therefore, we hypothesized that a strain with a defect in growth at high temperature should also have a significant leakage and show biparental mtDNA inheritance. To test this hypothesis, we crossed strains MCC3 and YZX1 and incubated them at different temperatures following the procedure described above (see “Temperature treatments”). In this experiment, four temperatures were examined: 22, 26, 28, and 33 $^{\circ}$ C. In addition, aside from the ND5 marker, an additional polymorphic locus, ND2, capable of discriminating the mtA and mtD genomes were also included to screen the entire zygote populations (Yan et al., 2004).

2.4. Vegetative fitness determination

Fitness of zygotes was determined using a protocol similar to that described by Xu (2004a). Briefly, zygote colonies were randomly picked from among the mating products on SD medium and zygote cells were resuspended in sterile distilled water through vigorous vortexing. These cells were then streaked onto two different agar media, YPD and SD, for single colonies with two repeats for each medium. Plates were then incubated for 48 h at two different temperatures, one set of plates at 25 $^{\circ}$ C and another set at 37 $^{\circ}$ C. In total, the fitness of each randomly picked zygote was examined in four different environments: YPD/37 $^{\circ}$ C, YPD/25 $^{\circ}$ C, SD/37 $^{\circ}$ C, and SD/25 $^{\circ}$ C. Five random colonies were measured for each zygote in each of the four incubating environments using an ocular meter under a microscope. The mean, standard deviation and co-efficient of variation are calculated

for each treatment in each testing environment according to the methods described in Xu (2004a).

3. Results

3.1. UV irradiation increases leakage and biparental mtDNA inheritance in *C. neoformans*

Similar to results from previous studies, the mating mixtures not exposed to any UV irradiation produced progeny containing mitochondrial genome predominantly from the MATa parent (Table 2), with about 8% leakage from the MAT α parent. However, mating mixtures exposed to UV irradiation had significantly increased leakage, similar to what was observed for the inheritance of chloroplast DNA in *C. reinhardtii* (χ^2 test, $p < 0.01$, Table 2). In addition, there seemed a positive correlation between UV exposure and mtDNA leakage within the tested range of exposure times. In pairwise comparisons in the degree of leakage between different exposure times, the 11 s exposure had a significantly greater leakage than no UV exposure. In turn, both the 22 and 45 s exposures had greater leakages than both the 11 s exposure and no exposure treatments ($p < 0.01$ for all χ^2 test). However, no difference was observed between UV exposure times of 22 and 45 s. These two exposure times resulted in almost equal proportions of progeny with mtDNA from the MATa and MAT α parents (Table 2).

3.2. 5-*adc* has no effect on mitochondrial inheritance

In *Chlamydomonas*, the use of methylation inhibitor 5-*adc* at concentrations around 200 μ M during mating significantly altered the chloroplast inheritance pattern from uniparental to biparental (Umen and Goodenough, 2001). However, in this study, we found that 5-*adc* treatment at a similar concentration had little effect on mitochondrial

inheritance in *C. neoformans* ($p > 0.1$, χ^2 test). Reducing the 5-*adc* concentration to 125 μM or increasing its concentration to 500 μM did not change the original mtDNA inheritance pattern (Table 2).

3.3. Ammonium chloride has no effect on mitochondrial inheritance

Sutovsky and his colleagues (2000) reported that the use of 10 mM of ammonium chloride in the fertilized mammal eggs could prevent the degradation of sperm mitochondria. However, our results showed that ammonium chloride had little effect on mtDNA inheritance in *C. neoformans* ($p > 0.2$, χ^2 test). Progeny from mating on V8-juice medium containing various concentrations of ammonium chloride inherited predominantly mtDNA from the MAT α parent (Table 2). Increasing the concentration of ammonium chloride to ten times of that effective in mammalian eggs (100 mM) did not change the mtDNA inheritance pattern in *C. neoformans* (Table 2).

3.4. High temperature treatment results in increased leakage and biparental mtDNA inheritance

Four different temperatures were tested for their effects on mtDNA inheritance: 13, 22, 28, and 33 °C. A pilot experiment identified that temperatures at 34 °C and above did not produce any zygotes (Yan and Xu, 2005). Our results identified that mating at 33 °C produced a zygote progeny population with a significantly increased mtDNA leakage compared to those from mating at 22 to 28 °C (χ^2 test, $p < 0.01$ in both comparisons). Similarly, the 28 °C environment produced a zygote population with a slightly higher mtDNA leakage than that in the 22 °C environment ($p < 0.05$, Table 2). In contrast to the increased leakages observed at high temperatures, the low temperature environment of 13 °C produced progeny with a similar rate of leakage as that at 22 °C ($p > 0.1$, Table 2).

3.5. Abundant mtDNA recombinants from a heteroplasmic zygotic colony

To examine whether mtDNA recombination occurs in above crosses that resulted in heteroplasmic zygotes, we isolated 50 mitotic segregants from a heteroplasmic colony and determined their mtDNA genotypes at two additional polymorphic loci, ND2 and ND4 based on protocols

described previously in Yan et al. (2004). Our analysis identified that at these two loci, among the 50 mitotic segregants, seven had mtDNA from the MAT α parent, 20 were from the MAT α parent and 23 were recombinants. Thus, this result indicates that biparental mtDNA inheritance as revealed by the ND5 marker gene described above includes mtDNA recombination.

3.6. Crosses involving a high temperature-sensitive mutant show biparental mtDNA inheritance and recombination

Since high temperature environments showed significantly increased leakage and biparental mtDNA inheritance, we hypothesized that strains with a defect in high-temperature growth should show increased leakage and biparental mtDNA inheritance even at low temperatures. Indeed, our experiment confirmed that mating involving a high-temperature sensitive strain MCC3 showed biparental mtDNA inheritance at three of the four tested temperatures (22, 26, and 28 °C) (Table 3). In this cross (MCC3 \times YZX1), the 33 °C mating environment produced no zygote in our experiment. In all three treatments that produced zygotes, recombinant mitochondrial genotypes were also observed (Table 3).

3.7. Fitness consequences of biparental mtDNA inheritance

To examine the potential consequences of biparental mtDNA inheritance in *C. neoformans*, we determined the vegetative fitness of progeny zygotes from crosses conducted at two different temperatures that showed significant differences in mtDNA leakage: 28 and 33 °C temperature environments. Progeny from treatments with UV irradiation were not examined here because UV irradiation is mutagenic and that different doses can have differentially adverse effects on progeny fitness. Our analysis identified that in three of the four fitness-testing environments (YPD/37 °C, YPD/25 °C, and SD/25 °C), the mean vegetative fitness for progeny zygotes from mating at 33 °C was greater than those from mating at 28 °C (Table 4). The reverse was true for testing environment SD/37 °C. Interestingly, the co-efficient of variation was greater in all four fitness-testing environments for the progeny population derived from mating at 33 °C than that from mating at 28 °C (Table 4). Though not all of these differences were statistically significant, our results are consistent with the hypothesis that and biparental mtDNA inheritance and

Table 3
mtDNA inheritance from crosses between a high temperature-sensitive mutant MCC3 and YZX1 at different temperatures

Temperature (°C)	No. progeny with mtDNA from the MAT α parent	No. progeny with mtDNA from the MAT α parent	No. progeny with mtDNA from both parents	No. progeny with recombinant mtDNA genotypes	Percent of heteroplasmic and recombinant progeny
22	15	17	0	1	3.03
26	12	18	5	1	16.67
28	15	15	4	2	16.67
33	0	0	0	0	N/A

Table 4
Fitness distribution of zygote populations from crosses conducted in two different temperatures between strains JEC43 and YZX2

Fitness testing environments	Mating environment			
	28 °C (N = 39) ^a		33 °C (N = 29)	
	Mean (±SD) ^b	CV ^c	Mean (±SD)	CV
YEPD/37 °C	2.0418 (±0.3754) ^d	0.1838	2.2400 (±0.4327) ^{ns}	0.1931
YEPD/25 °C	1.5927 (±0.2567)	0.1612	1.9667 (±0.3192) ^{***}	0.1623
SD/37 °C	1.4179 (±0.2142)	0.1511	1.1793 (±0.1965) ^{**}	0.1666
SD/25 °C	0.4834 (±0.1362)	0.2817	0.8310 (±0.2608) ^{***}	0.3138

^a N, the number of progeny zygotes analyzed.

^b SD, standard deviation.

^c CV, co-efficient of variation that refers to the ratio of standard deviation over mean.

^d Values are in ocular units ($\times 10$) measured under the microscope with 100 \times magnification.

^{ns} Not significantly different between 28 and 33 °C mating environments, $p > 0.05$.

^{**} Significantly different between 28 and 33 °C mating environments, $p < 0.01$.

^{***} Significantly different between 28 and 33 °C mating environments, $p < 0.001$.

recombination are adaptive in certain natural environments.

4. Discussion

This study examined whether certain external physical and chemical factors could influence mitochondrial inheritance in the model basidiomycete yeast *C. neoformans*. The results demonstrated that among the four tested environmental factors, UV irradiation and high temperature increased leakage of mtDNA from the MAT α parent into the progeny population. In several treatments, there were almost equal representations of both the MAT α and MAT α mtDNA genotype in the progeny population. To our knowledge, this is the first demonstration that environmental physical and chemical factors can influence mtDNA inheritance in fungi.

In a previous study, we proposed that during mating in *C. neoformans*, mitochondrial DNA from the MAT α parent might be selectively tagged and destroyed soon after cell fusion to result in uniparental mtDNA inheritance from the MAT α parent (Yan and Xu, 2003). Methylation and ubiquitination are two common pathways involved in the recognition and degradation of subcellular materials. In *Chlamydomonas* and mammals, methylated DNA or ubiquitinated organelles were found selectively degraded while those not methylated or ubiquitinated were maintained in the progeny (Sutovsky et al., 2000; Umen and Goodenough, 2001). Surprisingly, we found neither the methylation inhibitor 5-*adc* nor the ubiquitination inhibitor ammonium chloride had any effect on mitochondrial inheritance in *C. neoformans*. The result thus suggests one of three possibilities: (i) that selective tagging and degradation does not play a role in mtDNA inheritance in *C. neoformans*; (ii) that selective tagging and degradation may play a role but unlike those for algae and mammals, these two compounds are ineffective at preventing methylation or ubiquitination in *C. neoformans*; and (iii) there might be an alternative mechanism different from those in algae and mammals for the selective tagging and degradation of mitochondria or mtDNA in *C. neoformans*.

In contrast to the results from treatments with 5-*adc* and ammonium chloride, treatments with high temperature and UV irradiation were found to significantly increase leakage of mtDNA from the MAT α parent into the zygote progeny population. The effect of high temperature on mtDNA inheritance was further supported by results from crosses involving a high temperature-sensitive mutant MCC3 that had a deletion in the gene coding for calcineurin subunit A. Interestingly, calcineurin is also required for conjugation tube formation and for hyphal elongation during mating (Cruz et al., 2001). On filamentation agar inoculated with wild type MAT α and MAT α cells in close proximity to each other, in response to pheromones from opposite mating types, MAT α cells may enlarge and occasionally form short conjugation tubes while MAT α cells form long conjugation tubes (Cruz et al., 2001). After cell fusion, the MAT α nuclei migrate into MAT α cells while the nuclei from the MAT α cells do not move (McClelland et al., 2004). Whether a similar process happens in a mating mixture is not known. However, it is possible that during this process, most of the mitochondria from the MAT α cells were left behind and not completely mixed with the mitochondria from the MAT α parent, similar to those observed in several filamentous basidiomycetes (Yan and Xu, 2005). If so, the diploid zygotes would contain mitochondria from only the MAT α parent (Yan and Xu, 2003). Hyphal elongation of the newly formed zygote away from the fusion zone could further reduce the cytoplasmic contribution of the MAT α parent to the zygote, thus contributing to uniparental mtDNA inheritance from the MAT α parent. Consequently, defects in both conjugation tube formation and hyphal elongation would contribute to leakage and biparental mtDNA inheritance (Yan et al., 2004). Strain MCC3 is unable to undergo hyphal elongation after mating (Cruz et al., 2001). Therefore, any cytoplasmic contribution from the MAT α parent might be maintained in the newly formed zygote. Consistent with the hypothesis, crosses involving a strain with a deletion in the *SXII α* gene had seriously impaired hyphal growth during mating (Hull et al., 2002) and also showed biparental mtDNA inheritance (Yan et al., 2004). In addition, we were unable to observe any conjugation tubes or

hyphal elongation in crosses performed at 33 °C or with UV irradiation in our tested time periods (<24 h).

Aside from the environmental factors discussed above, other genetic and potentially non-genetic factors may also contribute to leakage and biparental mtDNA in *C. neoformans* and other fungi. For example, in the study by Toffaletti et al. (2004), different strain combinations of *C. neoformans* showed different patterns of mtDNA inheritance. Specifically, in the cross between JEC20 (serotype D, MAT α) and H99 (serotype A, MAT α), all 20 hybrid progeny inherited mtDNA from the MAT α parent JEC20. This result was identical to those observed by Xu et al. (2000) in that all 570 progeny from six independent crosses between JEC20 and six serotype A, MAT α strains inherited mtDNA from the MAT α parent JEC20. Crosses between typical strains of serotype D also showed uniparental mtDNA inheritance (Yan and Xu, 2003). However, in a cross between JEC21 (an isogenic strain of JEC20 but is MAT α) and KN99a (an isogenic strain of H99 but is MAT α), among the 22 analyzed hybrid progeny, 16 inherited mtDNA from the MAT α parent, 2 from the MAT α parent, and 4 were recombinants (Toffaletti et al., 2004). This laboratory result by Toffaletti et al. (2004) was different from the analyzed natural hybrids in that all 21 natural serotype AD hybrids inherited mtDNA only from the MAT α parent, regardless of their serotype and mating type combinations (Xu et al., 2002; Yan and Xu, 2003).

Regardless of the potential underlying molecular mechanisms for uniparental and biparental mtDNA inheritance, our observation that external physical factors can influence the relative frequencies of uniparental and biparental mtDNA inheritance suggests a potentially adaptive role of mtDNA inheritance for *C. neoformans* in natural environments. Indeed, we found that the mating environment (33 °C) with a greater mtDNA leakage showed a greater variation in vegetative fitness in the zygote progeny population than that from an environment (28 °C) with a lower level mtDNA leakage. Since the progeny zygotes analyzed here were selected directly from among the mating products without going through meiosis, each zygote should contain the full nuclear genome complements from both mating partners. As a result, the nuclear genomes of all analyzed zygotes should be identical and the observed differences in fitness variation must have come from the mitochondrial genome or the interaction between the mitochondrial genome and the environments. The greater fitness variation seen among zygote progeny from the 33 °C mating environment should allow more efficient natural selection and faster rate of adaptation in different environments (Fisher, 1958).

Evolutionarily, a mixture of uniparental and occasionally biparental mtDNA inheritance and recombination might help avoid Muller's Ratchet of irreversible fitness loss due to mutation accumulation in completely asexual genomes (Xu, 2004a; Xu, 2004b). In stressful environments such as UV irradiation, biparental inheritance and recombination in the mitochondrial genome might be

particularly adaptive and advantageous because these environments could elevate mutation rate and generate significant deleterious mutations. Having biparental mtDNA inheritance and recombination would allow mutations accumulated in different lineages to be combined into the same lineage so that deleterious mutations may be purged more quickly and advantageous mutations may be combined and selected more efficiently (Xu, 2004b). Indeed, this seemingly adaptive mitochondrial inheritance strategy found here in *C. neoformans* might be widespread among many groups of eukaryotes. In recent years, an increasing number of studies have reported mitochondrial recombination in natural populations of fungi (Saville et al., 1998), plants (Städler and Delph, 2002) and animals (Zouros et al., 1992; Ladoukakis and Zouros, 2001; Kraysberg et al., 2004) (for a recent review, see Xu, 2005).

Acknowledgments

This research is supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada, the Premier's Research Excellence Award, Genome Canada, the Canadian Foundation for Innovation, and the Ontario Innovation Trust.

References

- Cruz, M.C., Fox, D., Heitman, J., 2001. Calcineurin is required for hyphal elongation during mating and haploid fruiting in *Cryptococcus neoformans*. *EMBO J* 20, 1020–1032.
- Fisher, R.A., 1958. *The Genetic Theory of Natural Selection*. Dover Publications, New York.
- Gillham, N.W., 1994. *Organelle Genes and Genomes*. Oxford University Press, New York.
- Hull, C.M., Davidson, R.C., 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.
- Kraysberg, Y., Schwartz, M., Brown, T.A., Ebralidse, K., Kunz, W.S., Clayton, D.A., Vissing, J., Khrapko, K., 2004. Recombination of human mitochondrial DNA. *Science* 304, 981.
- Ladoukakis, E.D., Zouros, E., 2001. Direct evidence for homologous recombination in mussel (*Mytilus galloprovincialis*) mitochondrial DNA. *Mol Biol Evol* 18, 1168–1175.
- Litter, J., Keszthelyi, A., Hamari, Z., Pfeiffer, I., Kucsera, J., 2005. Differences in mitochondrial genome organization of *Cryptococcus neoformans* strains. *Antonie Van Leeuwenhoek* 88, 249–255.
- McClelland, C.M., Chang, Y.C., Varma, A., Kwon-Chung, K.J., 2004. Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends Microbiol* 12, 208–212.
- Odom, A., Muir, S., Lim, E., Toffaletti, D.L., Perfect, J.R., Heitman, J., 1997. Calcineurin is required for virulence in *Cryptococcus neoformans*. *EMBO J* 16, 2576–2589.
- Sager, R., Ramanis, Z., 1967. Biparental inheritance of nonchromosomal genes induced by ultraviolet irradiation. *Proc Natl Acad Sci USA* 58, 931–937.
- Saville, B.J., Kohli, Y., Anderson, J.B., 1998. mtDNA recombination in a natural population. *Proc Natl Acad Sci USA* 95, 1331–1335.
- Städler, T., Delph, L., 2002. Ancient mitochondrial haplotypes and evidence for intragenic recombination in a gynodioecious plant. *Proc Natl Acad Sci USA* 99, 11730–11735.

- Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C., 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., Perfect, J.R., 2004. *Cryptococcus neoformans* mitochondrial genomes from serotype A and D strains do not influence virulence. *Curr Genet* 46, 193–204.
- Umen, J.G., Goodenough, U.W., 2001. Chloroplast DNA methylation and inheritance in *Chlamydomonas*. *Genes Dev* 15, 2585–2597.
- Xu, J., 2002. Mitochondrial DNA polymorphisms in the human pathogenic fungus *Cryptococcus neoformans*. *Curr Genet* 41, 43–47.
- Xu, J., 2004a. Genotype-environment interactions of spontaneous mutations for vegetative fitness in the human pathogenic fungus *Cryptococcus neoformans*. *Genetics* 168, 1177–1188.
- Xu, J., 2004b. The prevalence and evolution of sex in microorganisms. *Genome* 47, 775–780.
- 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., Mitchell, T.G., 2000. Uniparental mitochondrial transmission in sexual crosses in *Cryptococcus neoformans*. *Curr Microbiol* 40, 269–273.
- Xu, J., Luo, G., Vilgalys, R., Brandt, M.E., Mitchell, T.G., 2002. Multiple origins of hybrid strains of *Cryptococcus neoformans* with serotype AD. *Microbiology* 148, 203–212.
- Yan, Z., Xu, J., 2003. Mitochondria are inherited from the MATa parent in crosses of the basidiomycete fungus *Cryptococcus neoformans*. *Genetics* 163, 1315–1325.
- Yan, Z., Xu, J., 2005. Fungal mitochondrial inheritance and evolution. In: Xu, J. (Ed.), *Evolutionary Genetics of Fungi*. Horizon Bioscience, Wymondham, Norfolk, UK, pp. 221–252.
- Yan, Z., Hull, C.M., Heitman, J., Sun, S., Xu, J., 2004. *SXI1alpha* controls uniparental mitochondrial inheritance in *Cryptococcus neoformans*. *Curr Biol* 14, R743–R744.
- Zouros, E., Freeman, K.R., Ball, A.O., Pogson, G.H., 1992. Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel *Mytilus*. *Nature* 359, 412–414.