**Mitochondrial DNA Inheritance in the Human Fungal Pathogen *Cryptococcus gattii***

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**ABSTRACT**

 The inheritance of mitochondrial DNA (mtDNA) is predominantly uniparental in most sexual eukaryotes, including the human pathogenic basidiomycetous yeast *Cryptococcus neoformans*. In this study, we examined the mitochondrial inheritance pattern of *Cryptococcus gattii,* a closely - related sister species of *C. neoformans* responsible for the recent and ongoing outbreak of Cryptococcalinfections in the US Pacific Northwest and British Columbia (especially Vancouver Island) in Canada. Using molecular markers, we analyzed the inheritance of mitochondrial DNA in 9 crosses between strains within and between divergent lineages in *C. gattii*. Our analyses identified significant variations in mtDNA inheritance patterns among strains and crosses. For two of the crosses that showed uniparental mitochondrial inheritance in standard laboratory conditions, we further investigated the effects of the following environmental variables on mtDNA inheritance: UV exposure, temperature, exposure to the methylation inhibitor 5-aza-2’-deoxycytidine, and exposure to the ubiquitination inhibitor ammonium chloride. Interestingly, one of these crosses showed no response to these environmental variables while the other exhibited diverse patterns ranging from complete uniparental inheritance of the *MATa* parent mitochondria, to biparental inheritance, and to complete uniparental inheritance of the *MATalpha* parental mitochondria. Our analyses indicated that the mtDNA inheritance pattern in *C. gattii* differs significantly from its closely related species *C. neoformans.* We discussed possible mechanisms underlying these divergent mitochondrial DNA inheritance pattern.

**THESIS FORMAT**

This thesis is a result of Amanda Wilson and Zixuan Wang’s cooperative work. Amanda Wilson analyzed most of the crosses in standard laboratory conditions.I took responsibility for detecting whether environment factors can influence mitochondrial DNA inheritance in *C. gattii.*

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**INTRODUCTION**

The mitochondrion is the principal energy-producing organelle within most eukaryotes. Mitochondrion is the power plant of the cell and houses many important biosynthetic pathways (Newmeyer and Ferguson-Miller 2003). The most significant function of this organelle is the production of adenosine triphosphate (ATP) from chemical energy via oxidative phosphorylation.

The importance of mitochondrial genome is manifested by its link to many important biological characters including aging (Miquel 1980; Basse 2010), apoptosis (Green and Reed 1998), drug resistance (Brun 2005; Cheng 2007), virulence (Olson and Stenlid 2001), nuclear genome stability (Dirickert al. 2013), metabolite homeostasis and male sterility (Saumitou-Laprade et al. 1994). In human, mitochondrial DNA mutation contribute to the pathogenesis of many degenerative diseases (Taylor and Turnbull 2005) and have been widely observed in many diabetes (Lowell and Shulman 2005), neurodegenerative disorders (Lin and Beal 2006), and cancers ( Chatterjee *et al.* 2006; Brandon *et al.* 2006; Fliss *et al.* 2000).

In the majority of sexual eukaryotes, a zygote will usually inherit equal contributions of paternal and maternal genetic makeup for nuclear genes (Birky 2001; Xu 2005). Unlike other organelles, mitochondrion contains its own genome and mitochondrial inheritance is predominantly uniparental, with the zygote receiving mitochondrial DNA (mtDNA) from the maternal parent (Xu 2005). Although uniparental mitochondrial inheritance is the predominant pattern, paternal and biparental patterns of inheritance have also been observed in some organisms (Jannotti - Passos *et al.* 2001; Schwartz and Vissing 2002; Xu 2005; Yan and Xu 2005). For example, in the model organism, *Sacharomyces cerevisiae*, progeny can inherit mtDNA from both parents. At present, the detailed genetic mechanisms govern mtDNA inheritance remain poorly understood.

There are 37 species in the fungal genus *Cryptococcus,* of which only two (*C*. *gattii* and *C*. *neoformans*) are relevant to the majority of clinical infections in humans (Casadevall and Perfect, 1998; Heitman et al., 2011; Perfect and Casadevall 2011). These basidiomycete yeasts infect hosts by first colonizing the lungs of susceptible individuals and then spreading to multiple organs, with dissemination to the central nervous system causing the most problematic symptoms, especially in immune-compromised patients. The majority of cryptococcosis cases are caused by *C. neoformans*, and this species is distributed worldwide, often found in association with bird guano (Jarvis and Harrison, 2007; Mitchell and Perfect, 1995). There are two serotypes (A and D) or varieties (var. *grubii* and var. *neoformans*) in *C. neoformans* and they have become model organisms for fungal molecular genetics and pathogenesis research. In contrast, a closely related species, *C. gattii* (serotypes B and C), is clinically rare, causing <1% of global cryptococcosis cases and is largely limited to tropical and subtropical regions (Chaturvedi et al., 2011; Springer et al., 2012). Compared to *C. neoformans*, relatively little is known about *C*. *gattii,* until recently when it was found to cause a significant number of infections in humans as well as both wild and domesticated animals in the temperate region of the west coast of North America.

Historically, *Cryptococcus gattii* was considered as a varietal form of its closest relative, *Cryptococcus neoformans*. However based on differences in ecology, epidemiology, biochemical and molecular characteristics, *C. gattii* was defined as a separate species from *C. neoformans* (Kwon-Chung and Varma 2006) and together the form the *Cryptococcus neoformans* species complex CNSC (Xu 2014). One of the defining differences between *C. gattii* and *C. neoformans* is the susceptible host range. *C. gattii* is capable of infecting healthy humans, whereas *C. neoformans* is mostly found to infect immune-compromised individuals, and as such *C. neoformans* is commonly referred as an opportunistic pathogen (Rozenbaum and Goncalves1994) while *C. gattii* a primary pathogen.

Within *C. gattii* there are four well-established lineages or four distinct molecular types (VGI, VGII, VGIII and VGIV), which are genetically distant from each other. These evolutionary divergent lineages were established through multi-locus sequence typing. Among the four lineages, VGI and VGIII are the most closely related with VGIV being the next closely related to them and VGII being the most distantly related to all other lineages (Bovers *et al.* 2008). The VGII lineage is prevalent in the environments of South America, the Pacific Northwestern United States and Vancouver Island, Canada (Kidd *et al.* 2007). VGII is also the second most prevalent lineage in Australia, but mostly in the northern and western regions (Ellis *et al.* 2000). The VGI lineage is the most prevalent in Australia and India accounting for the majority of environmental and clinical isolates (Ellis *et al.* 2000; Chowdhary *et al.* 2011). VGI has also been found in northwestern United States and Canada (Kidd *et al.* 2007). VGIII is found commonly in Columbia and India as well as southwest United States, while VGIV is rare and found mainly in Africa and Central America (Campbell *et al.* 2005; Meyer *et al.* 2003; Xu *et al.* 2011).

Mitochondrial inheritance in *C. neoformans* is uniparental under normal mating conditions (Yan and Xu 2003). In *C. neoformans*, there are two mating types: *MATa* and *MATalpha* (Kwon-Chung, 1976). Progeny from mating between wild type strains within and between the two varieties predominantly or exclusively inherit mitochondrial DNA from the *MATa* parent (Xu et al. 2000; Yan and Xu 2003), with the sex-determining genes *sxi1*α and *sxi2a* playing determining roles. Similar to *C. neoformans*, mating in *C. gattii* is also controlled by a single locus with two alleles, *MATa* and *MATalpha* (Kwon-Chung 1976). However, the pattern of mtDNA inheritance has not been examined in *C. gattii*. Given their close evolutionary relationship, we hypothesize that mtDNA in *C. gattii* should also be inherited from the *MATa* parent. In addition, experiments conducted by Yan et al. (2007) showed that environmental factors such as high temperature and UV irradiation could impact mitochondrial inheritance in *C. neoformans*. These factors were found to increase leakage from the MATα parent, which in some cases resulted in biparental mtDNA inheritance (Yan *et al.* 2007). These environmental factors can be considered as stressors to the organisms and the increased leakage and biparental mtDNA inheritance was hypothesized to be a response by the organism to increase mitochondrial genetic variation among the progeny (Yan *et al.* 2007). Adaptive evolution is shaped by the interaction of population genetics, natural selection and essential network and biochemical constraints. Variation can be generated by mutations (Olson et al. 2012). This observation also raised an intriguing possibility that mitochondrial inheritance may play a role in the adaptive evolution of CNSC (*Cryptococcus neoformans* Species Complex), including its spread in natural environments and its virulence in humans. The recent outbreaks of *C. gattii* infections in British Columbia in Canada and the Pacific Northwest of the United States (Datta *et al.* 2009) also highlight the importance of understanding the mechanism(s) for the range of expansion and virulence in *C. gattii* and other emerging pathogens.

In this study, we examined the mitochondrial inheritance in *C. gattii* using genetically diverse strains that belonged to different evolutionary lineages. We examined crosses within and between lineages of *C. gattii.* Crosses between these lineages are analogous to hybrid crosses since the strains found in one lineage of *C. gattii* are genetically different from those in another lineage (Litvintseva *et al.* 2011).

In addition, we examined the effects of four environmental factors (temperature, UV irradiation, methylation inhibitor 5-aza-2’-deoxycytidine, ubiquitination inhibitor ammonium chloride on their mtDNA inheritance. Two of these four factors, UV irradiation and temperature were found to influence mtDNA inheritance in *C. neoformans.* However, 5-adc and ammonium chloride showed no effect at a wide range of concentrations that have previously shown to influence mtDNA inheritance in animals. We hypothesized that the mtDNA inheritance pattern in *C. gattii* and its response to environmental factors should be similar to that in *C. neoformans*. However, our analyses identified significant differences between these two closely related species in their mtDNA inheritance.

**2. MATERIALS AND METHODS**

**Strains**

Twelve *Cryptococcus gattii* strains were used in mitochondrial inheritance analyses (Table 1). Strains B4545, B4492, and B4495 belong to the VGI lineage; strainsLA55n, LA61n, and R265 belong to the VGII lineage; and the remaining six strains (B4544, B4546, B4499, ATCC32608, JF101, and JF109) belong to the VGIII lineage. The B4544, B4492, B4499, LA61n, JF101, and R265 strains are of the *MATalpha* mating type while the remaining six strains have the *MATa* allele. All strains except JF109 and JF101 were wild type strains isolated from clinical samples. Strain JF101 is a derivative of a clinical strain NIH312 with the crg1 gene deleted (NIH312 *crg1::NAT*) while strain JF109 is a derivative of another clinical strain B4546 with the crg1 gene deleted (B4546 *crg1::NEO*). The deletion of the crg1 gene allows the strains to mate more efficiently (Fraser et al. 2003). The information about the parental strains arranged for crosses in this study is shown in Table 1.

**Media, growth, and mating of strains of *C. gattii***

Yeast cultures were typically grown and maintained on regular Yeast extract-Peptone-Dextrose agar (1%Bacto Yeast Extract, 2% Bacto Peptone, 2% Dextrose; 1.8% agar) at 22°C. Prior to mating, fresh cultures were grown on YEPD at 22 °C for 2-3 days. Fresh cells from each parental strain were then scraped off the agar surface via toothpicks and re-suspended in 600μL of sterile distilled water through vortexing. Cell densities were adjusted to OD600=0.5. For each cross, equal volumes (300μL) of cell suspensions of the two parental strains were transferred into another 1.5mL micro-centrifuge tube and cells were mixed thoroughly via vortexing. 20μl of the mixed suspension was spotted on the mating V8-juiceagar media [5% V8 vegetable juice (Campbell Soup Co., Etobicoke, Ontario, Canada), 0.5 g/l KH2PO4, 4% Difco agar and pH 7.2] as one repeat of the cross. Each agar plate (10cm diameter) was divided into eight sections, with two sections spotted with each of the two parental strains (also 20μl) individually as negative controls. The remaining six sections were each spotted 20µl of the same mixed suspensions, representing six repeats of the same cross. The mating plates were incubated in the dark at 25°Cfor 7-30 days, checked once a week for hyphal production and evidence of mating. Successful mating was indicated by hyphal growth at the periphery of the mating spots that contain yeast cells of the two parents.

For those that failed to mate the first time, up to four additional attempts were made, first on V8pH7 agar again and then on V8pH5 agar.

For successful crosses, spores and hyphae were collected from the periphery of the mating spot (i.e. away from the parental yeast cells) and suspended in 300uL sterile water for isolation of progeny via one of two methods.

The first was through random spore germination and isolation. In this method, 100uL of this suspension was spread on 3 YEPD agar plates and incubated at 30°C for 2-3 days. Each colony germinated from either a basidiospore or a hyphal fragment was treated as one progeny in our analyses. Single yeast colonies were then randomly picked, scraped off the agar medium, and sub-cultured onto new YEPD agar plates at 30°C for 2 -3 days for DNA extraction and mitochondria genotype determination (See below). For each successful cross, we picked 96 individual colonies for genotype analyses.

The second method was through micro-dissection operating a micromanipulator (Singer MSM 300, UK). In this method, the spore and hyphae suspension was first vortexed then centrifuged for 1min at 13 000 rpm then 200uL of supernatant was removed and the pellet was re-suspended in the remaining liquid. 50uL of this suspension was then placed on a small area of a YEPD plate then individual spores were dissected from the source. The plate was incubated at 30°C for 2 -3 days to allow the dissected cells to form colonies. Progeny obtained through this method allowed the calculation of spore germination rate (number of germinated spores/number of spores micro-dissected). Similar to the first method, for each successful cross, 96 colonies were sub-cultured onto YEPD at 30°C for 2 -3 days for subsequent DNA extraction and genotyping.

**Testing the effects of environmental factors on mtDNA inheritance**

The following four environmental factors were tested for their effects on mtDNA inheritance for two crosses that were found to have uniparental mitochondrial inheritance: UV irradiation, 5-adc treatment, ammonium chloride, and temperature. The two crosses were JF109 x B4544 and LA55n x B4544. The protocols largely followed those described in Yan et al. (2007). We briefly describe the treatments as below.

 **UV irradiation treatment**: To determine the effects of UV irradiation on mtDNA inheritance, cell suspensions were plated on V8-juice agar, a common medium for mating in *C. gattii*. Fluorescent lamps as sources of simulated natural UV were used (Douglas Brown et al. 2000). 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 administered: 0, 10, 20, and 40 seconds. 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) and mtDNA inheritance in *C. neoformans* (Yan et al. 2007b). After UV exposure, the V8-juice agar plates were coffered by aluminum foil and incubated 22 °C for four weeks. The remaining procedures were as those described above.

 ***5-adc* treatment**: To check the effect of 5’-aza-2’-deoxycytidine (5-adc) on mtDNA inheritance in *C. gattii*, cells of B4544, JF109 and LA55n were prepared in a different fashion from those for UV irradiation. The parental cells were first grown separately on YEPD 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 500mM. The concentrations around 200mM 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. Plates were incubated at 22 °C for four weeks. The remaining procedures followed those described above for normal crosses.

 **Ammonium chloride treatment**: Similar to cell preparations during the treatment of 5-adc, strains B4544, JF109 and LA55n were first plated on YPD medium supplemented with selected concentrations of ammonium chloride (0, 10, and 100mM) and allowed to grow for 3 days at 22°C. These concentrations were chosen because Sutovsky and his colleagues (2000) reported that the use of 10mM of ammonium chloride to treat fertilized mammal eggs could prevent the degradation of sperm mitochondria. The treated cells were re-suspended 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 four weeks. The remaining procedures followed those described above for normal crosses.

 **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 14, 23 and 30°C, respectively, for four weeks. In a pilot experiment, different from that in *C. neoformans*, we found no mating occurred at temperatures of 30°C and above between our *C. gattii* strains.

**Determining progeny mtDNA genotype**

To identify mitochondrial DNA inheritance pattern for each cross, parental mtDNA genotypes need to be different. In this study, two types of markers were used. The first is restriction enzyme site polymorphisms, as identified based on their sequence differences at a variety of markers (Xu et al. 2009). Based on the success of crosses (see below in Results) and the patterns of restriction enzyme site polymorphisms among the strains, we selected ND4 and ND2 to differentiate parental mtDNA types in the progeny population. The second is based on PCR fragment length polymorphisms at the ND5 gene, due to the presence of an intron in some of the strains and absence in others (Xu et al. 2009). The PCR primers and allelic information for the parental strains are summarized in Table 2.

 To obtain progeny genotype, their genomic DNA was first extracted using lyticase, phenol-chloroform and isoamylic alcohol (Sigma-Aldrich, SP, Brazil) followed the protocol described previously (Xu et al. 2000). The extracted DNA was checked on 0.8% agarose gel stained with ethidium bromide and then visualized under UV light, to further demonstrate the success of extraction and to determine the relative quantity. Quantity and quality of DNA extract was indicated by the brightness of the bands. The extracted progeny DNA samples were each diluted to approximately 30ng/ml before being used as templates to amplify the mitochondrial NADH dehydrogenase subunits 2 (ND2), 4 (ND4) and/or 5 (ND5) gene fragments. The PCR primers for amplification of *ND2, ND4* and *ND5* genes used in this study were summarized in **Table 2.** Each PCR reaction contains 8μL the GO mixture (MgCl2, Taq polymerase and dNTPs), 4μL primer, 2μL water and 2μL DNA, in a total 16μL of volume. The PCR reaction conditions were 4 minutes initial denaturation at 94 °C; followed by 35 cycles of 60 seconds denaturation at 94 °C, 90 seconds at the annealing temperature of 50 °C, 2.5 minutes 1min/1000bp elongation time at 72 °C; and finally, 7 minutes of extension at 72 °C and 4°C until retrieval.

For the ND5 gene fragment, the length difference between the parental strains was visualized on 1% agarose gel and the PCR amplicon size from the progeny was used to directly score the inheritance pattern. For ND2 and ND4 fragments, 4μL of each PCR product was electrophoresed on 1% agarose gel in 1× TAE buffer and was stained with ethidium bromide to check for successful amplification. The remaining amplified PCR products were digested using appropriate restriction enzymes listed in Table 2. A typical restriction enzyme digest contained one unit of the enzyme, 12µl of PCR product, and appropriate amount of buffer (final concentration 1x) in a total volume of 20µl. The mixture was incubated for 3 hours at a temperature corresponding to the maximum activity of the specific enzyme. The digested PCR products were then run on a 1.5 % agarose gel in 1× TAE buffer. Parental DNA samples were always used as reference controls to allow for the identification of MATa parent mtDNA, MATalpha parent mtDNA, heteroplasmic mtDNA and recombinant mtDNA (in cases where at least two markers were used).

Progeny were considered heteroplasmic if for any marker we analyzed they showed mtDNA genotypes of both parents. Progeny were considered to have a recombinant mtDNA genome if they had inherited one allele at one locus from one parent and the other allele at a different locus from the other parent. To ensure that heteroplasmic and recombinant mtDNA genotypes had not resulted from partial digests, the mtDNA genotypes of these progeny were reconfirmed by repeated PCR and PCR-RFLP. Parental mtDNA and a standard primer were always used as internal and external controls to ensure that the DNA was being amplified properly. The additional analyses were done in addition to comparing the progeny mtDNA with parental mtDNA digestions in both digestion and gel electrophoresis to eliminate the possibility of partial digestion.

For the two specific parental combinations that we used to examine the effects of environmental influences on mtDNA inheritance, one marker was used for each cross, and three markers were used for previous crosses. The cross between strains LA55n and B4544 used ND5 gene fragment as the marker whereLA55n had a 425bp fragment while B4544 had a 750bp fragment. The crosses between strains JF109 and B4544 used the ND4 gene fragment where the amplified fragment size was the same, about 650bp for both strains. Parental mtDNA was always used as positive controls to ensure that a single fragment was not the result of a failed restriction digestion. However, strain B4544 could be cut by the restriction enzyme Sac1 into two fragments of about 150bp and 500bp respectively.

**RESULTS & DISCUSSION**

**Mating attempts and mating success**

A total of 36 strain-pairings (*MATa* x *MATalpha* crosses) were performed and screened for mating success in this study. Among these, 17 pairings were successful (Supplementary Table 1). There were nine crosses attempted among strains within the VGIII lineage, all nine mated successfully. Three crosses between strains of VGIII and VGII were successful (of 9 crosses attempted) and five crosses were successful between VGIII and VGI strains (out of 9 crosses attempted). Because of the limited amount of VGI and VGII strains, the less attempts were made within or between these two crosses. No cross was successful between strains within VGI (0 out of 2 crosses attempted) or those within VGII (0 out of 2 crosses attempted). No cross was successful between VGI and VGII strains (0 out of 5 crosses attempted). Previous reports have shown variable abilities of natural strains to mate (e.g. Yan et al. 2002). At present, the mechanism(s) for the variations in mating abilities among the examined strains is not known. An experimental evolution study in *C. neoformans* has shown that mating ability could decrease upon extended asexual reproduction (Xu 2002).

However, based on the limited number of crosses examined here, phylogenetic divergence doesn’t seem to play a significant role as crosses between strains within individual lineages did not show a statistically significant higher rate of mating success (9/14) than those between lineages (8/22) (Supplementary Table 1). But we can still infer that 1) some strains in the VGI or VGII lineage already lost the mating ability. 2) Because virulence traits can spread among strains through sexual reproduction and potentially creating novel hyper virulent genotypes (Voelz K et al. 2013), the high mating rate of VGIII strains implicates that strains belong to this lineage may rapidly generate novel virulent genotype through sexual crosses. Interestingly, though more hyphae were produced in mating using the crg1 mutants JF101 and JF109 (data not shown) than not using the two strains, JF109 did not show increased mating success over that of its wild type progenitor B4546 (both were 3/5=60%) when crossed with the same natural strains (Supplementary Table 1).

**Patterns of mtDNA inheritance**

 Of the 17 successful crosses, 14 produced sufficient hyphae to allow us analyze the mtDNA inheritance while the remaining three produced short stubby hyphae that we were unable to dissect for analyses. In this study, a total of 1692 progeny from the 14 successful crosses between strains from within and between lineages of *C. gattii* were examined for their mtDNA genotypes and compared to their parental types. The detailed results of mtDNA inheritance for all 14 crosses in standard laboratory mating conditions are summarized in Table 3. These crosses are grouped into within and between lineages of *C. gattii* for comparison (Table 3). A diversity of mtDNA inheritance patterns was found among the crosses, including complete uniparental from the *MATa* parent to a significant proportion of progeny containing mtDNA from the *MATalpha* parent. Of these 14 crosses, only one cross, B4544 x LA55n, showed complete uniparental mtDNA inheritance from the *MATa* parent in the standard laboratory mating condition. Other 13 crosses all showed at least one progeny inheriting mtDNA from the *MATalpha* parent with *MATalpha* mtDNA leakage rates ranging from 0% to 93.65%(Table 3).

We compared the leakage ratio between ingroup and outgroup crosses. Our results showed that the within lineage crosses (crosses 1-7) had similar mtDNA leakages of the *MATalpha* parent (average of 49.54% and range from 3.41% to 92.00%) as those observed in inter-lineage crosses (crosses 8-14) (average of 48.03% leakage and range from 0% to 93.65%). Comparing the intra-lineage crosses to the inter-lineage crosses in a T-test gives a non-significant p value of 0.647. The exact cause(s) for such varied mtDNA inheritance within C. gattii is not known. However, the data showed clear influences of increasing mtDNA leakages of the *MATalpha* parent, of both individual strains and strain-combinations contributing to the variable mtDNA inheritance patterns. The highly variable mtDNA inheritance pattern in *C. gattii* is consistent with the findings of mtDNA recombination in natural populations of *C. gattii* as found by Xu *et al.* (2009). The distinct mtDNA inheritance pattern between these two closely related species arise many questions. For example, why do they have different inheritance pattern? How do they control this distinct inheritance pattern? What genes caused this difference? Currently, the exact mechanisms causing such varied mtDNA inheritance are not pinpointed yet in this research. However, these two closely related species offered us a great opportunity to answer these questions.

**Effect of *crg1* mutation on mtDNA inheritance**

Since nine of the successful crosses here involved strains JF101 and JF109, the two strains with the crg1 deletion, we first examined whether the crg1 deletion had a directional effect on mtDNA inheritance pattern in *C. gattii*, in favor of either the *MATa* or the *MATalpha* parent. Our results showed no clear evidence of a directional effect of the *crg1* gene deletion on mtDNA inheritance in *C. gattii*. As JF109 was derived from B4546, crosses JF109 x B4499 and JF109 x B4544 were both directly compared to crosses B4546 x B4499 and B4546 x B4544 (Table 3). When comparing wild type strain B4546 to *crg1*-mutant strain JF109 in their mating with B4499 (crosses 1 and 2), the percentage of progeny inheriting mtDNA from the *MATalpha* parent (mtDNA leakage) increased from 7.69% to 42.04%. On the other hand, comparing the wild type to *crg1-*mutant mating with B4544 (crosses 3 and 4), the leakage decreased from 17.28% to 3.41%. Similarly, in the VGIII x VGII crosses involving *crg1*-mutant (crosses 9 and 10), more mtDNA leakage occurred than in the wild type cross #8. However, the VGIII x VGI crg1 mutant cross (cross# 14) has less mtDNA leakage than the wild type crosses (11, 12 and 13). While strain- and cross-specific effects of the crg1 deletion on mtDNA inheritance were observed, we did not find any directional bias. Taken together, the results suggest that while *crg1* geneis involved in mating, it does not have a directional effect on mitochondrial inheritance in *C. gattii*.

**Relevance to hybrid breakdown of uniparental mtDNA inheritance in other species**

Previous studies showed hybridization could break down uniparental mtDNA inheritance in animals, plants and fungi. In cases of hybridization between species or subspecies, the intraspecific uniparental mitochondrial inheritance pattern can be altered due to the breakdown of mechanisms governing uniparental inheritance and result in the presence of organelle DNA from the parent that usually does not contribute. This process is known as hybrid mtDNA leakage and can result in recombination of organelle genomes. Sometimes it is possible to obtain direct evidence for leakage of organelle DNA after hybridization either by lab crosses like in fruit flies (Kondo *et al.* 1990), pine trees (Wagner *et al.* 1991), mice (Gyllensten *et al.* 1991; Shitara *et al.* 1998), mussels (Zouros *et al.* 1998), and periodical cicadas (Fontaine *et al.* 2007), or by analysis of individuals from natural hybrid zones as in mussels (Rawson *et al.* 2006). In other cases, leakage can be inferred from evidence for recombination such as in the great tit, spruce trees, silk moths, and *C. gattii* (Kvist *et al.* 2003; Jaramillo & Bousquet 2005; Arunkmar *et al.* 2006; Xu *et al.* 2009)).

While the detailed mechanisms for hybrid breakdown of mtDNA inheritance remain to be elucidated, it has been shown in *Mytilus sp.* that with greater genetic distance between (sub-) species there is a greater amount of mtDNA leakage during their hybridization. However, the data on either C. neoformans (as shown before in Xu et al. 2000; Yan and Xu 2003) or C. gattii (Table 3 here) did not show a pattern similar to that observed in *Mytilus* sp. Our results re-enforce the uniqueness of mtDNA inheritance pattern in fungi as compared to those in plants and animals (Wilson and Xu 2012).

In a recent study of mitochondrial population genetics of two divergent lineages in *C. gattii* by Xu *et al.* (2009), evidence for recent recombination and hybridization in the mitochondrial genome was found within and between lineages respectively. The main evidence for hybridization was that the phylogenies constructed with different genes were not consistent with one another, indicating that there has been recent hybridization. Although several eukaryotic microorganisms have shown uniparental organelle inheritance patterns, as in chloroplasts of *Chlamydomonas reinhardtii* (Sager 1972), relatively few studies have critically examined mitochondrial population genetics and recombination in nature for eukaryotic microorganisms (Barr *et al.* 2005; Wilson and Xu 2012; Xu et al. 2009). With the rapidly reducing costs of DNA sequencing, we expect more such studies will emerge to allow a broader comparison of mtDNA inheritance pattern between eukaryotic microorganisms and complex organisms such as animals and plants.

In the presence of 5-adc or ammonium chloride at the specific concentrations, we were unable to observe any mating between the two strains B4544 and LA55n, despite repeated tries of six times over the course of a year with incubating lasting for up to two months each (detailed data not shown). For the other two environmental factors, successful mating was obtained between these two strains at selected treatments (all four UV irradiation dosages and three temperatures 14oC, 23oC, and 30oC). However, progeny from crosses between them at the tested environmental conditions that produced progeny showed no difference in their mtDNA inheritance pattern, with all 307 progeny inheriting mtDNA from the *MATa* parent (Table 3). In contrast, we were able to obtain progeny from crosses between strains B4544 and JF109 under more diverse environmental conditions and these crosses showed highly variable patterns for each of the four tested environmental conditions (Table 4). Below we describe and discuss the mtDNA inheritance patterns for the crosses between strains B4544 and JF109.

**Effects of environmental factors on mtDNA inheritance**

From the 14 crosses, we identified two (B4544 x JF109 and B4544 x LA55n) that showed uniparental mtDNA inheritance in standard lab conditions and these two crosses were further examined to study the potential effects of environmental factors on mtDNA inheritance in *C. gattii*. In *C. neoformans*, longer UV exposure and higher temperature could increase the leakage of *MATαlpha* mtDNA, while 5-adc and ammonium chloride had no influence in mtDNA inheritance. Similar mtDNA inheritance in *C. gattii* like that in *C. neoformans* was expected to observe. Surprisingly, our results showed that these two crosses differed significantly in their responses to these four physical (UV irradiation and temperature) and chemical (5-adc and ammonium chloride) factors. In the presence of 5-adc or ammonium chloride at the specific concentrations, we were unable to observe any mating between the two strains B4544 and LA55n, despite repeated tries of six times over the course of a year with incubating lasting for up to two months each (detailed data not shown). For the other two environmental factors, successful mating was obtained between these two strains at selected treatments (all four UV irradiation dosages and three temperatures 14oC, 23oC, and 30oC). However, progeny from crosses between them at the tested environmental conditions under standard laboratory conditions, that produced progeny showed no difference in their mtDNA inheritance pattern, with all 307 progeny inheriting mtDNA from the *MATa* parent (Table 3). In contrast, we were able to obtain progeny from crosses between strains B4544 and JF109 under more diverse environmental conditions and these crosses showed highly variable patterns for each of the four tested environmental conditions (Table 4). Below we focus on describe and discuss the mtDNA inheritance patterns for the crosses between strains B4544 and JF109. In addition, the observed responses in these two crosses also differed from those reported in *C. neoformans*. Below we compare our results with other studies and discuss the implications of our results in the evolution of *C. gattii*.

 **Effect of UV irradiation**: As expected, the mating mixtures without exposing to any UV irradiation produced progeny containing mitochondrial genome only from the *MATa* parent (Table 4). A similar result was obtained with 10 seconds of UV exposure where all examined progeny inherited mtDNA from only the *MATa* parent. However, those exposed to UV irradiation for 20 seconds and 40 seconds produced 31.8% and 32.6% of the progeny containing mtDNA from the *MATalpha* parent, respectively (Table 4).

**Effect of 5-adc treatment**: Of the three concentrations of 5-adc that we tested, all had progeny inheriting mitochondrial from the *MATalpha* parent, with the concentration of 250mM producing all 24 progeny containing the mitochondrial genomes from both the *MATa* and *MATalpha* parents. The other two concentrations (50mM and 500mM) also produced significant percentages of progeny with the *MATalpha* parental mtDNA genotype, at 24.1% and 81% respectively (Table 4). Interestingly, at the 500mM concentration of 5-adc, progeny with mtDNA from only the *MATa* parent was the minority, with 81% of the progeny containing mtDNA from only the *MATalpha* parent (Table 4).

 **Effect of ammonium chloride*:*** Two concentrations (10mM and 100mM) of ammonium chloride were tested for its effect on mtDNA inheritance in *C. gattii.* Sutovsky et al. (2000) reported that 10mM of ammonium chloride prevented the degradation of sperm mitochondria in fertilized mammalian eggs. In our study, 41.7% of the progeny were found to have mtDNA from the *MATalpha* parent when treated with 10mM ammonium chloride. There were progeny containing mtDNA from either the *MATalpha* parent alone or from both the *MATa* and *MATalpha* parents in this treatment. A similar result was found among the progeny from the crosses treated with 100mM ammonium chloride. However, the proportion of progeny containing the *MATalpha* parent mtDNA was significantly lower (18.8%) in the 100mM treatment than that in the 10mM treatment (41.7%).

 **Effect of temperature**: Four different temperatures were tested for their effects on mtDNA inheritance: 14, 23, 30, and 33 °C. Despite repeated attempts, we were unable to obtain any successful mating at the 33oC environment for either cross. At the 30°C environment, mating was successful for cross LA55n x B4544 and all progeny inherited mtDNA from the *MATa* parent. However, we were unable to mate JF109 and B4544 at 30°C. At the low temperature 14oC, mating was extremely infrequent and we were only able to obtain 10 progeny for analyses. Interestingly, 8 of the 10 progeny from the cross at 14°C environment contained mtDNA from the *MATalpha* parent (Table 4).

 At present, the mechanisms for the differences in mtDNA inheritance pattern between the two crosses under the diversity of treatments are not known. One possibility is that *crg1* deletion could have predisposed JF109 to be more prone to mtDNA leakage under environmental stress conditions. However, as shown in Table 3, the cross between B4544 and B4546 (the wild type progenitor of JF109) had greater leakage under normal lab condition than the cross between B4544 and JF109. Thus, other mechanisms were likely involved, like the disruption of *MATalpha* mtDNA degradation or other possibilities.

**Comparison between *C. neoformans* and *C. gattii***

When comparing the results of mtDNA inheritance (Tables 3 and 4) of *C. gattii* to those found in *C. neoformans* (Xu et al. 2000; Yan & Xu 2003) it is clear that there is much more leakage of *MATαlpha* mtDNA in crosses of *C. gattii* than was found in crosses of *C. neoformans.* Specifically, an average of 43.9% leakage was observed across all crosses between *C. gattii* strains, ranging from 0% to 93.65%, whereas Yan and Xu (2003) only found an average of 0.5% leakage across all crosses examined under standard laboratory conditions for *C. neoformans*. In addition, the mtDNA inheritance seen among strains and crosses in *C. gattii* is much more variable than in *C. neoformans*.The mechanisms controlling uniparental mitochondrial inheritance in *C. neoformans* might be disrupted in *C. gattii,* and that could be the difference. Comparative genomic analyses of the strains showing diverse mtDNA inheritance patterns may help identify the potential genetic and genomic changes associated with the differences in mtDNA inheritance.

 Previous studies of mtDNA inheritance in Chlamydomonas and mammals suggested that selective tagging and degradation were likely involved in uniparental mtDNA inheritance (Sutovsky et al., 2000; Umen and Goodenough, 2001). Methylation and Ubiquitination are two important factors contributing to 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). However, neither the methylation inhibitor 5-adc nor the ubiquitination inhibitor ammonium chloride affected mitochondrial inheritance in *C. neoformans* (Yan & Xu 2007). Surprisingly, we found both 5-adc and ammonium chloride had influence on mitochondrial inheritance in *C. gattii,* JF109 x B4544*.* Thus we hypothesized that selective tagging and degradation step may play a role in mtDNA inheritance in *C. gattii,* similar to those in algae and mammals. A completely different mechanism is present in *C. gattii* compared with *C. neoformans.*

Similar to the results from treatments with 5-adc and ammonium chloride, treatments with low temperature and longer UV irradiation exposure time were found to significantly increase leakage of mtDNA from the *MATalpha* parent into the zygote progeny population in cross JF109 x B4544. The environmental stress-related mtDNA inheritance might be evolutionary adaptive. For example, 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. gattii* 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; Kraytsberg et al., 2004; Xu, 2005; Xu et al. 2009; Cao et al. 2013).

**CONCLUSION**

*C. gattii* is widely considered a medically and ecologically relevant microorganism. Its known geographic distribution is continuously expanding, which clearly demonstrates that it is a versatile species. Mitochondria play critical roles in an organism's stress response and therefore have significant influences on host cells’ ability to survive under a diverse range of environmental conditions. Our study identified that *C. gattii* have highly variable mtDNA inheritance patterns that are influenced by individual strains, strain combinations, and/or a range of environmental factors. The mechanisms contributing to differences between *C. gattii* and *C. neoformans* remain to be elucidated.

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**Table 1.** *Cryptococcus gattii* strains used in mitochondrial inheritance analysis

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Strain Name | Molecular Type | Serotype | MAT | Wild type or crg1 knockout  | Strain Source |
| B4544 | VGIII | B | *MATalpha* | Wild type | Clinical, NY State, USA |
| B4545 | VGI | B | *MATa* | Wild type | Clinical, USA |
| B4546 | VGIII | C | *MATa* | Wild type | Clinical, NY State, USA |
| B4595 | VGI | B | *MATa* | Wild type | Clinical, Australia |
| B4499 | VGIII | B | *MATalpha* | Wild type | Clinical, Australia |
| LA55n | VGII | B | *MATa* | Wild type | Clinical, Brazil |
| LA61n | VGII | B | *MATalpha* | Wild type | Clinical, Brazil |
| ATCC32608 | VGIII | B | *MATa* | Wild type | Clinical, California, USA |
| JF101 | VGIII | C | *MATalpha* | *Crg1::NAT* | Lab, derivative of NIH312 |
| JF109 | VGIII | C | *MATa* | *Crg1::NEO* | Lab, derivative of B4546 |
| R265 | VGII | B | *MATalpha* | Wild type | Clinical, British Columbia, Canada |
| B4492 | VGI | B | *MATalpha* | Wild type | Clinical, California, USA |

**Table 2.** PCR fragment length polymorphism and restriction fragment length polymorphisms for the three mitochondrial markers used in this study**.**

|  |  |  |  |
| --- | --- | --- | --- |
| Gene | PCR amplicon size | Restriction enzyme | Alleles with different Digested fragment sizes |
| ND2 | 1150bp | *PvuII* | 1: 1150bp2: ~500, ~650bp3: 50bp, 500bp, 600bp |
| ND4 | 658bp | *SacI* | 1: 658bp2: 158, 500bp |
| ND5 | 437bp or 750bp | N/A | 1: 437bp2: 750bp3: 1250bp |

**Table 3.** Mitochondrial DNA inheritance among 14 crosses in the *Cryptococcus gattii* under standard lab conditions at 23oC, \*, progeny in this cross include all those in the environmental variable treatment crosses where no leakage was observed. See text for details

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |   |   |   |   |   |   |   |   |   |
|  No. | Cross | Parent | ND2 | ND4 | ND5 | Number of progeny with | Percent leakage |
| MATa | MATα | Recombinant |
| 1 | B4546 (VGIII) x B4499 (VGIII) | B4546 | 1150bp | 650bp |   | 86 | 7 | 0 | 7.69 |
| B4499 | 500bp, 650bp | 150bp, 500bp |   |
| 2 | JF109 (VGIII) x B4499 (VGIII) | JF109 | 1150bp | 650bp |   | 51 | 34 | 3 | 42.04 |
| B4499 | 500bp, 650bp | 150bp, 500bp |   |
| 3 | B4546 (VGIII) x B4544 (VGIII) | B4546 | 1150bp | 650bp |   | 67 | 1 | 13 | 17.28 |
| B4544 | 500bp, 650bp | 150bp, 500bp |   |
| 4 | JF109 (VGIII) x B4544 (VGIII) | JF109 | 1150bp | 650bp |   | 66 | 0 | 0 | 0 |
| B4544 | 500bp, 650bp | 150bp, 500bp |   |
| 5 | ATCC32608 (VGIII) x B4499 (VGIII) | ATCC32608 | 1150bp | 650bp |   | 17 | 51 | 1 | 75.36 |
| B4499 | 500bp, 650bp | 150bp, 500bp |   |
| 6 | B4546 (VGIII) x JF101 (VGIII) | B4546 | 1150bp | 650bp |   | 54 | 37 | 0 | 40.66 |
| JF101 | 500bp, 650bp | 150bp, 500bp |   |
| 7 | ATCC32608 (VGIII) x B4544 (VGIII) | ATCC32608 | 1150bp | 650bp |   | 6 | 69 | 0 | 92 |
| B4544 | 500bp, 650bp | 150bp, 500bp |   |
| 8 | LA55n (VGII) x B4544 (VGIII) | LA55n | 500bp, 650bp | 650bp  | 437bp | 307\* | 0 | 0 | 0 |
| B4544 | 500bp, 650bp | 325bp | 750bp |
| 9 | JF109 (VGIII) x LA61n (VGII) | JF109 | 1150bp | 650bp |   | 36 | 48 | 6 | 60 |
| LA61n | 50bp, 500bp, 600bp | 150bp, 500bp |   |
| 10 | LA55n (VGII) x JF101 (VGIII) | LA55n | 500bp, 650bp | 650bp |   | 38 | 4 | 49 | 58.24 |
| JF101 | 500bp, 650bp | 150bp, 500bp |   |
| 11 | B4495 (VGI) x B4499 (VGIII) | B4495 | 500bp, 650bp | 250bp, 400bp |   | 55 | 19 | 7 | 32.1 |
| B4499 | 500bp, 650bp | 50bp, 150bp, 450bp |   |
| 12 | B4495 (VGI) x B4544 (VGIII) | B4495 | 500bp, 650bp | 150bp, 500bp |   | 9 | 38 | 26 | 87.67 |
| B4544 | 500bp, 650bp | 650bp  |   |
| 13 | B4545 (VGI) x B4544 (VGIII)  | B4545 | 500bp, 650bp | 150bp, 500bp |   | 4 | 52 | 7 | 93.65 |
| B4544 | 500bp, 650bp | 650bp  |   |
| 14 | B4545 (VGI) x JF101 (VGIII) | B4545 | 500bp, 650bp | 150bp, 500bp | 750bp | 83 | 4 | 0 | 4.59 |
| JF101 | 500bp, 650bp | 150bp, 500bp | 1250bp |

**Table 4**. Effects of environmental factors on mitochondrial DNA inheritance in the *Cryptococcus gattii* cross JF109 x B4544

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Environmental factors | Treatments | Number of progeny with MATa mtDNA | Number of progeny with MATα mtDNA | Number of progeny with both MATa and MATα mtDNA | Percent progeny with MATα mtDNA (%) |
| UV exposure (in seconds) | 0 | 32 | 0 | 0 | 0 |
| 10 | 16 | 0 | 0 | 0 |
| 20 | 30 | 14 | 0 | 31.8% |
| 40 | 29 | 14 | 0 | 32.6% |
| Temperature | 14 | 2 | 7 | 1 | 80% |
| 23 | 32 | 0 | 0 | 0 |
| 30 | N/A | N/A | N/A | N/A |
| 33 | N/A | N/A | N/A | N/A |
| 5-adc | 0 | 32 | 0 | 0 | 0 |
| 50 mm | 22 | 1 | 6 | 24.1% |
| 250 mm | 0 | 0 | 24 | 100% |
| 500 mm | 7 | 30 | 0 | 81% |
| Ammonium chloride | 10µm | 14 | 6 | 4 | 41.7% |
| 100µm | 39 | 2 | 7 | 18.8% |

**Supplemental Table 1** Crosses attempted and mating success after up to 45 days of incubation on the respective media. “+”, successful mating; “-“ unsuccessful mating.

|  |  |  |  |
| --- | --- | --- | --- |
| *MATa* Strain | *MATalpha* Strain | Successful onV8 pH5 | Successful on V8pH7 |
| VGIII x VGIII |
|
| B4546 (VGIII) x B4499 (VGIII) | + | + |
| B4546 (VGIII) x B4544 (VGIII) | + | + |
| B4546 (VGIII) x JF101 (VGIII) | + | + |
| ATCC32608 (VGIII) x B4499 (VGIII) | + | --- |
| ATCC32608 (VGIII) x B4544 (VGIII) | + | + |
| ATCC32608 (VGIII) x JF101 (VGIII) | + | + |
| JF109 (VGIII) x B4499 (VGIII) | + | + |
| JF109 (VGIII) x B4544 (VGIII) | + | + |
| JF109 (VGIII) x JF101 (VGIII) | + | --- |
| VGIII x VGII |
| LA55n (VGII) x JF101 (VGIII) | + | + |
| LA55n (VGII) x B4544 (VGIII) | + | --- |
| LA55n (VGII) x B4499 (VGIII) | --- | --- |
| LA61n (VGII) x JF109 (VGIII) | + | -- |
| LA61n (VGII) x B4546 (VGIII) | --- | --- |
| LA61n (VGII) x ATCC32608 (VGIII) | --- | --- |
| R265 (VGII) x JF109 (VGIII) | --- | -- |
| R265 (VGII) x B4546 (VGIII) | --- | --- |
| R265 (VGII) x ATCC32608 (VGIII) | --- | --- |
| VGIII x VGI |
| B4495 (VGI) X B4499 (VGIII) | + | --- |
|
| B4495 (VGI) X JF101 (VGIII) | + | - |
| B4495 (VGI) X B4544 (VGIII) | + | + |
| B4545 (VGI) X B4499 (VGIII) | - | - |
| B4545 (VGI) X B4544 (VGIII) | + | + |
| B4545 (VGI) X JF101 (VGIII) | + | --- |
| B4546 (VGIII) X B4492 (VGI) | --- | --- |
| ATCC32608 (VGIII) X B4492 (VGI) | --- | --- |
| JF109 (VGIII) X B4492 (VGI) | --- | --- |
| VGII x VGI |
| LA55n (VGII) X B4492 (VGI) | --- | --- |
| B4495 (VGI) X LA61n (VGII) | --- | --- |
| B4495 (VGI) X R265 (VGII) | --- | --- |
| B4545 (VGI) X LA61n (VGII) | --- | --- |
| B4545 (VGI) X R265 (VGII) | --- | --- |
| VGII x VGII |
| LA55n (VGII) X LA61n (VGII) | --- | --- |
| LA55n (VGII) X R265 (VGII) | --- | --- |
| VGI x VGI |   |   |
| B4545 (VGI) X B4492 (VGI) | --- | --- |
| B4495 (VGI) X B4492 (VGI) | --- | --- |