HYBRIDIZATION IN *CRYPTOCOCCUS NEOFORMANS*

DEVELOPING A GENETIC LINKAGE MAP FROM AN INTERVARIETAL CROSS OF SEROTYPES A AND D AND THE ANALYSIS OF THE COSTS AND BENEFITS OF HYBRIDIZATION IN *CRYPTCOCCUS NEOFORMANS*

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Preface

This thesis consists of two chapters, and each chapter has been written as an independent unit. Chapter one generated a linkage map from a cross between serotype A and D strains of *Cryptococcus neoformans*, based on PCR-RFLP and RAPD markers. The second chapter analyzed potential outbreeding depression and heterosis of *C. neoformans* and explored putative mechanisms of these phenomena. Both chapters will be submitted for publication. My contribution to this research includes scoring and collecting data, data mining, finding suitable software and understanding the usage of it to generate the linkage map, and data analysis using statistical software. RAPD and PCR-RFLP were performed by Dr. Jianping Xu. The work was done under Dr. Xu's supervision.

Acknowledgements

Thanks to my supervisor, Dr. Jianping Xu, for his guidance, encouragement, support, and patience throughout this project. Thanks also to members of the Dr. Xu's lab for their helpful ideas and critiques. I also thank my parents, my sisters and brother for their generosity and unconditional love, and hope for the bright future of mine. Most of all, my thanks go out to my husband, Wayne, for staying beside me with his love, understanding, and support over all the years while I am attending school.

Table of Contents

List of Figures

List of Tables

Chapter 1 Developing a Genetic Linkage Map from an Intervarietal Cross of Serotypes A and D in *Cryptococcus neoformans*

1.1 Abstract

Intervarietal hybrids of *C. neoformans* have been found in nature and patients. To understand the genetic behavior of these hybrids, we analyzed 286 progeny between 2 strains, CDC92-15 of serotype A and JEC20 of serotype D. Two types of markers were used, co-dominant PCR-RFLP (Restriction Fragment Length Polymorphism) and random amplified polymorphic DNA (RAPD). 55 binary markers were produced and used to analyze the 286 progeny. A linkage map was generated using MAP MAKER, based on 43 of 55 markers. This map contained 4 linkage groups. The genetic distance of the linkage map is 1695.7 cM. The remaining 12 markers couldn't be placed on the map. These results are highly inconsistent with the number of chromosomes based on physical separation on pulse field gels. The analysis suggests a highly atypical meiosis in these hybrids, likely due to significant chromosomal non-disjunction.

1.2 Introduction

Cryptococcus neoformans is a fungal pathogen that can cause life-threatening infections in humans, especially in immunocompromised patients. It is reported that *C. neoformans* is the fourth most-common cause of lethal infections in AIDS patients (Mitchell & Perfect, 1995) and up to 15% of patients with AIDS are infected (Casadevall *et al.*, 1998; Mitchell & Perfect, 1995). Strains of *C. neoformans* have been divided into five distinct serotypes, A, B, C, D, and AD, distinguished by antigenic differences on cell surfaces. These serotypes represent divergent evolutionary lineages and three varieties have been proposed to reflect their historical divergence: serotype A strains as *C. neoformans* var. *grubii;* serotype D as *C. neoformans* var. *neoformans,* and serotypes B and C as *C. neoformans* var. *gatti*. Serotype AD strains have been identified as recent hybrids of serotypes A and D that are separated by ~18.5 million years of evolution (Xu *et al.* 2000a). Serotypes A and D and a hybrid AD serotype are found worldwide in nature and patients. However, serotypes B and C are mainly restricted to tropical and subtropical regions. Serotype A accounts for the majority of human infections and the majority of environmental isolates (Casadevall & Perfect, 1998; Franzot *et al.*, 1999). Mating system in *C. neoformans* is bipolar and controlled by a large locus with two alleles: MATa or MAT alpha (Kwon-Chung, 1975; 1976). For *C. neoformans* var. *neoformans* and *C. neoformans* var. *gatti*, both mating type alleles are found in natural and clinical samples (Casadevall & Perfect, 1998). However, most serotype A strains are mating type alpha (Mitchell & Perfect, 1995; Casadevall & Perfect, 1998; Franzot *et al.*, 1999; Heitman, 1999). *C. neoformans* var. *grubii* accounts for ~90% of clinical isolates

(Mitchell & Perfect, 1995; Casadevall & Perfect; 1998; Franzot *et al.*, 1999; Yan *et al.* 2002). Experimental studies demonstrate that MAT alpha is more virulent than are MAT *a* congenic strains in serotype D in a murine model of Cryptococcosis (Kwong-chung *et al.*, 1992). Some of serotype AD strains are less virulent in comparison to the serotype A strain in a murine model (Lengeler *et al.*, 2001). However, in a recent study, virulence tests with rabbit and murine models of cryptococcal meningitis showed that the serotype A congenic MAT*a* and MATα strains had similar virulence in animal models (Nielsen *et al.*, 2003).

Reproduction of all serotypes of *C. neoformans* is primarily asexual. In the laboratory, strains of opposite mating types can be crossed to produce sexual progeny. Because sexual crosses are possible, two tester strains, JEC20 and JEC21, from *C. neoformans* var. *neoformans* are typically used for the identification of mating types of natural strains and for genetic analysis of traits of interests. Although strains of serotype A and D have been shown to be genetically distinct, it is possible to obtain mating between isolates from the two different serotypes (Kwon-Chung, 1975). Because intervarietal sexual cross is feasibile in the laboratory, study of hybridization in *C. neofomrans* becomes possible.

C. neoformans has been studied intensely in aspects of ecology, epidemiology, and pathogenicity since the 1980s, due to the importance of this species as a human pathogen. However, there has been little study on the genetic structure of the *C. neoformans* genome. Genetic linkage analyses of *C. neoformans* would provide information about the genetic organization of this important pathogen and enable

important genes to be mapped. Forche *et al.* (2000) developed a genetic linkage map of *C. neoformans* var. *neoformans* based on a cross of B3501 (MATα) and B3502 (MATa), and several genes were mapped, including CAP64, CnLAC, and mating-type locus, using Amplified Fragment-Length Polymorphism (AFLP) markers. Schein *et al.* (2002) constructed fingerprinted bacterial artificial chromosome (BAC) clone physical maps for strains H99 (Serotype A) and JEC20 (Serotype D), as part of a genomics program for *C. neoformans*, to provide an initial comparison of the two genomes. A recent study constructed a genetic linkage map by a cross between MATα strain B3501 and MATa strain B3502. Twenty linkage groups were generated, based on restriction site polymorphisms, microsatellites, and mating-type associated markers *etc.* (Marra, 2004). These works provide a framework for further studies on *C. neoformans* genomes. However, there has been little analysis from cross/hybridization between *C. neoformans* var. *neoformans* and var. *grubii*, which could be crucial to understanding virulence and evolution of *C. neoformans*. Here, we are interested in the patterns of inheritance of genetic markers in such crosses. Molecular-marker-based genetic linkage mapping provides an effective method to study the interactions of differentiated genes and genomes in a hybrid genetic background. Two types of markers are used: one is co-dominant, based on PCR-RFLP, the other is dominant, based on RAPDs. Developing a genetic linkage map from crosses of *C. neoformans* var. *neoformans* and var. *grubii* and searching for the patterns of inheritance of genetic markers from this cross will enhance our understanding of genomic, evolutionary, and ecological consequences of hybridization of *C. neoformans*.

1.3 Materials and Methods

1.3.1 Experimental design and implementation

1.3.1.1 Strains, crosses, and the isolation of progenies

Two strains, JEC20 and CDC92-15, were used in this study. Strain JEC20 is a laboratory tester strain and belongs to *C. neoformans* var. *neoformans.* It has the *a* allele at the mating type locus. Strain CDC92-15, which was obtained from the United States in 1992 through surveillance and belongs *to C. neoformans* var. *grubii,* has the alpha allele at the mating type locus. CDC92-15 was chosen because of its resistance to fluconazole (MIC = 64 μg/ml). Crosses between JEC20 and CDC92-15 were conducted on V-8 juice agar (Xu *et al.*,2000b). After incubation at 30°C for 2 -4 weeks, hyphal filaments were observed outside of the mating mixture. For isolation of progeny, single basidiospores were obtained from various regions outside the mating mixture to maximize sampling of different mating events (Xu *et al.*, 2000b). A total of 286 basidiospores were randomly picked for genetic analysis.

1.3.1.2 Development of genetic markers

To identify polymorphic markers for genetic analysis, two methods were used: the first was to screen oligo-10mer primer sets A and B from an Operon set using two parents to identify reliable polymorphic markers. The second was to use codominant PCR-RFLP markers developed based on sequences from an earlier study (Xu *et al.*, 2000c).

1.3.1.2.1 Developing RAPD markers to generate the genetic linkage map

Two-hundred and eighty-six sexual offspring from the cross of JEC20 (Da) and CDC92-15 (Aα) were available from previous work. DNA was extracted from each parental strain and meiotic progeny, according to a protocol established by Xu *et al.* (2000). RAPD markers were generated by using single oligonucleotide primers. Eleven arbitrary10-nucleotide primers named OPA17, OPB01 to OPB08, OPB10, and OPB12 (Operon Technologies Inc. Alameda, CA; Kits A and B) were found to generate easily scorable polymorphisms between two parental strains. These primers were then used to genotype all 286 sexual offspring. PCRs were carried out in a total volume of 10 μl, where each PCR mixture contained 4μl *d*d H_2O, 1μl 10x buffer (contains $MgCl_2$), 1μl deoxyribonucleotide triphosphate (10x), 1μl primer, 0.1 μl Taq DNA polymerase, and 3 μl of DNA. The following PCR conditions were used: 5 min at 94° C, followed by 45 cycles of 1 min at 94° C, 1 min at 36° C, 2 min at 72° C, and 7 min at 72° C for final extension. Reaction ended with an infinite hold at 4° C. Each of the eleven arbitrary primers was used individually for PCR for each of the 286 isolates and parental strains. PCR products were electrophoresed on 1.5% agarose gel with ethidium bromide in 1 X TAE for 4 or 5 h at 80 volts, and then visualized under UV light. Fluoro Chem 8800 (Canberra Packard) was employed to photograph amplified PCRs (Figure 1-1). Markers were manually scored as described below. To help scoring, each gel included both parental strains as markers.

Figure 1.1 Example of RAPD band segregation in F1 populations. To produce molecular markers adjacent to a gene of interest or in the specific region of the genome, RAPD markers were amplified by using single oligonucleotide primers. Eleven arbitrary10-nucleotides primers were used to test the polymorphisms between the parental strains and among sexual offspring in RAPD analysis. Each fragment that showed polymorphism between the parents and segregated in the progeny was treated as an independent genetic marker. Segregating markers were scored as "1" for presence in one parent and "0" for absence in the other. Ambiguous and missing markers were scored as "-".

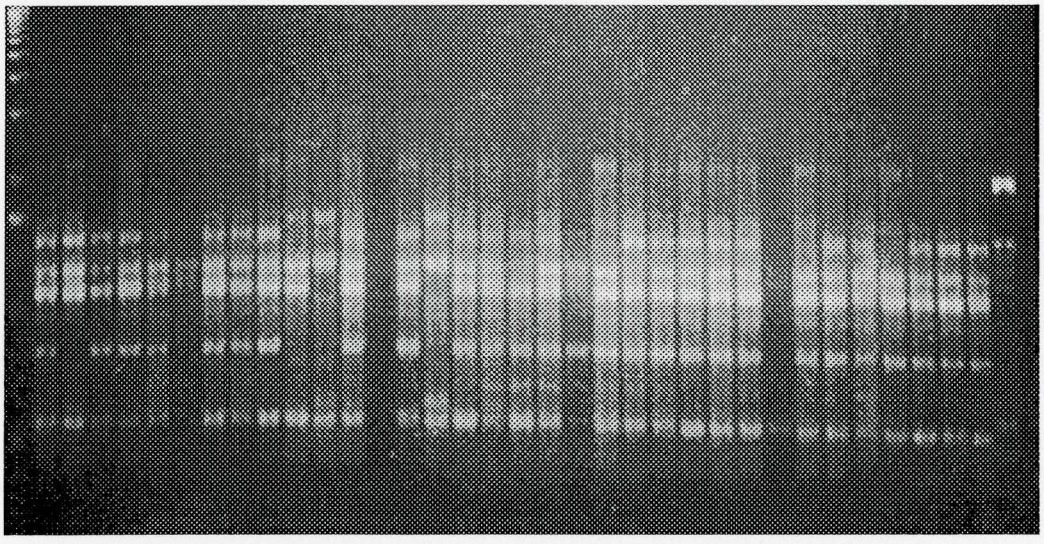

Figure 1-1 Example of RAPD band segregation in F1 populations

1.3.1.2.2 PCR-RFLP markers to genes LAC (laccase) and URA5

Two genes, URA5 and LAC (Laccase), were amplified. Oligonucleotide primer sequences amplifying a region of genome at which LAC and URA5 are located were (5' – 3'): ggcgatactattatcgta (forward) and ttctggagtggctagagc (reverse) for LAC (Williamson, 1994); and acgcctgcctgttacttaa (forward) and ggacatgatgattggagt (reverse) for URA5 (Casadevall *et al.*, 1992).

For amplifying LAC, each PCR was performed in 15 μl reaction mixture containing 3 μl of dd H_2O, 1.5 μl *of* 10x buffer (contains $MgCl_2$), 1.5 μl of 10x deoxyribonucleotide triphosphate, 0.5 μl of each primer, 0.1 μl of Taq DNA polymerase, and 8 μl of DNA template. The amplification was performed in a thermal cycler under the following conditions: 3 min at 97° C for initial denature, followed by 40 cycles of 30 s at 93° C, 30 s at 50° C, 30 s at 72° C, and 6 min at 72° C for final extension. Reaction ended with an infinite hold at 5° C. PCR products from each isolate were digested with the enzyme BsmI in the mixture of 2 μl of NEBuffer, 0.1 μl of BsmI, 11 μl of dd H_2O, and 7 μl of PCR products. The mixtures were sealed well and incubated at 65° for 4-5 h.

For amplifying URA5, the amount of each component in each PCR mixture was the same as that of LAC. The following PCR conditions were used: 3 min at 94° C, followed by 40 cycles of 1 min at 94° C, 1 min at 50° C, 1 min at 72° C, and, lastly, 7 min at 72° C for final extension. PCR products from each isolate were digested with the enzyme HindIII. The amount of each component of

digesting mixture was the same as that of LAC. The mixture was incubated at 37° C for 3 hours.

Digested PCR products were separated by electrophoresis in a 1.5% agarose gel with ethidium bromide in 1 X TAE for 4 or 5h at 8 volts, and then visualized under UV light. Markers were manually scored.

1.3.2 Scoring and the coding of data

DNAs from the 286 progeny plus two parental strains (JEC20: Serotype D, MATa and CDC92-15: Serotype A, MATα) were used for RAPD fingerprinting with 11 primers. 51 segregating markers were obtained; each fragment that showed polymorphism between the parents and segregated in the progeny was treated as an independent genetic marker. Segregating markers were scored as "1" for the presence in one parent and "0" for the absence in the other. Ambiguous and missing markers were scored as "-" (Table 1-1). These genotypic data were presented in Appendix Table A.1 for generating the genetic linkage map by using the LinkageMap/exp3.0 computer program (Lincoln *et al.*, 1992).

LAC and URA5 were scored separately because they were co-dominant markers, in which both alleles contribute to the phenotype of the heterozygote. If a progeny inherited an allele from JEC20 (Parent1), that locus was recorded as '1' and its two genetic markers were '1' and '0'; the locus was recorded as '2' and two genetic markers on that locus were represented by '0' and '1' in order if the locus were inherited from CDC92-15 (Parent 2); the locus was recorded as ½ and genetic markers on that locus were treated as '1' and '1' if the locus came from both parents

(Table A 2). At the end of scoring, two genetic markers were generated for one locus and total genetic markers were 4 from LAC and URA5 genes, based on RFLP. Two markers from LAC gene were represented by LAC1 and LAC2; URA5-1 and URA5-2 stand for two markers of URA5 gene.

Possible genotypes from putative dihybrid crosses between LAC and URA5 genes were scored and examined. LAC genes inherited from JEC20 was scored as "1" and those inherited from CDC92-15 were scored as "2". Conversely, URA5 genes that came from JEC20 were scored as "1' "and those from CDC92-15 were scored as " 2' ". Progeny with both parental alleles as "1/2 "or "1'/2' "and missing data was represented by "-". Genotypic classes from dihybrid cross were represented by following symbols: (1, 1'), (2, 2'), (1/1', 2/2'), (1/1', 2'), (1', 2/2'), (1/1', 2), (1, 2/2') (1, 2'), (2, 1'). Symbols (1, 1') and (2, 2') stand for parental genotypes and symbols (2, 1') and (1, 2') represents recombinant genotypes. The rest of the symbol displayed represent heterozygosis at one (aneuploid) or two loci.

Table 1.1. RAPD fingerprinting data for 286 progeny. 55 segregating markers were obtained; each fragment that showed polymorphism between the parents and segregated in the progeny was treated as an independent genetic marker. Segregating markers were scored as "1" for presence in one parent and "0" for absence in the other. Ambiguous and missing markers were scored as "-". The first column displays 55 segregating markers obtained from RAPD fingerprinting with 11 primers and RFLP For genes LAC and URA5. A single digit of each row represents a genotype of an individual of 286 progeny on that locus.

Markers	progeny genotypes(286)
1	1100000000110-0100010110011101011111….
0	000001000100-0000001100000110010000….
1	
0	011001000110-00000000000010000000..
0	010001000110-01000000000010000000..
0	111101101110-11100000011011001100..
1	000010000011-01000000000000000000..
0	000000000000-00000000000010000000..
1	010001000110-01000000000010000000..
0	1000000000000-00000000000010000000..
0	11001000110-00000000000010000000..
0	010001000110-01000000000010000000..
.	11010001-000100-111010101000000-00001-10000..
.	000010000011-01000000000000000000.
LAC1	011001000110-00000000000010000000..
LAC2	011001000110-00000000000010000000..
URA5-1	010001000110-01000000000010000000..
URA5-2	010001000110-01000000000010000000..

Table 1-1 RAPD and RFLP fingerpringting data for 286 progeny

1.4 Results

The experimental design was treated as F2 backcross because Mapmaker does not deal with haploid- haploid cross data. The threshold LOD value and Recombination Frequency (RF) for determining linkage groups were 5 and 0.2 respectively. Two adjacent markers analysis was used to generate the linkage map. Map construction was based on the "Haldane" mapping function, stating that for more than two markers, a simplifying assumption is that recombination between any two of them is independent of recombination between any other non-overlapping two (no interference).

During the process of generating the linkage map, various combinations of LOD thresholds and recombination fraction values were tested to determine the optimal parameters to generate the framework of the linkage map. Under recombination values between 0.2 and 0.26 with the LOD values from 3 to 5, the linkage groups were stable. With LOD = 5 value and RF = 0.2 as the optimal parameters, four linkage groups were generated. For unassigned markers left from initial analysis of the linkage map, one marker was added at a time to the stable linkage groups. If the added marker changed the order of linkage groups or the composition of the map, the marker was excluded from the linkage groups as an unlinked marker (Forche *et al.*, 2000).

The final linkage map included 43 of the 55 markers, containing 39 RAPD and 4 RFLP markers. Four major linkage groups were generated (Figure 1-2). The map is 1695.9 cM long and the average distance between markers is of 40.38 cM. The largest linkage group (LG 1) covers 1020 cM, equivalent to 60% of the length of the map.

Twenty markers are widely spread out over LG 1 with average distance of 53.68 cM between adjacent markers; 4 markers (L011, L016, L038, and L008) are closely linked with an average distance of 7.7 cM. The second large group (LG 2) is 408.5 cM long in size and contains 13 markers with the average distance of 34.04 cM. Interestingly, LAC1 and URA5_1 markers are two of six linked (L051, L013, LAC1, URA5_1, L037, and L015) markers, and they lie adjacently on LG2 with a distance of 16.2 cM between them. The third linkage group contains 7 markers with the length of 239.7 cM, and the average distance is 39.95 cM; LAC2 and URA5_2 are close to each other with a distance of 16cM in this group. The smallest linkage group contains 3 markers and is 27.7 cM in length; the mean distance between markers is 9.2 cM, on average (Table 1-2).

Figure 1.2. Linkage Groups of *C. neoformans* in Graphic Format. Genetic linkage map of *Cryptococcus neoformans* based on 43 of 55 markers from the sexual cross of var. *neoformans* (Da) and var. *grubii* (Aα) at LOD = 5.0 and RF = 0.2. Four linkage groups were identified: the largest LG1 contains 20 genetic markers, the second largest one has 13 markers, and the smallest one includes 3 markers. LAC and URA5 genes were linked in the linkage groups. Digital numbers on the left sides of maps present the distances among markers and numbers on the right side of the map display markers.

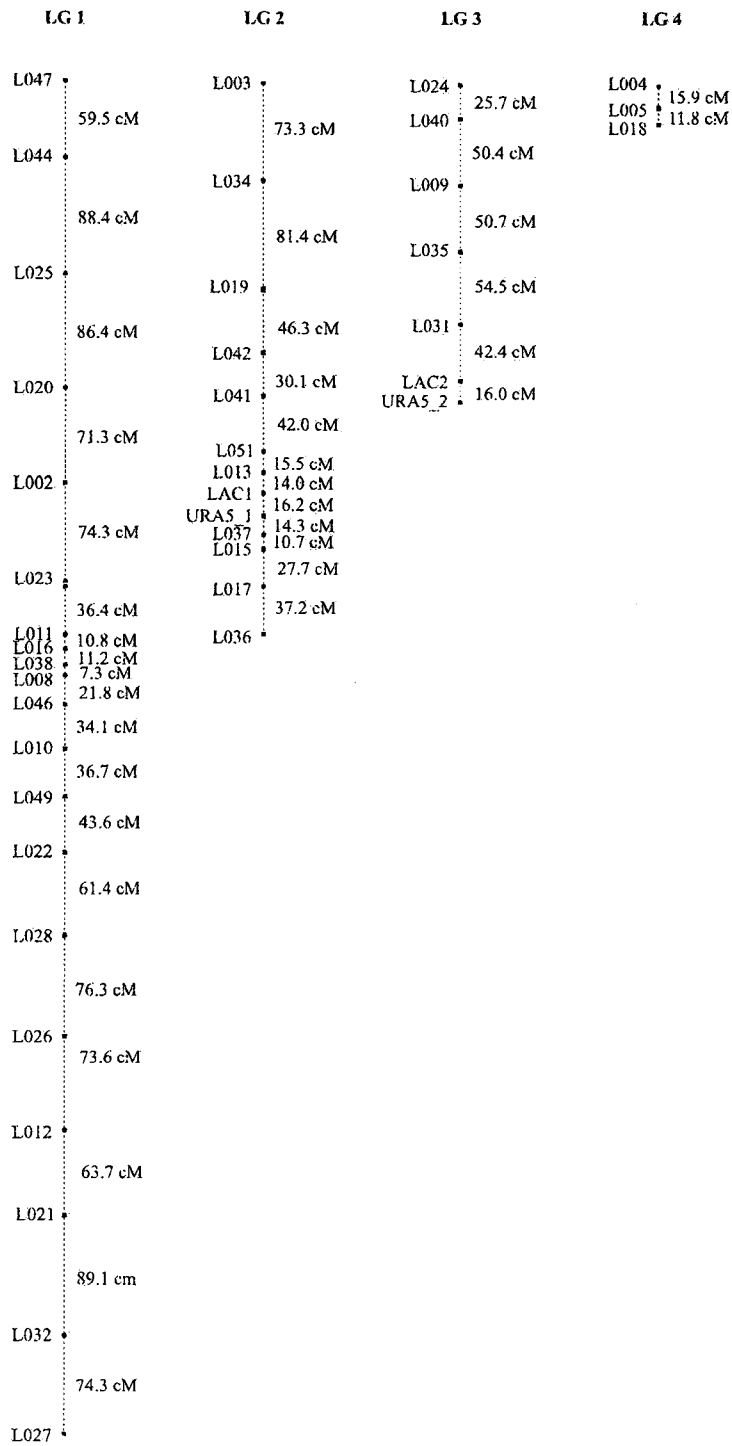

Figure 1-2 Linkage Groups of *C. neoformans* in Graphic Format

Table 1.2. Linkage Groups based on the cross between *C. neoformans* var. *neoformans* & *C. neoformans* var. *grubii*. Four linkage groups of *C .neoformans* were generated, based upon RAPD and RFLP markers. The map is 1695.9 cM long in size and the average distance is 40.38 cM between markers. Columns display the number of genetic markers in each LG, the size of each LG, and the average distance between markers in each LG.

LG Name	Num. of markers	Size of LG (cM)	Average distance between markers(cM)
LG 1	20	1020	53.68
LG 2	13	408.9	34.1
LG 3	7	239.7	39.95
LG 4	3	27.7	13.85

Table 1-2 Linkage Groups based on the cross between *C. neoformans* var. *neformans* & *C. neoformans* var. *grubii*

Three genotypic classes each were identified for URA5 and LAC genes, respectively. For each marker, one class was identical to JEC20; second one identical to CDC92-15; and a third includes alleles from both parental strains. 59.4% and 65.8% progeny were heterozygous at LAC and URA5 loci, respectively (Figure 1-3). 34.3 % progeny were homozygous at LAC locus in which 11.9% was from JEC20 (serotype D) and 22.4% was from CDC92-15 (serotype A). Missing data of the LAC gene accounts for the remaining 6.3%. For URA5, 33.5% progeny was homozygous at URA5 locus in which 17.8% and 15.7% inherited from serotypes D and A strains respectively. Missing data of URA5 accounts for 0.8%. Over half of the progeny were heterozygous at both loci and more than 30% were homozygotes of these two loci in our study.

Putative dihybrid crossing displayed 9 possible genotypes of progeny. Numbers of 9 genotypes were variable. Of the 286 hybrids, 144 showed heterozygosis on both loci (1/1', 2/2'); 61 displayed heterozygosis at one locus and homozygosis at the other locus symbolized by (1/1', 2'), (1', 2/2'), (1/1', 2), and (1, 2/2'); 61 were homozygous at both loci and symbols (1, 2'), (2, 1'), (2,2'), and (1,1') stand for homozygote. Of all homozygotes, 57 progeny have identical genotypes to one of their parents ((1,1'), (2, 2')). Only 4 were recombinants ((1,2') and (2,1')) (Figure 1-4). From this hypothetically dihybrid cross, progeny from this cross would be mostly diploid or aneuploid.

Figure 1.3. Genotypic classes in gene URA5 and LAC. Three genotypic classes were identified for URA5 and LAC genes of progeny, respectively. One class was inherited from JEC20; a second one came from CDC92-15; and a third was from both parental strains. 59.4% and 65.8% progeny were heterozygous at LAC and URA5 loci separately, and both the serotype A and D alleles of each gene are often present in serotype AD strains. 33.5 % progeny were homozygous at URA5 loci, where 17.8% was from serotype D strain and 15.7% was from serotype A strain. For LAC, 34.3 % progeny was homozygous, and 11.9% and 22.4% came from serotype D and A strains, respectively. The column presents the frequency of each genotype.

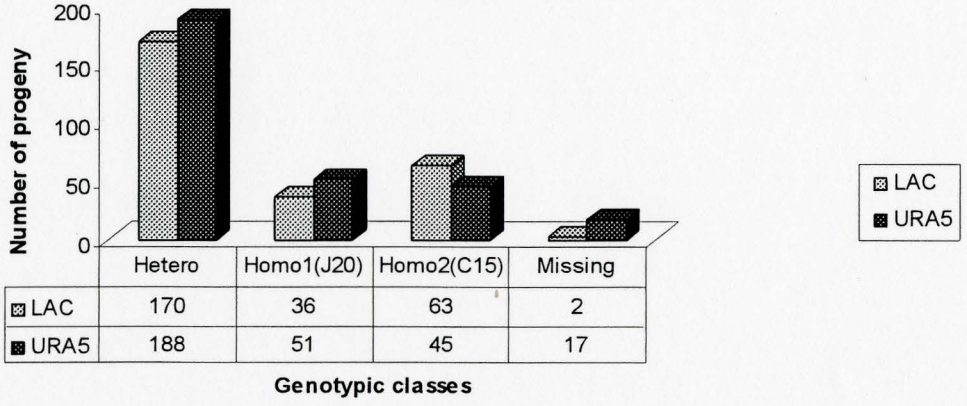

Figure 1-3 Genotypic classes in genes URA5 and LAC

Figure 1.4. Polymorphism of combination between genes URA5 and LAC. 9 genotypic classes were identified. The column presents the frequency of each genotype. 50% progeny displays heterozygosis on the combination of URA5 and LAC genes. Frequencies of 9 genotypes were variable. Of the 286 progeny, more than half showed heterozygosity on both loci (1/1', 2/2'); Of all homozygote, only 4 were putative haploid recombinant ((1, 2'), and (2, 1')). From this hypothetically dihybrid cross, progeny from this cross would be mostly diploid or aneuploid. The first row stands for the genotypic class and the second row is the number of isolates of a single genotypic class. The bar chart presents the frequency of each genotype.

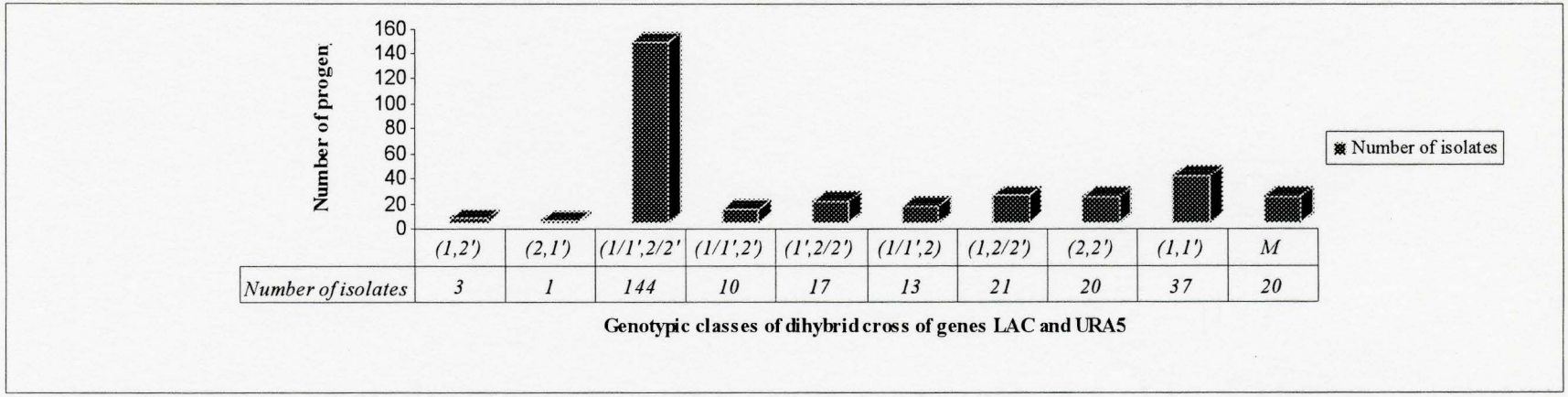

Figure 1-4 Genotypic classes of dihybrid cross of genes LAC and URA5

1.5 Discussion

Our study displayed four linkage groups in this intervarietal cross. This result differs from previous karyotype-analyses. Several studies on have shown chromosome size variability in *C. neoformans* within a variety or serotype and among varieties and serotypes (Table 1-3) (Perfect *et al.* 1989, 1993, Wickes *et al.*, 1994, Boekhout *et al*, and Maria *et al.* 2001). Wickes *et al.*'s study on CHEF panels of *C. neoformans* karyotypes showed differences between var. *neoformans* and var. *grubii* with respect to the mean of number of chromosome and the size of common band. Their study also displayed the difference of the size of band among individuals within the serotype (Figure 1-5 from Wickes *et al.* 1994). These studies reveal that the average number of chromosomes and ranges of chromosome number between *C. neoformans* var. *neoformans* and var. *grubii* are different. Forche *et al.* developed a genetic linkage map of *Cryptococcus neoformans* var. *neoformans* using amplified fragment length polymorphisms and other markers (Forche *et al.*, 2000) and displayed 14 major linkage groups. A new genetic linkage map of *C. neoformans* var. *neoformans* was constructed recently based mainly on restriction site polymorphisms and microsatellites markers. This linkage map consists of 20 linkage groups (Marra *et al.*, 2004). Another study of physical map based on the number of contigs, compared two strains, H99 (serotype A) and JEC21 (serotype D): 15 for H99 and 17 for JEC21 (Schein *et al.*, 2002). In contrast, the study we conducted was between different varieties and resulted in a different outcome from previous studies. Unlike the many studies on plant hybridization, there are few studies of fungal hybridization.

Figure 1.5. Contour-Clamped Homogeneous Electric Field Electrophoresis (CHEF) panels of *C. neoformans* var *neoformans* and var. *grubii*. (a) Serotype A *C. neoformans* var. grubii. Sc, *Sacch. cerevsiae*; lane 1, NIH966; lane 2, NIH971; lane 3, NIH288; lane 4, NIH386; lane 5, NIH302; (b) lane 1, Serotype D *C. neoformans* var. *neoformans*. Sc, *Sacch. cerevisiae;* lane1, NIH55; lane 2, NIH430; lane 3, B-3501; lane 4, NIH424; lane 5, NIH52.

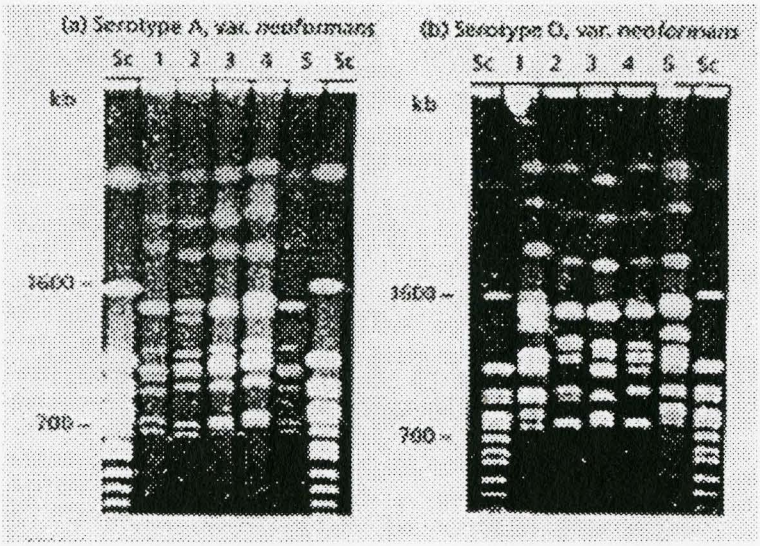

Figure 1-5 CHEF panels of *C. neoformans* karyotypes (serotype A, var. *grubii* and serotype D, var. *neoformans*)

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Like many hybrid plants, hybrids of *C. neoformans* also showed evidence of increased ploidy (diploid, aneuploid). *C. neoformans* var. *neoformans* (D) and var. *grubii* (A) diverged 18.5 million years ago and have large differences in genome structure and sequence, contributing to genetic incompatibility. Crosses between members of these varieties generate few viable spores (Lengeler, 2001). Reduced spore viability may be due to production of aneuploid spores as a result of nondisjunction, in which the unequal segregation of chromosomes occurs during meiosis. LAC and URA5 genes displayed a close link in the same linkage group in this study, a finding that is different from those obtained in other studies (Forche *et al.*, 2000), showing these two genes lie on different chromosomes. This result provides further evidence of failure of disjunction during meiosis, where improper alignment of chromosomes leads to defects in chromosome segregation. Experimental results reveal that hybrids from these crosses are heterozygous, diploid or aneuploid at loci URA5 and LAC. Progeny from these crosses showed strong diversity. A large number of genotypes would be generated when strains of serotypes A and D cross.

Experiences of studying plant hybridization will help us understand thoroughly hybridization of *C. neoformans*. One of the possible consequences of hybridization is speciation, a fundamental issue in evolutionary biology. Looking for specific genes that cause hybrid incompatibility will help us to investigate mechanism of hybrid incompatibility and establish the infrastructure to understand the evolution of *C. neoformans*.

Table 1.3. Several studies on karyotype of *C.neoformans* have shown chromosome size variability within a variety or serotype and among varieties and serotypes. These studies have revealed that the average number of chromosomes and ranges between *C. neoformans* var. *neoformans* and var. *grubii* are different.

Author	Date published	Mean of Chrom.	Range of Chrom.	Genome Size	Size of common band
Perfect *et al.*	1989	S- A: 10.5 S- D: 10 S -B: 12 S- C: 10	10-11	15000-17000kb	
Wickes *et al.*	1994	S- A: 12 S- D: 12.8 S- B: 12.4 S- C: 13.6	10-12 13-15 9-14 13-14		945-1125 700-1500 700-1400 680,1400
Maria *et al.*	2001	S- A&D: S-B&C:	9-13 7-12	17mb 10mb	
Boekhout *et al*	1997		11-14		

Table 1-3 Studies done by other groups on the chromosome size

1.6 Conclusion

1. We detected four linkage groups from a cross between strains of serotypes A and D.
2. LAC and URA5 genes were inherited together on the same linkage group.
3. Atypical meiosis with significant chromosomal non-disjunction.
4. More than half of progeny were heterozygous with alleles from both serotypes A and D strains
5. Most hybrids were likely aneuploids

Chapter 2 The Analyses of the Costs and Benefits of Hybridization in *Cryptococcus neoformans*

2.1 Abstract

Recent population genetic studies have revealed that there are multiple natural hybridizations among divergent lineages in the human pathogenic fungus *Cryptococcus neoformans*. However, the biological and phenotypic effects of such hybridization are little investigated. In this study, we constructed a laboratory cross between two genetically divergent strains and analyzed the vegetative fitness of their progeny. These two parental strains were chosen because they differed in their susceptibilities to the common antifungal drug fluconazole. Our analyses indicate that, overall, hybrids were less fit than were the parental strains. The fitness disadvantage was inversely correlated to fluconazole concentration, the highest when no fluconazole was present and the lowest at the highest tested drug concentration (64 *μg/ml*). With increasing drug concentration, the number of progeny exceeding the fitness of parental strains also increases. Using a previously constructed genetic linkage map based on random amplified polymorphic DNA markers, we identified that both vegetative fitness and drug susceptibility were associated with multiple genetic markers. Our results demonstrated environmentally dependent hybrid costs and benefits in this important human pathogenic fungus.

2.2 Introduction

Cryptococcus neoformans is a fungal pathogen that causes life-threatening infections in humans, especially in immunocompromised patients. Strains of *C. neoformans* have been divided into five distinct serotypes, A, B, C, D, and AD, distinguished by antigenic differences on cell surfaces. These serotypes represent divergent evolutionary lineages and three varieties have been proposed to reflect their historical divergence: serotype A strains with *C. neoformans.* var. *grubii;* serotype D with *C. neoformans* var. *neoformans,* and serotypes B and C within *C. neoformans* var.*gatti.* Serotype AD strains have been identified as recent hybrids of serotypes A and D. One study reported that serotype AD strains are moderately virulent in comparison to a serotype A strain H99 in a murine model (Lengeler, 2001). However, another study reveals that hybrid AD strains are highly pathogenic, with pathogenicity levels similar to that of the *C. neoformans* var. *grubii* type strain and unlike the low pathogenicity levels of *C. neoformans* var. *neoformans* strains (Chaturvedi, *et al.*, 2002). Though clinical isolates of serotype AD are not as common as serotype A strains, they occur in higher frequency than do other serotypes in certain geographic areas. For instance, 14% of isolates found from 1992 to 1994 in San Francisco were serotype AD, exceeding the combined number of isolates of serotypes B, C, and D from this region (Brandt *et al.*, 1996). Because of divergence between the varieties *neoformans* and *grubi*i and difference of serotype AD strains from strains of other serotypes, we are interested in the origin of

hybrid strains of *C. neoformans* (Xu, *et al.*, 2002) and outcomes of hybridization between these two varieties, especially the impact on the continuing evolution of *C. neoformans*.

Fundamentally important topics to many areas in evolutionary biology are: 1) the consequence of outcrossing between populations and 2) inbreeding within populations. This is because these phenomena can affect fitness and speciation (Lynch 1991; Grant and Grant 1996) and drive the evolution of mating systems, mate recognition, dispersal, and other behaviors (Greenwood 1980; Ralls et al. 1986; Pusey and Wolf 1996). Studies have shown that inbreeding that causes a shift in mean phenotype in a direction that causes a reduction in fitness and crossbreeding between populations often has positive effects on fitness-related traits. However, outcrossing does not always enhance fitness and outbreeding depression is sometimes observed in crosses between distant populations of the same species. These two hot topics have been studied intensively in animal and plant populations but rarely in fungal populations. The majority of papers concentrate on inbreeding depression and hybrid vigor. A recent literature search on PubMed found 334 papers on "inbreeding depression", 604 on "hybrid vigor", but only 31 relate to "outbreeding depression". None were identified in fungi.

Even less is known about how environmental factors influence outbreeding and inbreeding depression. Such knowledge is likely to be valuable for understanding fungal hybridization and speciation. *C. neoformans* is an excellent candidate species to examine these issues because of its short reproductive cycle, ease for sexual crossing in laboratory conditions, and significant divergence among lineages. To better understand the biological effects of hybridization between strains of serotypes A and D, we constructed a

laboratory cross and genetically analyzed 286 progeny. The aim of this study is to investigate possible outbreeding depression and hybrid vigor in vegetative fitness in hybrid progeny of *C. neoformans* between var. *grubii* serotype A (MATα) and var. *neoformans* serotype D (MAT*a*). We ask the following questions (1) Is there any evidence for outbreeding depression and hybrid vigor in this species with regard to vegetative fitness? (2) If they exist, are they environmentally dependent? (3) Is a single or are multiple genes involved in controlling vegetative fitness in various environments? (4) What are the relationships among these fitness-related genes?

2.3 Materials and Methods

Genetic cross and genotypic data are the same as those in Chapter 1. We re-processed these data to fit our analyse. Vegetative fitness was obtained as that described in Xu's paper (1998). The re-processing of genotypic data and the protocols for vegetative fitness determination are described below.

2.3.1 Classification of genetic markers

2.3.1.1 Classifying genetic markers into two different categories to analyse single marker association with vegetative growth at different drug concentrations.

The genetic markers, scored in the previous study were sorted and classified into two genotypic classes: the presence or absence of the band on a gel. Following classification of alleles, quantitative data for fluconazole-resistance that corresponded to the genetic markers was divided into two groups for each locus. For instance,

Marker L001 has two possible allelic classes represented by '0' for one parental and '1' for the other, respectively. The mean of vegetative fitness was calculated for each genotypic class of locus separately at each fluconazole concentration. The t-statistic was employed to test the null hypothesis that there was no significant difference for the two means between the two allelic classes at $\alpha = 0.05$. For LAC and URA5 genes, three possible allelic classes were identified, represented by '1','2', and '1/2', due to these genes' codominant character.

2.3.1.2 Classifying genetic markers into different categories to analyse single marker association with the minimum inhibitory concentration (MIC) at different fluconazole concentrations.

To understand whether a single gene is or multiple genes are associated with the MIC of progeny at different fluconazole concentrations, we categorized MICs into different classes 2, 4, 8, 16, 32 64, and 128 (*μg/ml)*. For each drug concentration, a single class of MIC was subdivided into a pair of subclasses in which one subclass is greater than the MIC value and the other is less than or equal to that level of MIC. Number of progeny whose MIC fell into two different subclasses was counted separately. For each genetic marker, we summed the number of progeny whose segregating marker scores with "1" and "0" independently. For instance, at 2 *μg/ml* MIC, the number of progeny whose MIC are less than or equal to 2 *μg/ml* is 20 and 14 progeny had allele "0" and 6 had allele "1"; 259 progeny has MIC which is greater than 2 *μg/ml*, and 149 progeny had allele "0" score and 110 progeny had allele "1" at locus 1. This procedure was repeated for 7 different MIC levels for each of the 51

markers (Table A 4). The same procedure was used to classify genotypic classes of LAC and URA5 genes (Table A 5).

2.3.2 Test strains for Resistance to Fluconazole

Two parental strains (JEC20 and CDC92-15) and a total of two hundred and eighty six progenies from the cross of those parental strains were examined for resistance to fluconazole with varying fluconazole concentrations. We chose fluconazole because it is one of the most widely used antifungal drugs for long-term maintenance treatment of Cryptococcus infections.

In this study, the following fluconazole concentrations were use: 0.0 μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, and 64 μg/ml. Each isolate was tested at all concentrations of fluconazole separately by two methods: the colony size method and the microbroth dilution based on the National Committee for Clinical Laboratory Standards (NCCLS, 1997). Since the rapid and statistically analyzable colony size method is comparable to the NCCLS method for *C. neoformans* (Xu, *et al.*, 1998), only colony size data were used. In contrast, NCCLS data were categorical data, not suitable for quantitative analyses, and therefore were used only to confirm colony size data.

To maximize representation of measurements, ten independent colonies were measured at each concentration and the mean of these 10 measurements was calculated (Table A 3). Briefly, for each strain, a single colony was suspended in 200 μl of sterile water by vortexing and 1 μl of liquid suspension was streaked onto YMA (Yeast Morphology Agar) plates containing 0.0, 0.5, 1, 2, 4, 8, 16, 32,

and 64 μg of fluconazole per mL respectively. The plates were incubated for 48 h at 37 °C and examined for evidence of growth under a microscope with 100x magnification (Xu *et al.*, 1998).The degree of drug-resistance of strains was measured according to the change of colony size after the treatment of fluconazole on the isolate. MICs of fluconazole, the lowest concentration of fluconazole that prevents visible growth of *C .neoformans* in broth dilution susceptibility tests, were determined by a standard broth microdilution method, M-27A, recommended by NCCLS (1997). If a strain showed no growth on the plate with certain concentration of fluconazole, this no-growth phenotype was necessary for estimating MIC to this level of fluconazole concentration.

2.4 Data Analyses

2.4.1 t-test on single marker association with vegetative fitness at different drug concentrations (Quantitative analysis)

To determine whether a single is or multiple genes are involved in vegetative fitness of hybrids under fluconazole - stressful condition, we performed a two-sample t-test on a single marker based on its genetic class information. If means of vegetative fitness of two genetic classes of a single marker are not significantly different ($p>0.05$, $df = 284$), that indicates the genetic marker is not involved in vegetative fitness at tested fluconazole concentration. t-test was performed for each marker at each fluconazole concentration.

2.4.2 Chi-Square test for association between a single marker and the Minimum Inhibitory Concentration for different drug concentrations (Qualitative analysis)

A 2x2 contingency table was employed to test potential association between a marker and the MIC because MIC comprises categorical data and each MIC class and allelic class have two categories respectively. If the frequencies of the two allelic classes of a single marker have different MIC, a statistically, significant association between the vegetative fitness at that fluconazole concentration and the genetic loci. 2X3 contingency table was used for LAC and URA5 genes because they are co-dominant and each of them has three allelic classes.

2.4.3 Comparison of vegetative fitness between parental lines and hybrids at different concentrations of fluconazole

The objectives of comparing mean vegetative fitness between parents and progeny is to examine whether there is the evidence for outbreeding depression, and if so, the relationships between outbreeding depression and fluconazole concentration. The mean vegetative fitness among all testing conditions were compared. A graphical overview of vegetative fitness distribution was used to present the number of individuals that fall into different intervals of vegetative fitness.

2.4.4 Regression analysis of vegetative fitness *vs.* fluconazole concentration

To investigate the relationship between fluconazole concentration and vegetative fitness, Fitted Line Plot procedure of MINTAB (version 13.20) was performed to look for better lines or curves to fit the data. Residual Analysis and

Regression Diagnostics procedures were employed to assess if the residuals appear random and normally distributed. Linear regression was the method to find the relationship between a response variable Vegetative Fitness (Y) and a predictor variable Fluconazole Concentration (X) by extending the simple linear regression model to the linear regression of natural logarithm (LN) value of vegetative fitness vs. fluconazole concentration through transformation of data until the best model is found.

2.4.5 Pearson correlation between concentrations of fluconazole

To examine the strength of a relationship between vegetative fitness at different concentrations of fluconazole, we calculated the Pearson product moment correlation coefficient between each pair of concentrations and performed hypothesis test that there is zero correlation between a pair of concentrations (MINTAB 13.20)

2.5 Results

2.5.1 Multiple markers are associated with vegetative fitness at different drug concentrations

Results from t-test show that 74.3% of the 51 mapped RAPD markers showed no influence on vegetative fitness at all concentrations. The exception were markers L047, L020, L008, L046, L010, L049, L026, L032, L013, L015, and L005 (Table 2-1). Alleles of markers L020 and L005 displayed significantly different effects on vegetative fitness from 2 to 16, and 0.5 to 8 μg/ml fluconazole respectively. Marker L010 showed significant effect at 16, 32, and 64 μg/ml of fluconazole. Marker L013

had strong effect on vegetative fitness from 0.5 to 2 μg/ml of fluconazole. Marker L047 displayed significant difference at 16 and 32 μg/ml and the rest of marker showed significant influence at one fluconazole concentration.

Results from the t-test show that LAC gene had significant influence on vegetative fitness at most fluconazole concentrations with the exception of 64 μg/ml. URA5 gene displayed no influence on vegetative fitness at lower fluconazole concentrations but showed significant influence at higher concentrations but not at 64 μg/ml (Table 2-2).

Results from t-test of 36 combined genotypic classes, based on 9 possible genotypic classes from the dihybrid cross of LAC and URA5, displayed that 47.2% of 36 genotypic classes had significant influence on vegetative fitness at different fluconazole concentrations. Interestingly, if an allelic class is associated with the vegetative fitness, it will influence the vegetative fitness at almost all fluconazole concentrations (Table 2-3).

2.5.2 Multiple markers are associated with Minimum Inhibit Concentration at different drug concentrations

Based on the results of classifications of genetic markers, data of each pair of MIC subclasses were summarized in a frequency table in which two variables, MIC and genetic marker, were presented simultaneously. Because data were discrete, a contingency table was constructed by listing all the levels of one variable MIC as rows and the other variable genetic marker as columns and then found the joint products for each cell. The products were then summed across both rows and columns.

The sums are placed in the margins, the values of which are called marginal frequencies. Since each variable has two levels, hypothesis tests were performed on 2x2 contingency tables in order to decide whether or not relationship between MIC and genetic marker are present. We conducted Chi-square tests on all different levels of MIC for each genetic marker and found that vegetative fitness and fluconazole resistance are controlled by multiple genes and different genes influence different levels of resistance at $p < 0.05$ with 1 degree of freedom (Table 2-4). At lower and higher fluconazole concentrations (less than 4 or greater than 32 μg/ml), a few genes were associated. Interestingly, a large number of genes were linked to vegetative fitness at middle levels of concentrations of fluconazole (4 to 32 μg/ml). For instance, three and four genes are involved in vegetative fitness of hybrids at 4 and 64 *μg/ml,* respectively; and fifteen and twelve genes were involved in the control of vegetative fitness at 8 and 16 μg/ml separately. No significant results showed participation of genes in controlling vegetative fitness at 0, 0.5, 1, and 128 μg/ml fluconazole.

Both LAC and URA5 genes were linked to vegetative fitness at middle levels of fluconazole concentrations (8 to 16 μg/ml and 4 to 16 μg/ml). None of them was associated with vegetative fitness at lower or higher fluconazole concentrations (Table 2-5).

Table 2.1. Comparison of Contribution to vegetative fitness of *C. neoformans* between genotypic classes on each RAPD marker. Two sample t-test for two genotypic marker classes for each marker at all levels of concentration of fluconazole. t-statistic is for the hypothesis that two classes have no significant difference on effect on fluconazole-resistance of *C. neoformans*. The column p contains the probability of significant difference between markers on effect of vegetative fitness of *C. neoformans.* Significance at the 5% and 1% levels are indicated by *, and **, respectively

		Con_0		Con_0.5		Con_1	Con_2		Con_4		Con_8		Con_16		Con_32		Con_64	
M	A	mean	P	mean	P	mean	P	mean	P	mean	P	mean	P	mean	P	mean	P	mean	P
L047	0	41.23	0.193	40.74	0.153	39.59	0.143	37.19	0.138	35.46	0.143	32.78	0.062	27.57	**0.032***	14.73	**0.006****	6.47	0.165
	1	37.69		36.93		35.66		33.12		31.34		27.53		22.38		11.17		5.56	
L044	0	40.68	0.279	39.96	0.287	38.50	0.367	35.69	0.495	33.52	0.684	30.96	0.354	26.02	0.217	12.99	0.510	6.09	0.604
	1	37.79		37.18		36.12		33.86		32.40		28.40		23.06		12.13		5.76	
L025	0	43.54	0.344	42.15	0.504	40.56	0.530	38.40	0.508	36.60	0.454	32.40	0.783	27.22	0.669	13.65	0.733	5.76	0.514
	1	40.30		39.90		38.45		36.11		33.96		31.41		25.88		13.07		6.34	
L020	0	41.17	0.113	40.68	0.089	39.44	0.105	37.56	**0.034***	35.84	**0.034***	32.77	**0.019***	26.81	**0.037***	13.39	0.158	6.01	0.657
	1	36.97		36.26		35.21		31.93		30.04		26.36		21.87		11.56		5.72	
L002	0	40.62	0.093	39.69	0.167	38.65	0.117	35.63	0.275	33.73	0.328	30.43	0.302	25.17	0.269	12.84	0.347	5.75	0.673
	1	35.97		35.93		34.36		32.57		30.91		27.44		22.40		11.56		6.04	
L023	0	38.64	0.620	37.91	0.528	36.68	0.524	34.02	0.457	32.16	0.445	28.97	0.619	24.04	0.768	12.92	0.367	6.09	0.434
	1	40.09		39.71		38.51		36.20		34.47		30.48		24.81		11.63		5.53	
L011	0	39.38	0.053	38.67	0.092	37.55	0.110	34.95	0.128	33.19	0.108	29.65	0.332	24.72	0.140	12.85	0.087	5.99	0.248
	1	30.60		31.33		30.53		28.14		25.78		25.15		18.82		9.11		4.73	
L016	0	38.80	0.436	38.19	0.504	37.01	0.684	34.45	0.656	32.57	0.771	29.11	0.817	24.17	0.915	12.61	0.475	5.92	0.555
	1	34.93		34.93		35.00		32.21		31.06		30.32		23.69		10.86		5.20	
L038	0	38.88	0.592	38.30	0.549	37.13	0.411	34.52	0.335	32.65	0.375	29.41	0.250	24.24	0.337	12.56	0.265	5.91	0.959
	1	34.93		33.98		31.16		27.35		25.85		20.58		17.84		8.47		6.00	
L008	0	39.23	0.574	38.60	0.566	37.35	0.647	34.66	0.863	32.84	0.853	29.57	0.642	24.44	0.470	12.56	0.300	5.90	0.513
	1	35.86		35.23		34.64		33.61		31.69		26.67		20.52		9.51		4.94	
L046	0	39.10	0.871	38.40	0.929	37.26	0.802	34.78	0.796	32.84	0.945	29.53	0.933	24.33	0.968	12.24	0.343	5.58	**0.014***
	1	38.50		38.07		36.34		33.81		33.10		29.21		24.20		13.96		7.76	
L010	0	39.28	0.244	38.95	0.125	37.95	0.096	35.45	0.081	33.47	0.141	30.33	0.107	25.53	**0.019***	13.31	**0.010****	6.22	**0.028***
	1	35.43		33.97		32.51		29.63		28.42		24.78		18.62		9.18		4.46	
L049	0	36.13	0.194	35.97	0.258	35.88	0.456	35.12	0.914	34.29	0.644	32.14	0.340	28.36	0.092	15.22	0.054	8.47	**0.001****
	1	41.20		40.50		38.98		35.77		33.62		29.67		23.84		12.08		5.22	
L022	0	38.91	0.836	38.13	0.607	36.89	0.515	34.44	0.668	32.63	0.686	29.60	0.904	24.32	0.971	12.25	0.479	5.74	0.422
	1	39.62		39.87		39.10		35.93		34.08		29.17		24.21		13.44		6.42	
L028	0	38.30	0.448	37.69	0.434	36.41	0.380	33.49	0.278	32.00	0.465	28.68	0.466	23.10	0.186	11.90	0.203	5.60	0.271
	1	40.42		39.84		38.84		36.55		34.13		30.82		26.44		13.63		6.34	
L026	0	41.88	0.574	42.69	0.779	40.86	0.756	40.88	0.912	39.30	0.831	34.58	0.973	29.25	0.828	13.67	0.695	6.72	**0.028***

	1	42.51		41.56		40.05		37.62		35.62		32.60		27.18		13.72		6.24	
L012	0	37.94	0.445	37.34	0.416	36.28	0.438	33.68	0.418	31.82	0.411	28.31	0.247	23.60	0.402	12.43	0.805	5.70	0.360
	1	40.24		39.74		38.60		36.14		34.39		31.94		25.87		12.79		6.37	
L021	0	39.90	0.444	39.32	0.407	37.97	0.477	35.26	0.577	33.61	0.494	30.28	0.441	24.84	0.557	12.92	0.481	6.21	0.282
	1	37.83		37.12		36.08		33.75		31.69		28.12		23.41		11.98		5.50	
L032	0	42.92	0.060	42.16	**0.028***	40.59	0.105	38.25	0.108	36.39	0.099	33.07	0.143	26.98	0.341	13.36	0.533	6.20	0.411
	1	34.38		33.51		34.27		31.80		29.61		26.96		23.54		12.16		7.06	
L027	0	43.83	0.601	42.78	0.760	40.94	0.898	38.61	0.785	37.14	0.811	34.30	0.895	28.13	0.885	14.21	0.915	6.57	0.852
	1	38.78		39.84		39.71		35.87		34.69		32.90		29.40		13.69		6.09	
L003	0	37.38	0.593	37.08	0.732	35.73	0.626	32.59	0.526	31.03	0.557	28.29	0.723	23.05	0.629	12.33	0.879	6.09	0.697
	1	38.91		38.05		37.13		34.44		32.80		29.36		24.30		12.11		5.81	
L034	0	43.17	0.296	42.63	0.184	41.50	0.141	38.59	0.198	37.39	0.091	34.72	0.061	28.22	0.160	13.95	0.193	6.46	0.954
	1	38.17		36.38		34.56		32.38		29.11		25.36		22.20		10.97		6.39	
L019	0	35.72	0.137	35.53	0.183	35.01	0.299	32.42	0.310	30.97	0.405	27.28	0.334	22.58	0.391	11.63	0.445	5.02	0.120
	1	40.21		39.47		38.10		35.51		33.58		30.31		24.91		12.76		6.16	
L042	0	39.96	0.450	39.50	0.309	37.93	0.483	35.29	0.513	33.66	0.387	30.59	0.308	25.17	0.379	13.17	0.139	5.92	0.917
	1	37.76		36.60		35.92		33.37		31.05		27.51		22.87		11.07		5.85	
L041	0	39.94	0.667	38.55	0.987	36.91	0.806	34.54	0.932	31.70	0.494	28.63	0.554	23.43	0.479	11.73	0.316	5.66	0.546
	1	38.76		38.59		37.57		34.78		33.65		30.33		25.18		13.07		6.07	
L051	0	39.17	0.972	38.97	0.804	37.70	0.818	34.96	0.899	33.32	0.859	31.02	0.523	24.52	0.911	11.94	0.631	5.84	0.925
	1	39.07		38.23		37.01		34.57		32.76		29.00		24.22		12.65		5.91	
L013	0	30.99	0.070	31.50	**0.039***	30.68	**0.050***	28.64	**0.080**	27.07	0.104	24.01	0.119	18.70	0.059	10.08	0.128	4.65	0.121
	1	39.80		39.05		37.92		35.25		33.38		30.10		25.07		12.89		6.07	
L037	0	35.85	0.231	35.55	0.273	34.27	0.264	31.94	0.314	29.57	0.237	28.44	0.710	21.88	0.318	11.29	0.377	5.01	0.186
	1	39.82		39.13		37.93		35.31		33.64		29.72		24.86		12.72		6.08	
L015	0	30.59	0.053	30.47	**0.041***	30.13	0.070	27.08	0.057	26.60	0.130	23.67	0.161	20.02	0.240	12.40	0.992	7.43	0.118
	1	40.15		39.45		38.17		35.67		33.67		30.25		24.76		12.38		5.72	
L017	0	33.62	0.081	32.90	0.066	32.09	0.085	29.98	0.128	29.44	0.300	27.52	0.564	22.69	0.567	12.22	0.854	6.19	0.634
	1	39.60		39.08		37.93		35.24		33.13		29.59		24.46		12.54		5.79	
L036	0	36.19	0.180	36.06	0.248	35.38	0.373	32.78	0.396	30.30	0.275	28.57	0.706	22.01	0.274	11.11	0.260	5.18	0.271
	1	40.24		39.49		38.04		35.37		33.74		29.76		24.99		12.75		5.97	
L024	0	38.84	0.612	38.25	0.627	37.21	0.676	34.60	0.714	32.93	0.762	30.80	0.797	24.74	0.820	12.32	0.651	6.31	0.465
	1	40.29		39.63		38.40		35.68		33.85		30.02		25.34		12.97		5.79	

L040	0	38.63	0.785	37.92	0.750	36.68	0.739	34.34	0.834	32.45	0.807	30.36	0.695	24.33	0.989	12.39	0.934	6.06	0.699
	1	39.41		38.82		37.62		34.94		33.17		29.20		24.36		12.50		5.80	
L009	0	39.90	0.444	39.32	0.407	37.97	0.477	35.26	0.577	33.61	0.494	30.28	0.441	24.84	0.557	12.92	0.481	6.21	0.282
	1	37.83		37.12		36.08		33.75		31.69		28.12		23.41		11.98		5.50	
L035	0	41.72	0.598	41.40	0.791	40.09	0.783	37.34	0.859	36.29	0.804	33.48	0.530	27.25	0.670	13.32	0.703	6.25	0.465
	1	43.91		42.49		41.23		38.08		35.23		30.75		25.65		14.12		7.08	
L031	0	40.10	0.383	39.11	0.328	37.87	0.364	35.79	0.473	34.13	0.530	30.76	0.460	26.33	0.945	13.33	0.902	6.31	0.905
	1	42.73		42.00		40.56		37.97		36.09		33.11		26.14		13.15		6.41	
L004	0	39.88	0.275	39.37	0.198	38.00	0.308	35.26	0.426	33.18	0.697	29.85	0.680	24.33	0.912	12.54	0.936	5.85	0.690
	1	36.36		35.30		34.76		32.66		31.87		28.46		24.65		12.41		6.17	
L005	0	37.98	0.056	37.33	**0.049***	36.12	**0.050***	33.35	**0.028***	31.37	**0.016***	28.22	**0.042***	23.44	0.123	12.06	0.209	5.77	0.581
	1	44.81		44.24		43.01		41.26		40.28		35.79		28.44		14.27		6.26	
L018	0	38.50	0.362	37.95	0.421	36.72	0.415	34.10	0.376	32.01	0.198	28.75	0.299	23.78	0.379	12.31	0.444	5.88	0.735
	1	41.65		40.68		39.49		37.18		36.62		32.47		26.50		13.61		6.16	

Table 2-1 Comparison of contribution to vegetative fitness of *C. neoformans* between genotypic classes on each RAPD marker

Table 2.2. Comparison of contribution to vegetative fitness of *C. neoformans* among genotypic classes of LAC and URA5 codominant markers. Two sample t-test for any two genotypic marker classes for each mark at all levels of concentration of fluconazole. t-statistic is for the hypothesis that two classes have no significant difference on effect on fluconazole-resistance of *C. neoformans*. The column of p is the probability of significant difference between markers on effect of vegetative fitness of *C. neoformans.* Significance at the 5% and 1% levels are indicated by *, and **, respectively.

		Con_0		Con_0.5		Con_1		Con_2		Con_4		Con_8		Con_16		Con_32		Con_64	
Gene	**Allele**	**mean**	**P**	**mean**	**P**	**mean**	**P**	**mean**	**P**	**mean**	**P**	**mean**	**P**	**mean**	**P**	**mean**	**P**	**mean**	**P**
LAC	1	19.78	**0.001****	20.08	**0.003****	19.70	**0.009****	17.54	**0.008****	15.93	**0.007****	13.56	**0.007****	10.95	**0.005****	7.09	**0.045***	4.06	0.113
	2	32.10	**0.001****	31.45	**0.004****	29.90	**0.013***	27.8	**0.01***	26.83	**0.010***	24.35	**0.012***	20.71	**0.001****	10.97	0.108	5.39	0.210
	1/2	45.55	**0.001****	44.70	**0.01****	43.32	**0.031***	40.59	**0.02***	38.54	**0.026***	34.79	**0.037***	28.62	**0.023****	14.14	0.174	6.45	0.279
URA5	1	29.54	**0.030***	28.61	**0.031***	26.78	0.052	24.32	0.068	23.45	0.077	21.78	**0.011***	18.58	**0.007***	8.96	**0.026***	4.702	0.10
	2	20.69	0.052	20.23	0.058	19.12	0.097	17.09	0.117	16.01	0.132	11.61	**0.024***	9.27	**0.014***	5.69	**0.033***	3.475	0.13
	1/2	47.86	0.067	47.16	0.088	45.90	0.142	43.12	0.165	40.81	0.213	37.63	0.060	30.96	**0.040***	15.66	0.082	7.083	0.15

Table 2-2 Comparison of contribution to vegetative fitness of *C. neoformans* among genotypic classes of LAC and URA5 codominant markers

Table 2.3. Comparison of contribution to vegetative fitness of *C. neoformans* among genotypic classes of dihybrid cross of LAC and URA5 codominant markers. Two sample T-test for any two genotypic marker classes for two genes at all levels of concentration of fluconazole. T statistic is for the hypothesis that two classes have no significant difference on effect on fluconazole-resistance of *C. neoformans.* The column Gen_class stands for 36 combined genotypic classes. The column of p is the probability of significant difference between genotypic classes on effect of vegetative fitness of *C. neoformans.* Significance at the 5% and 1% levels are indicated by *, and **, respectively.

Gen_Class	Con_0(p)	Con_0.5(p)	Con_1(p)	Con_2(p)	Con_4(p)	Con_8(p)	Con_16(p)	Con_32(p)	Con_64(p)
11' 22'	0.343	0.346	0.378	0.405	0.404	0.419	0.401	0.456	0.422
11' 12'	0.257	0.228	0.293	0.315	0.547	0.815	0.959	0.962	0.986
11' 21'	**0.007****	**0.009****	**0.018***	**0.018***	**0.05***	0.060	0.068	0.222	0.390
11' 1/21'/2'	**0.016***	**0.020***	**0.026***	**0.018***	**0.038***	**0.041***	**0.043***	0.074	0.162
11' 11'/2'	**0.001****	**0.001****	**0.001****	**0.001****	**0.003****	**0.004****	**0.007****	**0.016***	**0.035***
11' 21'/2'	**0.013***	**0.015***	**0.030***	**0.037***	**0.056**	0.091	0.125	0.189	0.255
11' 1/21'	**0.001****	**0.002****	**0.007****	**0.010****	**0.028***	**0.038***	**0.041***	0.113	0.303
11' 1/22'	**0.004****	**0.006****	**0.015***	**0.012***	**0.041***	0.142	0.192	0.282	0.459
22' 12'	0.521	0.565	0.568	0.564	0.501	0.453	0.401	0.449	0.384
22' 21'	0.913	0.880	0.925	0.959	0.976	0.818	0.958	0.976	0.775
22' 1/21'/2'	0.234	0.224	0.248	0.275	0.304	0.198	0.316	0.274	0.584
22' 11'/2'	0.342	0.298	0.294	0.358	0.358	0.232	0.373	0.185	0.289
22' 21'/2'	0.426	0.410	0.456	0.481	0.472	0.359	0.498	0.276	0.617
22' 1/21'	0.588	0.602	0.701	0.767	0.791	0.666	0.793	0.778	0.905
22' 1/22'	0.789	0.793	0.866	0.919	0.910	0.935	0.764	0.878	0.704
12' 21'	**0.006****	**0.008****	**0.016***	**0.021***	**0.019***	**0.012***	**0.009****	**0.016***	**0.046***
12' 1/21'/2'	**0.001****	**0.0001****	**0.001****	**0.0001****	**0.0001****	**0.0001****	**0.0001****	**0.0001****	**0.001****
12' 11'/2'	**0.001****	**0.0001****	**0.001****	**0.0001****	**0.001****	**0.001****	**0.002****	**0.010***	**0.006****
12' 21'/2'	**0.001****	**0.0001****	**0.001****	**0.0001****	**0.0001****	**0.0001****	**0.0001****	**0.001****	**0.004****
12' 1/21'	**0.002****	**0.003****	**0.01****	**0.021***	**0.024***	**0.014***	**0.012***	**0.022***	0.089
12' 1/22'	**0.003****	**0.004****	**0.005****	**0.004****	**0.003****	**0.015***	**0.028***	0.067	0.155
21' 1/21'/2'	**0.0001****	**0.001****	**0.0001****	**0.0001****	**0.001****	**0.003****	**0.005****	**0.0001****	**0.011***
21' 11'/2'	**0.031***	**0.016****	**0.012***	**0.025***	**0.045***	0.063	0.106	**0.055**	0.036*
21' 21'/2'	**0.036***	**0.041****	0.059	0.056	0.070	0.139	0.162	0.037*	0.107
21' 1/21'	0.261	0.325	0.461	0.549	0.602	0.694	0.663	0.507	0.739
21' 1/22'	0.654	0.745	0.826	0.879	0.817	0.472	0.369	0.758	0.806
1/21'/2'	0.382	0.474	0.681	0.532	0.778	0.851	0.850	0.523	0.290
1/21'/2'	0.057	0.061	0.070	0.090	0.232	0.225	0.328	0.916	0.958
1/21'/2'	**0.032***	**0.018***	**0.016***	**0.021***	**0.047***	**0.040***	0.065	0.053	0.146
1/21'/2' 11'/2'	**0.001****	**0.0001****	**0.0001***	**0.0001****	**0.0001****	**0.0001****	**0.0001****	**0.0004****	**0.016***
11'/2' 21'/2'	0.622	0.470	0.346	0.514	0.587	0.516	0.634	0.623	0.336
11'/2' 1/21'	0.304	0.164	0.095	0.142	0.175	0.158	0.243	0.124	0.076
11'/2' 1/22'	0.079	**0.032***	**0.016****	**0.026***	0.053	**0.014***	**0.023***	**0.043***	**0.030***
21'/2' 1/21'	0.488	0.399	0.348	0.309	0.311	0.343	0.399	0.154	0.271
21'/2' 1/22'	0.115	0.088	0.076	0.054	0.081	**0.016***	**0.020***	**0.027***	0.091
1/21' 1/22'	0.482	0.491	0.564	0.609	0.716	0.290	0.217	0.381	0.614

Table 2-3 Comparison of contribution to vegetative fitness of *C. neoformans* among genotypic classes of dihybrid cross of LAC and URA5 codominant markers

Table 2.4. RAPD genetic markers that are associated with MIC at different concentrations of fluconazole. Chi-square tests on all different levels of MIC for each single marker found that vegetative fitness and fluconazole resistance are controlled by multiple genes and different genes influence different levels of resistance at $p < 0.05$ with 1 degree of freedom.

	MIC (*ug/ml*)						
	2	**4**	**8**	**16**	**32**	**64**	**128**
Marker	32*,35, 49**	9**,10**, 11**,13*, 16**,17**, 20*,5**, 46*,49**	5*,9**, 16*,17**, 18**,20**, 26*,33*, 34*,35**, 36*,38*, 39*,46**, 49**	2**,9**, 10**,17*, 18**,20*, 33*,35**, 36**,39*, 46**,49**	9**,3*, 32*, 4*, 35**,41**	9*,35**, 41**,	

Table 2-4 RAPD Genetic Markers that associated with MIC at different concentrations of fluconazole

Table 2.5. RFLP of co-dominant LAC and URA5 genetic markers that associated with MIC at different concentrations of fluconazole. Chi-square tests on all different levels of MIC for each single marker found that vegetative fitness and fluconazole resistance are controlled by LAC and URA5 genes and different genes influence different levels of resistance at $p < 0.05$ with 1 degree of freedom.

	MIC (μg/ml)						
Gene	**2**	**4**	**8**	**16**	**32**	**64**	**128**
LAC			**	**			
URA5		**	**	**			

Table 2-5 RFLP codominant genetic Markers of LAC and URA5 that associated with MIC at different concentrations of fluconazole

2.5.3 Evidence of outbreeding depression within serotype AD strains

Hybrids of *C. neoformans* show the lowest vegetative fitness compared to that of two parental strains when progeny were not exposed to fluconazole stress (Figure 2-1). Vegetative fitness of progeny fell into neither of the range of that of parental strains. Crosses between two varieties resulted in a significant reduction in vegetative fitness of progeny. This indicates that outbreeding depression occurs in *C. neoformans*

Overall, outbreeding reduced vegetative fitness and fluconazole resistance. As the concentration of fluconazole increased from 0 to 2 $\mu g/ml$, vegetative fitness and drug resistance of hybrids were reduced (Figure 2-2). However, with increasing drug concentration, the relative vegetative fitness of progeny increased, indicating that the level of outbreeding depression decreased as well.

Figure 2.1. Means of the vegetative fitness of parents and offspring are different in the absence of fluconazole. Hybrid of *C. neoformans* shows the lowest vegetative fitness compared to that of two parental strains at non-fluconazole state. Cross between two varieties resulted in a significant reduction in vegetative fitness of progeny. This evidence indicates that outbreeding depression occurs within hybrid population of *C. neoformans.* Means of growth grate are represented by bars.

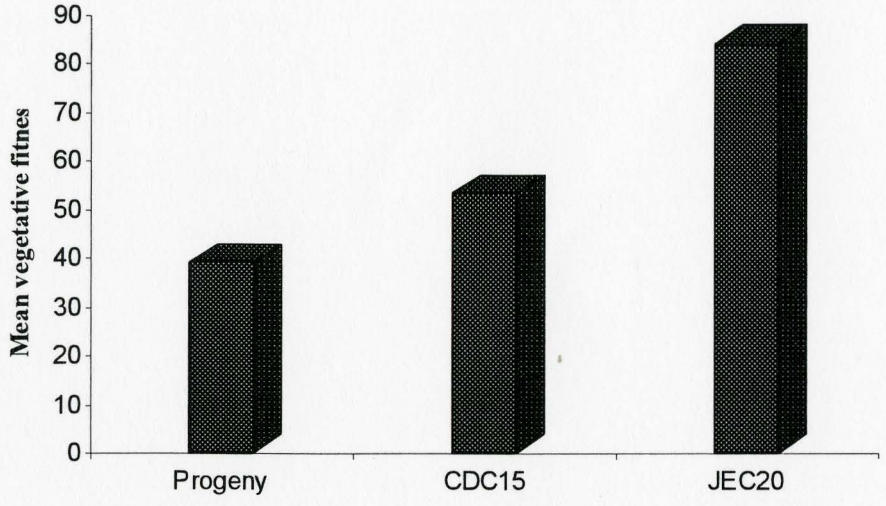

Figure 2-1 Means of vegetative fitness of progeny and parents in the absences of fluconaole

Figure 2.2. Fluconazole-concentration-dependent outbreeding depression in *C. neoformans*. Lines in the graph represent the trends of distribution of vegetative fitness vs. fluconazole concentration of progeny and parents.

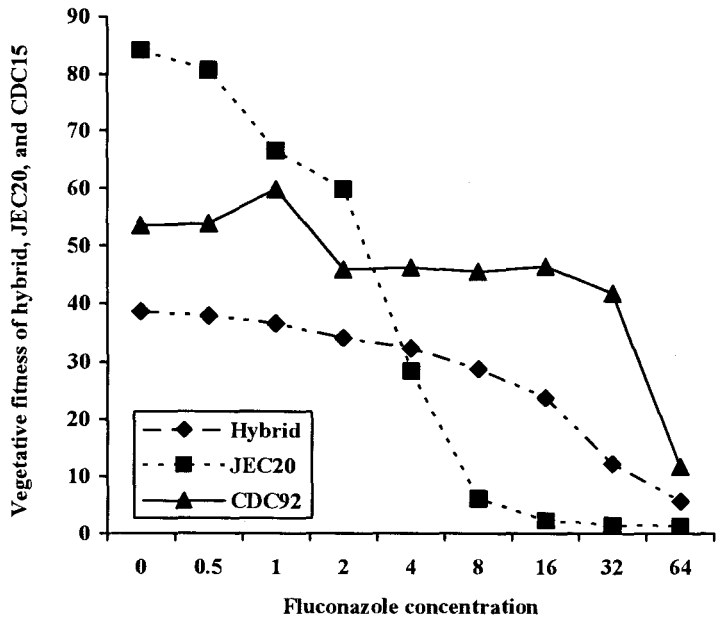

Figure 2-2 Vegetative fitness of hybrid, cdc92-15, and jec20 at different fluconazole concentrations

2.5.4 Evidence of heterosis within hybrid progeny

The histogram of vegetative growth of hybrids displays two distinct peaks, indicating a bimodal distribution of vegetative fitness of hybrids in the absence of fluconazole (Figure 2-3). Interestingly, we also discover the mean of vegetative fitness of hybrids fall into neither parental strain. With increasing fluconazole concentration, the peak with greater vegetative fitness decreases and progeny fitness is unimodally distributed (Figure 2-4 to Figure 2-11). However, some progeny displayed greater fluconazole-resistance than parental strains and the number of progeny with higher fitness increases with increasing fluconazole concentration (Figure 2-11). Specifically, 10.84% of progeny has greater vegetative fitness than both parents at 64 *μg/ml* fluconazole concentrations. Only 1.75% of progeny with fitness is greater than parents at 32 *μg/ml* (Table 2-6). These increased numbers of progeny with greater drug resistance at the highest drug concentration suggest potential hybrid vigor.

Figure 2.3. Distribution of vegetative fitness of hybrids in the absence of fluconazole. Vegetative fitness of the hybrid displays a bimodal distribution. Arrows indicate means of vegetative fitness of parents and hybrids, respectively.

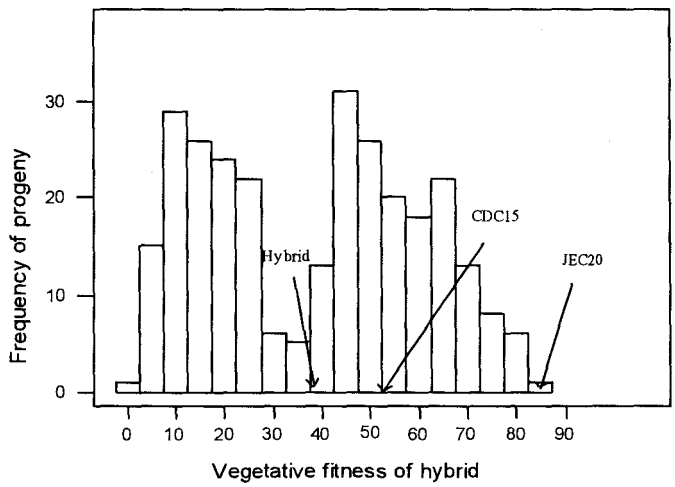

Figure 2-3 Distribution of vegetative fitness of hybrids in the absence of fluconazole

Figure 2.4. Distribution of vegetative fitness of hybrids for 0.5 *μg/ml* fluconazole.

Vegetative fitness of the hybrid exhibits a bimodal distribution for 0.5 *μg/ml* fluconazole.

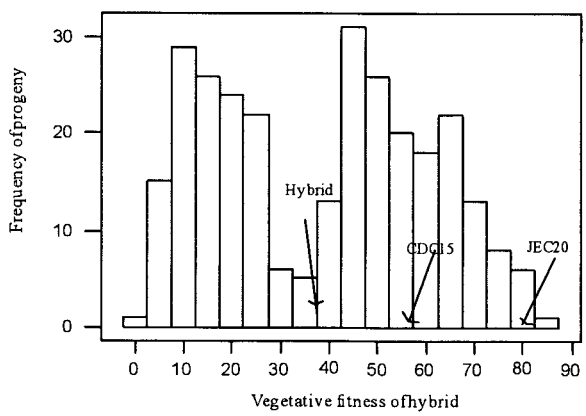

Figure 2-4 Distribution of vegetative fitness of hybrids for 0.5 µg/ml fluconazole

Figure 2.5. Vegetative fitness of the hybrid shows a bimodal distribution. One mode exhibits reduced kurtosis for 1.0 $\mu g/ml$ fluconazole and the distribution pattern is slightly skewed to the right.

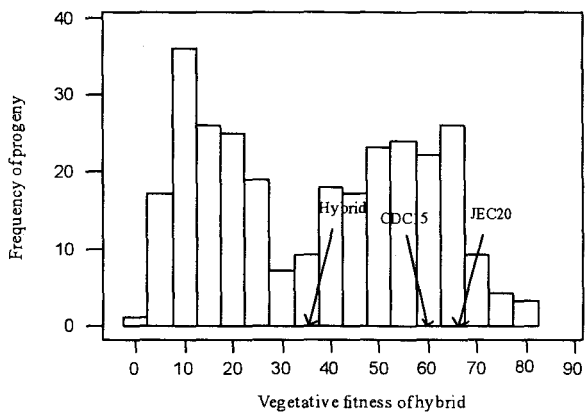

Figure 2-5 Distribution of vegetative fitness of hybrids for 1.0 µg/ml fluconazole

Figure 2.6. Vegetative fitness of the hybrid maintained a bimodal distribution pattern for 2.0 *μg/ml*. Shape of distribution is similar to that for 1.0 *μg/ml* fluconazole. Distribution displays a right tail skew. Bimodal distribution still can be observed.

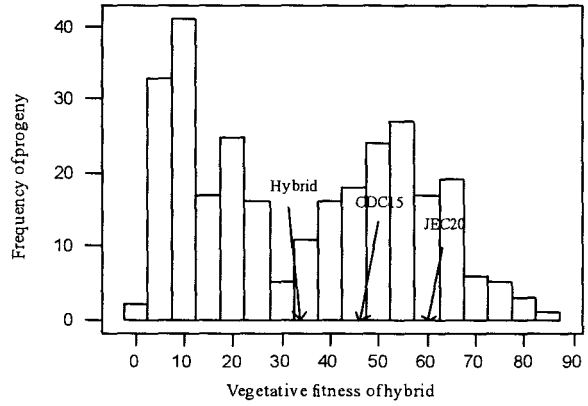

Figure 2-6 Distribution of vegetative fitness of hybrids for 2.0 μg/ml fluconazole

Figure 2.7. Vegetative fitness of hybrids exhibits a slightly bimodal distribution. One mode displays reduced kurtosis for 4.0 *μg/ml* compared with one for 2.0 *μg/ml* and the pattern of distribution is skewed to the right.

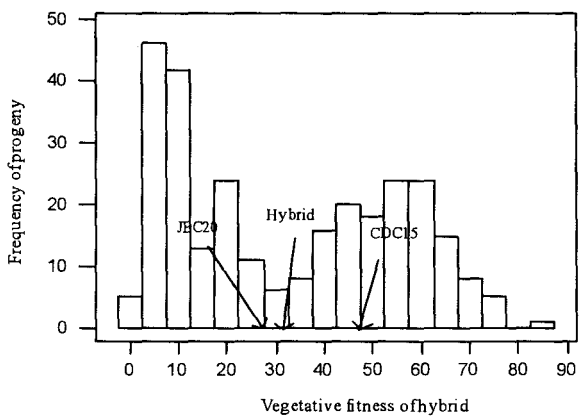

Figure 2-7 Distribution of vegetative fitness of hybrids for 4.0 μg/ml fluconazole

Figure 2.8. Vegetative fitness of the hybrid exhibits a right-skewed distribution and the bimodal distribution disappeared for 8 $\mu g/ml$ fluconazole.

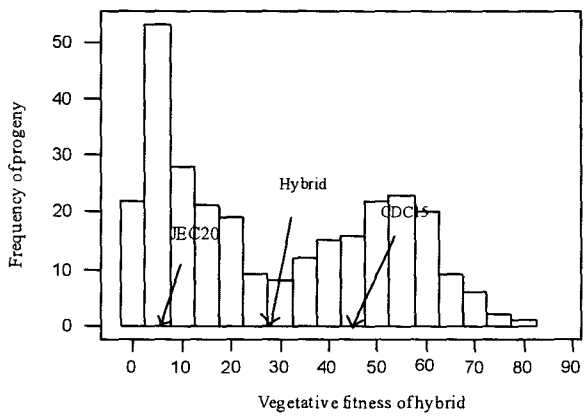

Figure 2-8 Distribution of Vegetative fitness of hybrids for 8.0 μg/ml fluconazole

Figure 2.9. Distribution of vegetative fitness of the hybrid displays a strong right-skewed distribution and no bimodal distribution was observed for 16 *μg/ml* fluconazole.

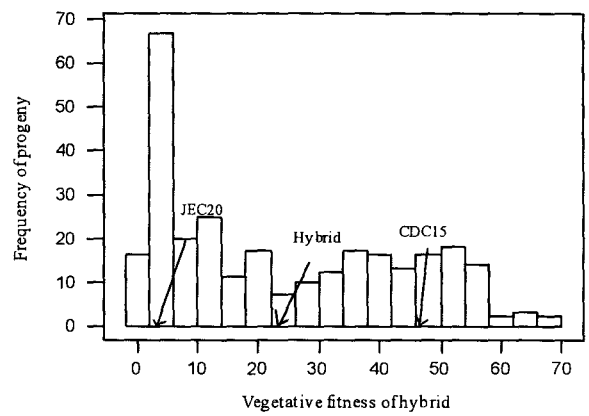

Figure 2-9 Distribution of vegetative fitness of hybrids for 16 µg/ml fluconazole

Figure 2.10. Vegetative fitness of the hybrid has a stronger right-skewed distribution and a few progeny demonstrate higher fluconazole-resistance for 32 $\mu g/ml$ fluconazole.

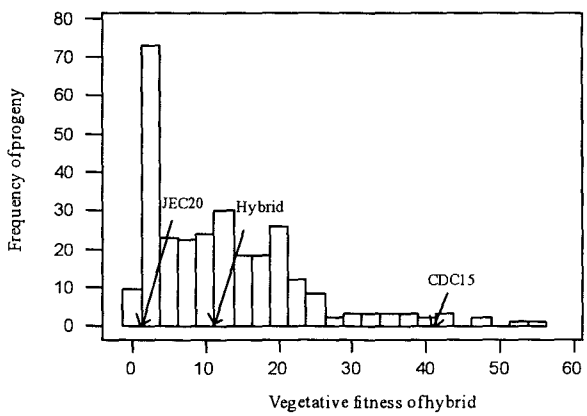

Figure 2-10 Distribution of vegetative fitness of hybrids for 32 μg/ml fluconazole

Figure 2.11. Vegetative fitness of the hybrid displays the strongest right-skewed distribution and the number of progeny demonstrates the highest fluconazole-resistance for 64 *μg/ml* fluconazole suggesting hybrid vigor among progeny. This hybrid vigor was likely environmentally dependent. Heterosis had been shown in the highest drug concentrations only but not in non- fluconazole environment.

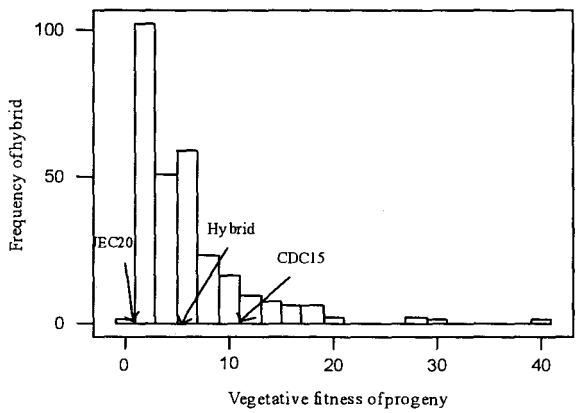

Figure 2-11 Distribution of vegetative fitness of hybrids for 64 µg/ml fluconazole

Table 2.6. Summary information for percentage of progeny with fitness lower, higher, and between both parents at different fluconazole concentrations. The number of progeny with higher fitness increases with increasing fluconazole concentration. Specifically, 10.84% of progeny has greater vegetative fitness than both parents at 64 *μg/ml* fluconazole concentrations. Only 1.75% of progeny with fitness is greater than parents at 32 *μg/ml*. These increased numbers of progeny with greater drug resistance at the highest drug concentration suggest potential hybrid vigor.

Drug concentration	Mean of JEC20	Mean of CDC15	Mean of progen	% of progeny with fitness lower than both	% of progeny with fitness higher than	% of progeny with fitness between both
0	84.13	53.44	38.59	69.58%	0.00%	30.42%
0.5	80.67	53.78	37.86	70.63%	0.70%	28.67%
1	66.44	59.8	36.55	80.77%	6.64%	12.59%
2	59.8	45.86	34.11	63.29%	15.73%	20.98%
4	28.4	46.2	32.34	49.30%	36.01%	14.69%
8	6.1	45.5	28.76	24.13%	30.07%	45.80%
16	2.3	46.4	23.75	11.89%	18.53%	69.58%
32	1.5	41.8	12.17	6.64%	1.75%	91.61%
64	1.3	11.7	5.67	10.49%	10.84%	78.67%

Table 2-6 Summary information for percentage of progeny with fitness lower, higher, and between both parents at different fluconazole concentrations

2.5.5 Linear regression of fluconazole concentration vs. vegetative fitness

Hybrids of *C. neoformans* showed different degrees of growth on different fluconazole concentrations. In general, the vegetative fitness is lower in higher drug concentration media (Figure 2-12). After the concentration of fluconazole reached 16 μg/ml, the mean vegetative fitness fell rapidly. Stable vegetative fitness from 0.5 to 16 μg/ml suggest that these drug concentrations have very little or no significant effects on *C. neoformans* vegetative fitness.

The goal of regression analysis is to determine the values of parameters for a function that best fit a set of data. To find the best model to fit the set of data, value of vegetative fitness was transformed to natural logarithm and significance of fit was found to be very high ($p < 0.001$). Vegetative fitness of *C. neoformans* displayed a linear relationship vs. the concentration of fluconazole. The linear model appears to provide a good fit to the data. Regression equation is: VegetativeFitness = 3.61 - 0.03 Concentration. From the analysis, we also obtain R^2, describing how much variation in dependent variable is explained by variation in the independent variable. Our analysis indicates that the concentration of fluconazole accounts for 99.1% of the variation in vegetative fitness of the hybrid in this cross.

Figure 2.12. Vegetative fitness of *C. neoformans* displays a linear relationship with fluconazole concentrations. Growth is expressed as the mean of colony sizes of 286 progeny at each concentration of fluconazole. Regression equation: VegetativeFitness = 37.5097 - 1.07544 Concentr. + 0.0090329 Concentr.2. The R^2 = 99.4% indicates that concentration of fluconazole accounts for 99.4% of the variation in colony size of the hybrid. The line labeled Plot 1 Regr. is the mean growth. The upper and lower error bars are 95% limits for the vegetative fitness of *C. neoformans*.

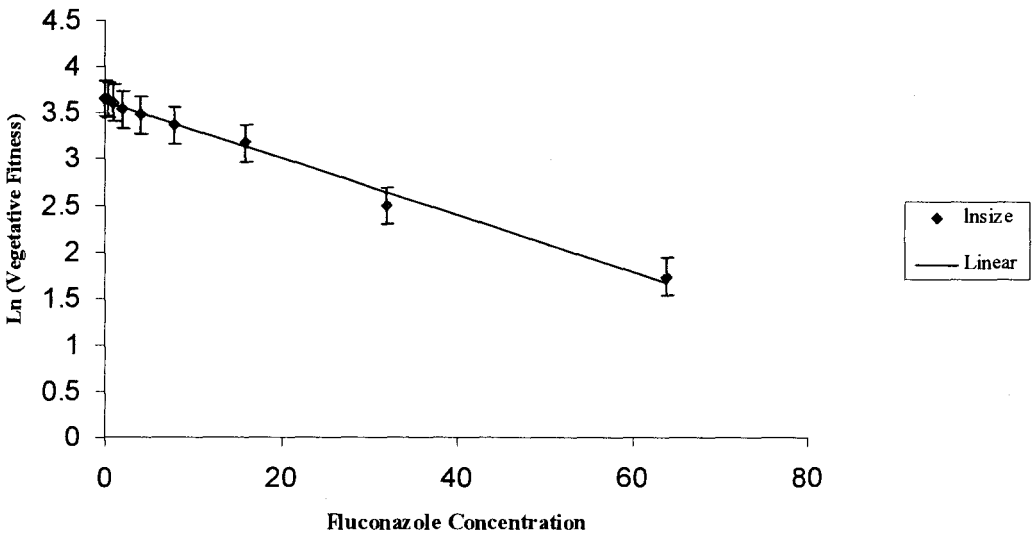

Figure 2-12 Linear regression of vegetative fitness of *C. neoformans* at different concentrations of fluconazole

2.5.6 Correlation between vegetative fitness at different concentrations of fluconazole

The tests on correlation between two fluconazole concentration showed that all the p-values were smaller than 0.01 and there was sufficient evidence at $\alpha = 0.01$ that the correlations were not zero, indicting that growth between all pairs at different concentrations of fluconazole were significantly correlated. The Pearson correlation coefficient between any pair of concentrations was positive. However, the degree of correlation varied (Table 2-7). Specifically, we observed the following: the closer the two concentrations, the higher correlation coefficient it is. For instance, the coefficient between concentrations 0 *μg/ml* and 0.5 *μg/ml* was greater than that between concentrations 0 *μg/ml* and 1 *μg/ml* ($r = 0.99$ vs $r = 0.96$).

Table 2.7. Correlation coefficient between vegetative fitness at different fluconazole concentrations. The titles of the columns and rows represent the concentration of fluconazole. The closer the two concentrations are, the higher correlation coefficient it is. As concentration increases, R decreases.

	C_0	C_0.5	C_1	C_2	C_4	C_8	C_16	C_32
C_0.5	0.99							
C_1	0.96	0.98						
C_2	0.91	0.93	0.97					
C_4	0.88	0.90	0.94	0.98				
C_8	0.83	0.85	0.90	0.94	0.97			
C_16	0.77	0.80	0.84	0.89	0.92	0.96		
C_32	0.49	0.52	0.56	0.61	0.66	0.71	0.80	
C_64	0.21	0.23	0.26	0.31	0.35	0.41	0.51	0.81

Table 2-7 Correlation coeffcient between vegetative fitness for different fluconazole concentrations

2.6 Discussion

2.6.1 Multiple markers are associated with vegetative fitness at different drug concentrations

Markers L005, L010, L013, and L020 displayed significant effects on vegetative fitness of strains at more than three different levels of concentrations of fluconazole (Table 2-1). These four genetic markers may be tightly linked to major genes to that control vegetative fitness in *C. neoformans*. Markers L020 and L010 are genes in the same linkage group (LG1). Marker L013 is on LG2 and L005 is on LG4. LAC and URA5 genes have significant influence on vegetative fitness of *C. neoformans* at certain fluconazole concentrations. Specifically, LAC gene is linked to vegetative fitness at most of fluconazole concentrations (Table 2-2).

From analysis of histograms of vegetative fitness of hybrids of *C. neoformans*, we observed the bimodal distribution of vegetative fitness of hybrids (Figure 2-3) differed from these of parental strains. Together, these results suggest vegetative fitness of hybrids of *C. neoformans* is controlled by multiple genes including LAC and URA5 genes.

2.6.2 Multiple markers are associated with the Minimum Inhibit Concentration at different drug concentrations

In this cross, vegetative fitness and fluconazole resistance were governed by multiple genes including LAC and URA5, and different genes influence different levels of resistance. In lower concentrations of fluconazole, genes possessing the function of resistance to fluconazole might not be activated. At intermediate

concentrations of fluconazole, the number of genes involved in fluconazole-resistance increased because these levels of fluconazole concentration triggered the function of fluconazole-resistance of genes where genes started to fight against stressful environment to keep their internal environment consistent. Genes' function might be destroyed due to higher fluconazole concentrations and only a few genes might have the ability to survive at high concentration.

2.6.3 Putative mechanisms of outbreeding depression in *C.neoformans*

Our results provide the first evidence of outbreeding depression of fungi. Several mechanisms may contribute to outbreeding depression in *C. neoformans*. The first is differences in chromosomal number, size, and structure. Wickes *et al.* used pulsed-field-gel electrophoresis to elucidate sizes and numbers of chromosomes of serotypes A and D in *C.neoformans*. From their electrophoretic karyotypes, they found that the average number of chromosomes was 12 for serotype A and 12.8 for serotype D. The smallest size of chromosomes was from 700 to 770 kb in serotype A and 700 kb in serotype D. The largest chromosomes were greater than 2.2 Mb for both serotypes. All isolates had common bands approximately 945-1125 kb in size in serotype A and 770 kb-1500 kb and in size in serotype D (Figure 2-13 from Wickes *et. al* 1994). The other study revealed the number of chromosome of serotype A and D is between 10 and 12 (Perfect *et. al.*, 1989). In addition, we have CHEF gels of these two parental strains showing significant differences. These can be linked to a common proposed mechanism: chromosomal incompatibility. Incompatible karyotypes of the parental isolates cause significant problems in a sexual cross of

these varieties: one chromosome of parental strain cannot pair with one from the other parental strain and homologous chromosomes can't segregate properly due to the lack of the homology. Difference in chromosome may also cause chromosome mismatching.

Chromosomal nondisjunction occurs during meiosis and causes a failure of the normal separation of homologous chromosomes to opposite poles at nuclear division. This can result in aneuploidy. In a true diploid, the ratio of genes on any one chromosome to genes on other chromosomes is 1:1. In contrast, in an aneuploid, the ratio of genes on the aneuploid chromosome to genes on the other chromosomes differs from wild type by 50 percent. Thus, the aneuploid genes are out of balance (Griffiths *et al.* 2000) and progeny viability and fitness could be reduced from the intervarietal cross. Thus, chromosome incompatibility could contribute to outbreeding depression in *C. neoformans.*

Two other conventional mechanisms have been proposed to explain outbreeding depression in animals and plants. These mechanisms could be applicable to explain outbreeding depression in fungi. The first is the disruption of local adaptive genes and the second is the disruption of coevolved gene complexes (Templeton 1986, Waser 1993, Lynch & Walsh 1998). If hybrid individuals express genes or mixed genomes that are not adapted to local conditions or either of the parental environments, it will result in the failure of hybrids in these environments or local conditions. Outbreeding depression in *C.neoformans* may relate to disruption of

Figure 2.13 CHEF panels of *C. neoformans* var *neoformans* and var. *grubii*. (a) Serotype A *C. neoformans* var. grubii. Sc, *Sacch. cerevsiae*; lane 1, NIH966; lane 2, NIH971; lane 3, NIH288; lane 4, NIH386; lane 5, NIH302; (b) lane 1, Serotype D *C.neoformans* var. *neoformans*. Sc, *Sacch. cerevisiae;* lanel, NIH55; lane 2, NIH430; lane 3, B-3501; lane 4, NIH424; lane 5, NIH52.

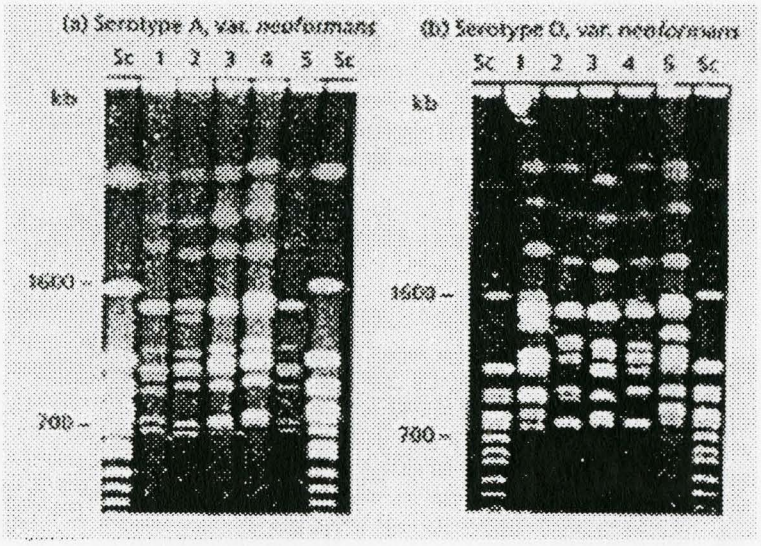

Figure 2-13 CHEF panels of *C. neoformans* karyotypes (serotype A, var. *grubii* and serotype D, var. *neoformans*)

coevolved gene complexes. Two populations of var. *grubii* and var. *neoformans* have diverged for 18.5 millions years and may have evolved separate adapted gene complexes; it is possible that outbreeding depression will happen. Through the hybridization, locally coadapted gene complexes could have broken apart by segregation and recombination and genes may not be compatible in the mixed genome of hybrids consisting of genomes of two different populations.

Several studies have shown that serotype A (mating type alpha) strains accounts for the majority of human infections and the majority of environmental isolates, and serotype D strain is not common in clinical and environmental isolates (Casadevall & Perfect, 1998; Franzot *et al.*, 1999). Millions of years history of divergence (Xu *et al.*, 2000b) and significant differences in genome structure and sequence may lead to few viable spores and aneploid spores in sexual cross of *C. neoformans* var. *neoformans* (D) and *grubii* (A) (Lengeler, 2001) and outcrossing causes the disruptions of coevolved gene complexes and of local adaptations. These might be some of reasons that hybrids are not observed as commonly as serotype A strains in clinical and environment.

These two mechanisms of outbreeding depression could be activated at the same time or one of them could work independently. How do these mechanisms work together? Is there any interaction between them? And which one does play a major role in a particular population? These questions await answers.

Outbreeding depression of serotype AD of *C. neoformans* was genetically and environmentally dependent. When progeny were exposed to no fluconazole

environment, progeny expressed significant outbreeding depression. As drug concentrations increased, outbreeding depression decreased, suggesting environmentally dependent outbreeding depression.

2.6.4 Putative mechanisms of heterosis in *C. neoformans*

Evidence of heterosis came from the observation that some progeny exhibited greater fitness under lab conditions than either parent exhibited at the highest drug concentrations. Thus, hybridization between these two strains can have beneficial effects on fitness. Progeny might experience heterosis for fitness-related traits because recessive deleterious alleles contributed by one parent are more likely to be masked by alleles contributed by a parent from a different population with different alleles (Crow 1948; Paul 1992). When sexually crossing JEC20 (Da) and CDC15 (Aα), heterozygous and diploid hybrids were produced. In a heterozygous locus, one of the genes might be dominant, which means that the trait it codes for will be expressed and the trait coded for by the other gene (the *recessive* gene) will not be expressed because recessive deleterious allele was masked by dominant one. Ordinarily, a hybrid population contains many genes with heterozygous loci in which many of the detrimental recessives are covered by dominant alleles from the other parent and an increase in vigor is the consequence of the cumulative effects of dominant favorable genes (Crow, 1948). This dominance /recessive hypothesis was proposed by Bruce (Bruce, 1910).

Hybrid vigor of serotype AD of *C.neoformans* was environmentally dependent. Heterosis had been observed in the fluconazole environments only but not in non-fluconazole environment.

Outbreeding depression and heterosis occurred simultaneously in a hybrid population of *C. neoformans* in our study. While genes of hybrids from different populations may be incompatible or not adaptive to a local environment, masking of deleterious recessive alleles also occurs. Outbreeding resulted in the decreased fitness of hybrid strains but increased resistance to unfavorable conditions of the environment (fluconazole condition in our study).

2.6.5 The effects of fluconazole on the vegetative fitness of progeny at different fluconazole concentrations

Regression analysis showed a negative linear relationship between vegetative fitness and fluconazole concentration. The linear regression function could provide a potential model to predict the vegetative fitness of a single individual hybrid based upon fluconazole concentration. In particular, it is a useful guide in clinical trial of fluconazole-resistance test and medical practice in the treatment of AIDs patients with cryptococcal meningitis for a long term maintenance treatment. One of the key considerations for pharmaceutical manufacturers' is "be first to market". This requires shortening running study of development of a drug and, in the meantime, reducing cost of study. With the regression equation, predication of effect of fluconazole at a given concentration would be feasible and experiments of drug

resistance could be simplified. Consequently, it could reduce cost and shorten the time of study.

2.6.6 Correlation between vegetative fitness at different fluconazole concentrations

The correlation coefficient showed significant correlation between vegetative fitness in pairs of fluconazole concentrations. As the concentration of fluconazole decreases, the coefficients increase. The results here may be useful in evaluating the effect between fluconazole concentrations in clinical trials of drug-resistance. The prediction of the effect of a concentration of fluconazole could be based on similar concentration of fluconazole. In fluconazole-resistance test, it is possible for researchers not to implement experiments for all concentrations. This would shorten study time and reduce expense. In this study, the degree of correlation between similar levels of fluconazole concentrations decreased after 16 μg/ml, but they are still significantly correlated.

2.7 Conclusion

In this study, we found both outbreeding depression and condition-dependent heterosis. Extensive outbreeding depression detected from our study demonstrated environmentally dependent hybrid costs in this human pathogenic fungus and the levels of outbreeding depression are inversely related to fluconazole concentration. Hybrids had lower vegetative fitness than parental strains in low fluconazole environments. At highest drug concentration, a significant number of progeny showed evidence of hybrid vigor.

Vegetative fitness and fluconazole resistance are controlled by multiple genes, with different genes influencing different levels of resistance. Correlation and variation analysis among fluconazole concentrations suggests that the closer two concentrations are, the higher the correlation coefficient.

To our knowledge, this is the first study demonstrating both outbreeding depression and heterosis in fungi. Additional work could help identify specific genes influencing these two phenomenons in *C. neoformans*. Hybrid vigor, though relatively minor, could play a significant role in stressful environments and lead to the formation of new, more pathogenic species. Hypothetical mechanisms of outbreeding depression and heterosis of *C. neoformans* need to be further investigated.

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Appendix

Table A 1 Summary of data set scored and traits for 286 progeny. The experiment design was F2 backcross and 51 markers were used. File is in Mapmaker exp 3.0 format. Genetic classes of loci were represented by "0" or "1" and missing data was '-". First part of the file is the genotype of each progeny on a single locus and the second part is the phenotype of a progeny.

Table A 1 Summary of data set scored and traits for 286 progeny

data type f2 backcross
286 51 9 symbols 1=A 0=H

*L001
11-1110000100001110010111101100000000110-
01000101100111011111111111111111111-
11111110111011101001101000000000000000000000000100011000110101001000100011
11000001101000010101101011010101011110111000110011011101000000001000000000
000010001000100000011100100000000100001000000-000000100000000011
*L002
00-000000000000000000000000000001000100-
0000001100000100100000000000000001000-
00000010100000000000000000000000000000110000000100000110000000001000010001 1
00000110000000010111001001011110100001001111111100110010111111100111111 1
11110111001101100000010111111100100110001111-111011000001 1111
*L003
11-111111011111011111000000--------------------------------111111111101011-
01111110101111111111110011010000010010000011110101101111111111011011000
01011111111111110111111110110000100010001001000011011101000100111011111 1
1110011011111011111000001000000000100000000 1-1010110110100011
*L004
00-000001001100000000010000101100100011 0-
0000000000000000000000101001000010 0-
00000010111000110000000000001000001001100000000100100100000000000000000000100
0010000000000011101000000100001000000010001000000011000000000111000001100 00
0100000100000000000010000100000000000000000011-0110100110110010
*L005
00-000000000000000001000000100010001 10-
0100000000000000000000101001000000 0-
10000010100000010000000100000010000001001100000000100100100000000001000000000 0
000000000000000010010000001000010010001001100000011000000000000001000000000 0
010000110000010000000000010000000000100000000 0-0100000001011100
*L006
00-0011010100000000000111001111101101110-
111000000011010011001100100010011 01-
11111011111011111011011111111110101111111111111111110001001110101101111101 00
101111101011011011110111111111001001010110000100001000000110010100110100 00
011110001010000000101111110100000000100000000 0-1110100010101010
*L007
11-111010000011111111000110000010000011-
01000000000000000000111101100101010-
10000100100010101111110100010001100000000000000100111000000101001000000011

000000001010000000000000110110111111110000011111001111000110101111011101
110001010011011010111101111010000000000000-1001000011101000
*L008
00-00000000000000000000000000000000-
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000010000001000000000001000000001100000000000000001100000000010000000000
000000000010000000000000000000000000000000-0001000000000110
*L009
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111110110101111110111100101101100110111101111111101001011101100000111101 1
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*L010
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111100010100000000010000010000000100--000000001000100100100 1-100101-
0001111000-0-0-110010000-00100000110100000-00100000101000001110011001
*L011
100000001101000000000000000000000000000000000000000000000000000000000000
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001100000000000010000000000001000000--000000100001101000000 0-100001-
0000011000-1-0-000011000-00000000000000000-00000000000010010000010010
*L012
101000001111000000000110000000001101011001111111111000010000001110010101
100000000000100000000011000000010000000010000100000000000100010010000010
111100000000000000010000000000000111--100000000000001000000 1-000101-
0001010101-0-0-110010000-10000000010100000-00000110101000010000010101
*L013
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110111111111111110111111101111101101111111111111111111011101111111101111110
011111111111111101101111111111111101--11111101011111101111 01-111111-
1111101111-1-1-011111011-11011111111111111-11111111111101001011101010
*L014
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*L015
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111111111111111111111001111111111111111111111111111111111011110111101111111
111111000111111011011111111111111111--11111111011111011111 11-111110-
1111101111-0-1-101111111-11111111101011111-11011111010111011111111111

*L016
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0000000000000000000000000000000000000000000000000000000010000010010000000
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*L017
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1111110111-1-1-101111101-11110011111110110-1111100111111101101 1101000
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-0100000000011000001000000000000-00001001000001-
0000100000010011000000100010000100000000000000010-
0011000001000000000010000000001000000001010-000001001100
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1100000011111111000011101111110111111111111111111111111111111101111011110 10
-0011111111010111010111110111111 1-11111011111011-
0111011011111111100101101101110111111001100011101111-
0011101111111101011011001111100111111101111 10-101101001110
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0000000000010011111101001011101101100000000010110010010100010000111000 00
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0111111110111111101111111111110101111111101-011010110101
*L021
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1110001110111111101000000010101111101011011110100011011000100100101101 0000
-0000000000101100000100001000000 1-10011110000001-
1000100100010000000000010000000000010001011000101 0-
0000000010001000000000000000100111111110110-000001110000
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-0000000000001001010000101010010 0-01011000100000-
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0100100000000000100010000000000000000000000-000010101100
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-0000000000000000011101011000000 1-01000000000000-

000010000001111100111110010000111001110100110001 10-
011110100000000100101000001000100000100000 0-010000001000
*L024
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011000010111110000100110101001111111001011110 11-100--0-1111-0-01-
000001000010100000 1-100-000011010000010110111011-111111-1111-111-11111111-
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*L025
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010000011101110000001100000001000011011000000000000011100-
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110111010010110101 0-110-000010011100101110101111-110000-1111---1----------
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101000010000000000100010101001100100000010100 1-000--0-1001-1-01-
000100101010010000 0-100-000001010001010110110110-001111-0011---1----------
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111111111-111000111111010 10-100110100001000010110000 00-110111011-10000
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001001101-00000-10101100001-000010000010001001110010 00-110000001-01100
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000010010-00000-00000000000-001010000001001011100001 00-000001001-00011
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--1---------11---111----------11001--------
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1111101011111110101001101110001011111 0011-
10111111011111111010110111111011001111010001001011101111101111110110 1----
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*L035
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01100000110100100101111101111001110111 11-
11111111101111011111111111111011111111011111111011111111111111111111111---
------011111111010110------------------------
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01110101111101101111010111111101111111011101111111111111101111111101111111
11110101111111111011111111001010111010100 1-
11110010111101111001111110100100100000100011100000111111110111100101111-
-------1111011101110000100100101111101101110 1 0
*L037
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1111111-1111111-111-11111111111-1-1111111111111111111-1-111111111-11111111-
---111111111--11-111111-1111-11111-1111111111-111----11-1-1111--1-1111111--
1111-11-1111-111111-1111111111--1-111-1111111111-11-11-111--
*L038
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000000000000000000000000000000000000000000-000000000000000000-
000000000000000000000000000000000000000000000000000000000000-000000000000-000--
000001100000000000000000111000000001000000000000000000010000000001000000
000000000000000---000000001000000
*L039
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0001011011010000000100100001101100111011111111000000100100101010111101100
00010010001000
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1111101001100100111111110111101101111110111111100100110101100111111011000
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*L044
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1101111010110001001110111111111101111111111110111100111111011110110111
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*L046
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*L049
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0000000000000001001010110010010010100000000000000000010000000000000010000
0011110000000000010100000000000000000000000010000000111110101110010001000
0000100000100101000001000001000000010001101000010001000010010000000
*L050
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1000000100111100101111100110111111001001001011001111--0001111011101
*L051
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0101011111111111111101111111011110011011111011111111111011101111111111111
1001111101111000110110111111010011111101011111110101111111001110011101011111
0111110111111011101011111111111010011101101111010111111—1001010101010
*LAC1
1111111101111111111111011111111101011101101111101011111101011101101010l
1100111111111111101111111101111111111111111111111111111011101111111111110
0111111111111101101111111111111111101001111101101011111101100001111111111
11111111-1111110101111111111111111101111111111010101l0-0010
*LAC2
1111111111111111111111111111111111111001110111111111110111111110111111110
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1111110111111101010l-1111001011111010001010011011111101011111101111-1101
*URA5_1 11111010-01-
1111111111111111111111111111101111111101111111111111111101111110111-
0111111111111101111111011111011111011-11-1111111011111111111101-
1111001111111111111111011111101111111111-10-1111100101111110111110011111-
111-1--1111111110111-0100111111111111111--1101111110-1-101110010
*URA5_2 11111111-11-
1111111111111111111111111001110111110111111001011111011101111011-
1011111010111110110111111111111111111-01-1111101111100111101010-
1110110001110000111111110100111011011O1-01-1111111111100101100111110111-
011-1--1011100011110-1111111101101110011O--0111010111-0-110101101

Table A 2 Summary of data set scored of LAC and URA5 genes for 286 progeny. LAC and URA5 were scored separately because they were co-dominant markers. If a progeny inherited an allele from JEC20 (Parent1), that locus was recorded as '1' and its two genetic markers were '1' and '0'; the locus was recorded as '2' and two genetic markers on that locus were represented by '0' and '1' in order if the locus were inherited from CDC92-15 (Parent 2); the locus was recorded as ½ and genetic markers on that locus were treated as '1' and '1' if the locus came from both parents. Columns 2 and 5 were original data form RFLP fingerprinting. Columns 3, 4, 6, and 7 were genetic markers based on the RFLP results.

Table A 2 Summary of data set scored of LAC and URA5 genes for 286 progeny

	LAC	LAC1	LAC2	URA5	URA5-1	URA5-2
JEC20	1	1		2	(0)	
CDC92-15	2		0	1		(1)
CR6-1	1/2	1	1	1/2	1	1
CR6-2	1/2	1	1	1/2	1	1
CR6-3	1/2	1	1	1/2	1	1
CR6-4	1/2	1	1	1/2	1	1
CR6-5	1/2	1	1	1/2	1	1
CR6-6	1/2	1	1	1/2	1	1
CR6-7	1/2	1	1	1	0	1
CR6-8	1/2	1	1	1/2	1	1
CR6-9	1/2	1	1	1	0	1
CR6-10	1/2	1	1			
CR6-11	2	0	1	1	0	1
CR6-12	1/2	1	1	1/2	1	1
CR6-13	1/2	1	1			
CR6-14	1/2	1	1	1/2	1	1
CR6-15	1/2	1	1	1/2	1	1
CR6-16	1/2	1	1	1/2	1	1
CR6-17	1/2	1	1	1/2	1	1
CR6-18	1/2	1	1	1/2	1	1
CR6-19	1/2	1	1	1/2	1	1
CR6-20	1/2	1	1	1/2	1	1
CR6-21	1/2	1	1	1/2	1	1
CR6-22	1/2	1	1	1/2	1	1
CR6-23	1/2	1	1	1/2	1	1
CR6-24	1/2	1	1	1/2	1	1
CR6-25	2	0	1	1/2	1	1
CR6-26	1/2	1	1	1/2	1	1
CR6-27	1/2	1	1	1/2	1	1
CR6-28	1/2	1	1	1/2	1	1
CR6-29	1/2	1	1	1/2	1	1
CR6-30	1/2	1	1	1/2	1	1
CR6-31	1/2	1	1	1/2	1	1
CR6-32	1/2	1	1	1/2	1	1
CR6-33	1/2	1	1	1/2	1	1
CR6-34	1/2	1	1	1/2	1	1
CR6-35	2	0	1	1/2	1	1
CR6-36	1/2	1	1	1/2	1	1
CR6-37	2	0	1	1/2	1	1
CR6-38	1	1	0	2	1	0
CR6-39	1	1	0	2	1	0
CR6-40	1/2	1	1	1/2	1	1
CR6-41	2	0	1	1	0	1
CR6-42	1/2	1	1	1/2	1	1
CR6-43	1	1	0	2	1	0
CR6-44	2	0	1	1/2	1	1
CR6-45	1/2	1	1	1/2	1	1
CR6-46	1/2	1	1	1/2	1	1
CR6-47	1/2	1	1	1/2	1	1

CR6-48	1/2	1	1	1/2		1	1
CR6-49	1/2	1	1		2	1	0
CR6-50	2	0	1		1	0	1
CR6-51	1/2	1	1	1/2		1	1
CR6-52	2	0	1	1/2		1	1
CR6-53	1/2	1	1	1/2		1	1
CR6-54	1/2	1	1	1/2		1	1
CR6-55	1	1	0		2	1	0
CR6-56	1/2	1	1		2	1	0
CR6-57	1/2	1	1	1/2		1	1
CR6-58	1/2	1	1	1/2		1	1
CR6-59	1/2	1	1		2	1	0
CR6-60	2	0	1	1/2		1	1
CR6-61	1/2	1	1	1/2		1	1
CR6-62	2	0	1	1/2		1	1
CR6-63	1/2	1	1	1/2		1	1
CR6-64	1/2	1	1	1/2		1	1
CR6-65	1	1	0		2	1	0
CR6-66	1/2	1	1	1/2		1	1
CR6-67	2	0	1		1	0	1
CR6-68	1/2	1	1	1/2		1	1
CR6-69	1/2	1	1		2	1	0
CR6-70	1/2	1	1	1/2		1	1
CR6-71	2	0	1	1/2		1	1
CR6-72	1/2	1	1	1/2		1	1
CR6-73	2	0	1		1	0	1
CR6-74	1	1	0		2	1	0
CR6-75	1/2	1	1	1/2		1	1
CR6-76	1/2	1	1	1/2		1	1
CR6-77	2	0	1				
CR6-78	2	0	1		1	0	1
CR6-79	1	1	0		2	1	0
CR6-80	1	1	0		2	1	0
CR6-81	1	1	0	1/2		1	1
CR6-82	1/2	1	1	1/2		1	1
CR6-83	1/2	1	1	1/2		1	1
CR6-84	1/2	1	1	1/2		1	1
CR6-85	1	1	0	1/2		1	1
CR6-86	1	1	0		2	1	0
CR6-87	1	1	0	1/2		1	1
CR6-88	1	1	0		2	1	0
CR6-89	1/2	1	1	1/2		1	1
CR6-90	1/2	1	1	1/2		1	1
CR6-91	1/2	1	1	1/2		1	1
CR6-92	1/2	1	1	1/2		1	1
CR6-93	2	0	1		1	0	1
CR6-94	1	1	0		2	1	0
CR6-95	1/2	1	1	1/2		1	1
CR6-96	1/2	1	1	1/2		1	1
CR6-97	1	1	0		2	1	0
CR6-98	1/2	1	1	1/2		1	1
CR6-99	1/2	1	1	1/2		1	1
CR6-100	1/2	1	1	1/2		1	1
CR6-101	1/2	1	1		1	0	1

CR6-102	2	0	1	1/2		1	1
CR6-103	1/2	1	1	1/2		1	1
CR6-104	1/2	1	1	1/2		1	1
CR6-105	1/2	1	1	1/2		1	1
CR6-106	1/2	1	1	1/2		1	1
CR6-107	1/2	1	1		1	0	1
CR6-108	1/2	1	1	1/2		1	1
CR6-109	1/2	1	1	1/2		1	1
CR6-110	1/2	1	1	1/2		1	1
CR6-111	1/2	1	1	1/2		1	1
CR6-112	1/2	1	1	1/2		1	1
CR6-113	1/2	1	1		1	0	1
CR6-114	1/2	1	1	1/2		1	1
CR6-115	1/2	1	1	1/2		1	1
CR6-116	1	1	0				
CR6-117	1	1	0		2	1	0
CR6-118	1/2	1	1	1/2		1	1
CR6-119	1/2	1	1				
CR6-120	1/2	1	1	1/2		1	1
CR6-121	1/2	1	1	1/2		1	1
CR6-122	1/2	1	1	1/2		1	1
CR6-123	1/2	1	1	1/2		1	1
CR6-124	1/2	1	1	1/2		1	1
CR6-125	1/2	1	1	1/2		1	1
CR6-126	1/2	1	1		2	1	0
CR6-127	1/2	1	1	1/2		1	1
CR6-128	1/2	1	1		1	0	1
CR6-129	2	0	1	1/2		1	1
CR6-130	1/2	1	1	1/2		1	1
CR6-131	1/2	1	1	1/2		1	1
CR6-132	1/2	1	1		2	1	0
CR6-133	2	0	1		2	1	0
CR6-134	1/2	1	1	1/2		1	1
CR6-135	1/2	1	1	1/2		1	1
CR6-136	1/2	1	1	1/2		1	1
CR6-137	1/2	1	1	1/2		1	1
CR6-138	1/2	1	1		2	1	0
CR6-139	1/2	1	1	1/2		1	1
CR6-140	1	1	0		2	1	0
CR6-141	1	1	0		1	0	1
CR6-142	1	1	0		2	1	0
CR6-143	1/2	1	1				
CR6-144	1/2	1	1	1/2		1	1
CR6-145	1/2	1	1	1/2		1	1
CR6-146	1/2	1	1	1/2		1	1
CR6-147	1/2	1	1		2	1	0
CR6-148	2	0	1		1	0	1
CR6-149	2	0	1		1	0	1
CR6-150	1/2	1	1		2	1	0
CR6-151	1	1	0		2	1	0
CR6-152	1	1	0		2	1	0
CR6-153	1/2	1	1	1/2		1	1
CR6-154	1/2	1	1	1/2		1	1
CR6-155	1/2	1	1	1/2		1	1

CR6-156	1/2	1	1	2	1	0
CR6-157	1/2	1	1	2	1	0
CR6-158	1	1	0	2	1	0
CR6-159	1	1	0	2	1	0
CR6-160	1/2	1	1	1/2	1	1
CR6-161	1/2	1	1	1/2	1	1
CR6-162	1/2	1	1	1/2	1	1
CR6-163	2	0	1	1/2	1	1
CR6-164	1/2	1	1	1/2	1	1
CR6-165	1/2	1	1	1	0	1
CR6-166	2	0	1	1/2	1	1
CR6-167	1/2	1	1	1/2	1	1
CR6-168	1/2	1	1	2	1	0
CR6-169	1/2	1	1	1/2	1	1
CR6-170	1/2	1	1	2	1	0
CR6-171	1	1	0	2	1	0
CR6-172	1/2	1	1	1	0	1
CR6-173	1/2	1	1	1/2	1	1
CR6-174	1/2	1		1/2	1	1
CR6-175	1	1	0	2	1	0
CR6-176	1	1	0	1/2	1	1
CR6-177	1/2	1	1	1/2	1	1
CR6-178	1	1	0	2	1	0
CR6-179	1/2	1	1	1/2	1	1
CR6-180	1/2	1	1	1/2	1	1
CR6-181	1	1	0	2	1	0
CR6-182	1/2	1	1	1/2	1	1
CR6-183	2	0	1			
CR6-184	1	1	0	2	1	0
CR6-185	2	0	1	1	0	1
CR6-186	2	0	1			
CR6-187	1/2	1	1	1/2	1	1
CR6-188	1/2	1	1	1/2	1	1
CR6-189	1/2	1	1	1/2	1	1
CR6-190	1/2	1	1	1/2	1	1
CR6-191	1/2	1	1	1/2	1	1
CR6-192	2	0	1	1	0	1
CR6-193	1/2	1	1	1	0	1
CR6-194	1/2	1	1	1/2	1	1
CR6-195	2	0	1	1	0	1
CR6-196	1/2	1	1	1/2	1	1
CR6-197	2	0	1	1/2	1	1
CR6-198	1/2	1	1	2	1	0
CR6-199	1/2	1	1	2	1	0
CR6-200	1/2	1	1	1/2	1	1
CR6-201	1	1	0	2	1	0
CR6-202	1/2	1	1	1	0	1
CR6-203	1/2	1	1	1/2	1	1
CR6-204	2	0	1	2	1	0
CR6-205	1/2	1	1	2	1	0
CR6-206	1/2	1	1	1/2	1	1
CR6-207	2	0	1	1	0	1
CR6-208	2	0		1	0	1
CR6-209	2	0	1	1/2	1	1

CR6-210	2	0	1		2	1	0
CR6-211	1/2	1	1	1/2		1	1
CR6-212	1/2	1	1	1/2		1	1
CR6-213	1/2	1	1	1/2		1	1
CR6-214	1/2	1	1				
CR6-215	1	1	0		2	1	0
CR6-216	1/2	1	1	1/2		1	1
CR6-217	1/2	1	1	1/2		1	1
CR6-218	1/2	1	1				
CR6-219	1/2	1	1	1/2		1	1
CR6-220	1/2	1	1				
CR6-221	1/2	1	1				
CR6-222	1/2	1	1	1/2		1	1
CR6-223	1	1	0		2	1	0
CR6-224	1/2	1	1	1/2		1	1
CR6-225	1	1	0	1/2		1	1
CR6-226	1/2	1	1	1/2		1	1
CR6-227	1	1	0		2	1	0
CR6-228	1/2	1	1		2	1	0
CR6-229					2	1	0
CR6-230	1/2	1	1	1/2		1	1
CR6-231	1/2	1	1	1/2		1	1
CR6-232	1/2	1	1		1	0	1
CR6-233	1/2	1	1	1/2		1	1
CR6-234	1/2	1	1	1/2		1	1
CR6-235	1/2	1	1	1/2		1	1
CR6-236	1	1	0		2	1	0
CR6-237	1	1	0				
CR6-238	2	0	1		1	0	1
CR6-239	1	1	0	1/2		1	1
CR6-240	2	0	1		1	0	1
CR6-241	1/2	1	1		1	0	1
CR6-242	1/2	1	1	1/2		1	1
CR6-243	1/2	1	1	1/2		1	1
CR6-244	1/2	1	1	1/2		1	1
CR6-245	1/2	1	1	1/2		1	1
CR6-246	1	1	0	1/2		1	1
CR6-247	1/2	1	1		2	1	0
CR6-248	1	1	0	1/2		1	1
CR6-249	1	1	0	1/2		1	1
CR6-250	1	1	0		2	1	0
CR6-251	1/2	1	1	1/2		1	1
CR6-252	1	1	0	1/2		1	1
CR6-253	1/2	1	1	1/2		1	1
CR6-254	1	1	0		2	1	0
CR6-255	1	1	0		2	1	0
CR6-256	1/2	1	1	1/2		1	1
CR6-257	1/2	1	1	1/2		1	1
CR6-258	1	1	0		2	1	0
CR6-259	2	0	1				
CR6-260	1/2	1	1				
CR6-261	1/2	1	1	1/2		1	1
CR6-262	1/2	1	1		2	1	0
CR6-263	1/2	1	1	1/2		1	1

CR6-264	1/2	1	1	1	0	1
CR6-265	1/2	1	1	1/2	1	1
CR6-266	1	1	0	2	1	0
CR6-267	1/2	1	1	1/2	1	1
CR6-268	1	1	0	2	1	0
CR6-269	1/2	1	1	1/2	1	1
CR6-270	1/2	1	1	1/2	1	1
CR6-271	1/2	1	1	1/2	1	1
CR6-272	2	0	1	1	0	1
CR6-273	1/2	1	1			
CR6-274	2	0	1	2	1	0
CR6-275	2	0	1	1	0	1
CR6-276	1	1	0			
CR6-277	2	0	1	1/2	1	1
CR6-278	1/2	1	1	1	0	1
CR6-279	1/2	1	1	2	1	0
CR6-280	2	0	1	1/2	1	1
CR6-281				2	1	0
CR6-282	2	0	1	1	0	1
CR6-283	1	1	0	2	1	0
CR6-284	2	0	1	1	0	1
CR6-285	1	1	0	2	1	0
CR6-286	2	0	1	1	0	1

Table A 3 Mean of vegetative fitness of each of 286 progeny was calculated at different concentrations of fluconazole. A single fluconazole concentration was treated as a trait. And total traits are 9. For each trait, 286 values of growth rate correspond with 286 progeny.

Table A 3 Summary of vegetative fitness of C.neoformans at different fluconazole concentrations on each marker

*trait00	79.50	79.30	78.60	82.50	44.70	61.70	71.00	64.10	80.00	43.60
47.80	52.00	51.70	27.20	24.20	57.00	74.20	67.20	62.40	69.80	75.80
32.70	78.90	58.90	4.50	67.40	10.20	65.10	17.90	67.10	54.40	58.60
50.30	45.20	19.40	54.80	10.40	12.30	58.90	18.00	51.80	19.10	25.10
64.90	44.30	74.20	47.80	67.80	12.40	45.80	25.70	64.90	49.90	64.00
52.10	42.60	59.90	45.00	47.50	9.90	25.40	66.10	8.30	8.50	13.90
44.80	43.60	12.60	35.40	23.80	41.70	35.00	54.60	41.90	56.70	15.80
13.30	15.10	56.70	64.20	51.80	59.50	7.00	69.10	31.20	52.20	47.40
52.70	74.90	7.30	11.00	55.90	43.80	11.70	31.50	68.40	72.20	24.90
50.60	77.50	48.60	25.90	62.90	31.30	26.50	55.90	61.60	37.80	60.90
70.80	63.50	56.30	14.80	8.00	25.90	42.90	46.70	51.20	44.10	42.00
51.90	13.40	55.60	14.80	57.90	66.00	65.60	20.90	72.40	67.20	67.90
68.90	64.60	57.80	47.40	19.00	11.13	4.86	11.00	24.50	53.75	58.67
9.11	47.13	13.38	14.63	9.56	15.25	20.67	58.43	45.83	11.38	7.38
2.90	28.25	41.14	71.63	10.20	26.33	62.14	19.43	42.75	69.17	24.75
53.56	22.50	20.14	50.00	19.00	4.90	6.20	34.29	56.00	22.88	25.60
21.75	10.80	54.44	41.75	23.56	10.20	53.88	52.60	43.78	11.13	49.00
56.10	33.71	56.10	43.13	12.40	70.22	38.33	19.20	14.20	72.50	5.10
13.70	26.10	5.30	8.80	42.43	21.10	5.40	62.44	11.80	45.63	20.56
57.90	68.10	13.80	46.90	58.63	60.11	51.89	44.88	47.14	47.71	10.78
58.50	50.44	62.63	10.56	36.75	12.44	28.11	46.13	18.70	41.10	39.22
41.78	9.10	15.00	50.56	44.50	65.00	31.50	63.22	13.90	23.80	20.00
41.11	44.00	42.75	61.40	10.60	71.33	19.10	45.63	17.00	5.70	13.80
44.80	17.70	6.30	10.70	73.50	50.56	20.40	18.10	47.60	20.20	75.89
26.89	21.30	54.60	53.78	59.38	13.30	13.20	10.30	15.10	22.10	17.67
51.25	43.40	23.80	15.40	15.20	6.30	15.70				

*trait05	81.70	56.10	78.40	78.40	44.70	58.30	79.20	61.10	78.90	44.20
41.50	54.80	47.50	25.20	24.00	72.10	72.30	58.50	66.30	67.50	69.90
32.60	81.30	64.80	4.40	75.20	10.50	63.00	18.00	65.90	54.40	55.50
49.10	41.30	18.70	53.90	10.30	12.60	63.30	17.40	49.00	20.60	26.20
66.60	45.40	74.30	49.20	57.00	12.30	44.60	24.80	65.20	48.20	61.80
57.70	40.70	64.20	46.80	44.20	8.40	25.10	62.00	8.50	8.70	14.60
44.50	43.10	12.00	35.40	23.00	38.00	40.10	51.60	41.40	50.50	15.70
15.10	18.00	52.80	60.90	53.30	58.50	6.80	64.10	33.10	50.20	46.20
43.50	76.40	7.30	10.20	54.20	42.00	10.60	30.63	72.80	69.00	26.00
50.10	66.60	49.90	24.60	53.30	28.20	29.00	53.30	60.80	36.50	61.20
64.10	59.20	61.10	14.60	9.10	25.70	45.20	57.60	55.80	45.70	42.10
50.90	13.20	51.70	14.70	53.00	64.50	63.60	20.70	70.80	68.60	70.00

65.10 59.10 56.60 44.80 19.50 10.43 3.86 11.00 25.13 52.75 58.33
10.11 55.00 12.00 14.38 9.63 15.00 20.40 59.83 44.17 11.88 7.38
3.00 26.29 36.17 62.38 10.40 23.40 54.88 11.00 41.63 72.14 24.38
52.75 22.20 20.25 51.67 13.11 4.90 7.50 38.33 55.88 17.22 23.30
21.11 10.30 53.00 40.63 25.17 9.50 57.11 59.90 48.00 11.33 44.29
55.13 29.38 55.13 42.44 11.40 63.89 37.75 19.40 14.00 66.50 5.00
14.00 24.20 5.30 7.70 41.75 20.90 6.40 59.38 9.10 46.10 20.00
57.50 68.43 18.50 45.22 57.44 58.71 47.14 44.57 48.25 46.43 11.78
68.86 54.38 60.38 10.33 37.44 12.11 27.56 51.00 19.10 40.70 38.75
41.00 8.80 18.90 50.78 44.22 63.20 30.20 63.33 14.80 22.60 33.78
46.00 42.70 43.60 57.44 10.67 67.13 18.50 50.50 16.89 6.60 13.50
42.10 17.10 6.40 10.70 72.40 40.22 17.00 19.10 48.00 16.60 67.33
27.78 20.00 53.60 54.89 60.57 13.00 12.90 10.00 15.10 21.50 14.00
50.88 38.70 23.00 16.00 14.30 6.40 16.50

*trait01 70.70 52.50 72.30 80.90 48.30 60.80 77.50 55.50 78.40 48.00
44.00 54.90 54.20 30.20 28.70 64.20 76.20 31.80 66.40 65.20 74.40
31.70 78.40 63.20 4.60 75.10 9.70 60.60 18.70 63.90 49.40 50.10
49.90 41.10 18.50 49.50 9.30 12.90 59.80 17.50 50.90 27.60 21.10
67.00 44.30 64.90 50.70 51.70 12.30 42.00 25.30 65.00 37.00 54.90
55.90 42.90 56.90 43.70 45.10 8.80 23.40 64.00 11.80 8.30 11.60
43.20 42.00 11.40 35.30 26.10 17.00 37.60 53.90 37.20 50.10 15.00
16.50 18.50 41.40 60.70 57.00 63.20 6.90 62.80 29.50 50.90 46.70
19.70 71.50 8.00 9.80 55.40 15.50 10.40 29.57 69.20 68.70 22.90
23.20 71.30 49.00 25.80 52.00 26.50 27.60 58.20 53.30 33.20 57.00
62.20 58.20 59.70 13.90 10.00 27.00 43.80 63.00 57.20 47.90 30.30
52.20 9.80 50.50 14.70 64.10 65.30 61.30 21.10 71.70 65.20 64.30
69.50 62.90 56.00 47.10 19.29 11.00 4.00 10.89 24.11 55.29 53.71
9.50 55.60 12.00 12.90 9.86 12.88 21.00 58.17 45.29 11.50 8.00
2.88 20.86 38.00 63.00 10.00 24.17 52.00 8.00 39.44 70.44 23.88
53.50 20.56 19.38 50.00 13.22 4.90 6.50 37.83 60.33 10.89 22.70
21.89 9.00 54.67 36.20 24.50 8.00 54.67 61.56 44.00 11.57 42.75
60.00 30.25 58.89 34.89 9.10 63.56 39.90 17.30 13.40 64.86 4.89
14.90 26.40 5.00 7.20 41.78 17.20 5.50 62.50 8.90 26.56 21.40
59.30 71.89 18.90 45.38 62.88 58.75 48.75 45.22 57.88 44.30 11.56
64.38 52.25 60.88 9.90 35.30 13.40 26.56 52.22 12.20 38.30 39.00
39.00 7.10 20.20 49.44 44.50 66.56 12.89 63.00 16.00 20.30 26.22
43.67 39.00 43.70 61.78 10.33 66.11 17.30 57.14 15.70 5.70 13.10
39.80 16.40 5.80 10.60 70.70 37.56 19.20 19.10 47.78 12.90 60.56
24.33 18.30 55.63 50.75 62.25 10.70 12.80 10.70 13.00 16.60 12.60
52.38 35.50 18.10 18.30 14.30 6.30 14.90

*trait02 81.60 50.30 74.50 79.80 45.30 62.90 72.90 53.20 77.30 44.00
40.30 53.80 49.40 25.00 24.60 70.30 74.60 7.80 65.30 55.00

64.20 30.60 76.70 60.10 3.90 76.00 9.10 66.40 16.70 64.40
37.70 53.90 58.60 44.80 17.90 47.30 8.50 13.60 59.90 15.60
51.90 22.90 21.80 65.50 51.20 69.70 52.10 45.40 12.80 41.50
25.90 66.00 35.00 44.60 53.70 38.90 46.70 42.90 49.30 8.90
25.10 58.00 8.60 8.30 18.10 28.10 41.80 11.10 34.20 24.50
5.70 39.40 45.90 42.60 50.80 14.70 13.00 17.80 18.00 66.30
66.00 57.10 7.10 58.90 26.10 53.70 44.90 6.20 73.30 6.90
6.00 52.20 6.10 10.20 29.57 68.90 66.00 22.30 8.60 62.20
46.70 24.10 55.60 27.70 27.90 56.10 56.30 33.90 51.50 63.60
58.80 60.00 9.30 11.90 15.80 38.60 62.40 56.50 25.40 9.20
51.10 8.70 51.60 14.70 50.00 64.70 60.20 20.90 70.90 68.60
63.50 59.10 59.00 53.40 43.50 19.86 10.00 3.63 11.38 20.25
55.86 51.50 7.63 51.33 12.43 7.80 10.50 11.56 21.17 56.50
42.75 10.80 5.89 2.56 21.00 33.17 64.00 8.40 23.50 52.00
6.00 39.00 68.57 21.75 54.00 21.11 18.38 42.63 13.11 4.80
7.20 36.00 55.88 5.00 22.00 19.67 4.80 53.14 34.67 21.88
5.00 51.44 56.44 22.78 10.71 40.38 54.11 32.13 54.11 35.89
9.00 54.71 40.22 12.70 13.40 64.83 5.30 7.60 25.40 5.10
7.60 39.63 7.20 3.80 62.88 4.40 11.40 23.00 61.90 64.67
9.40 46.78 58.22 57.14 44.00 43.17 55.56 6.90 11.00 60.63
53.63 50.00 5.50 33.25 14.30 22.25 51.80 6.30 39.20 32.63
41.40 6.20 21.50 45.33 43.50 63.11 4.00 52.89 15.80 11.90
25.33 33.44 38.60 33.67 59.67 4.80 54.22 10.60 50.25 15.70
1.60 10.60 36.20 8.20 6.00 6.10 62.50 37.33 16.60 17.60
45.40 10.20 57.33 25.44 17.90 57.63 53.89 61.78 9.90 13.30
10.30 11.00 10.00 8.30 51.38 31.50 10.30 17.70 14.50 6.30
10.50

*trait04 74.70 38.00 75.80 84.50 47.30 63.40 67.70 55.30 77.10 47.00
42.30 54.50 58.60 22.30 23.80 70.10 71.30 4.80 61.40 60.80 67.60
32.30 76.20 57.60 3.80 67.60 9.70 61.30 18.30 62.20 9.10 51.20
57.20 46.00 10.90 48.90 7.30 9.30 57.80 13.90 47.90 11.50 18.80
66.80 46.60 68.00 46.50 47.10 12.20 39.30 20.10 59.00 28.80 38.60
51.90 35.70 49.40 45.70 47.60 8.40 24.20 60.90 8.60 7.90 15.90
9.60 41.50 9.00 36.70 21.20 2.10 36.10 47.30 42.50 53.60 11.50
9.60 19.00 5.90 60.20 55.50 57.80 6.50 61.40 18.90 51.90 42.90
4.30 69.00 7.60 3.80 51.90 3.50 10.40 30.57 72.70 65.60 20.90
3.60 50.20 43.90 17.50 55.80 27.40 27.30 56.80 56.00 39.20 55.40
63.90 59.10 64.10 6.10 11.50 10.40 15.00 55.70 50.20 8.40 7.70
42.30 7.00 48.30 12.00 49.40 65.70 49.80 20.30 68.70 60.90 60.20
58.40 54.80 56.50 38.29 18.00 10.86 2.86 11.29 16.88 55.33 56.14
5.56 53.00 11.86 4.00 11.25 11.10 21.50 53.83 45.50 11.89 6.00
2.50 23.00 31.67 56.57 4.90 24.11 37.25 3.67 40.14 66.57 23.80
54.75 21.56 19.10 42.50 12.89 3.30 8.30 36.00 55.29 2.33 22.20

20.00 4.10 53.00 17.00 23.50 3.90 52.57 60.60 9.56 10.88 39.11
56.20 30.44 56.20 28.60 10.20 51.50 40.89 7.60 13.20 66.71 5.10
2.60 27.20 4.60 6.10 22.38 4.40 3.40 65.11 4.30 5.00 25.22
60.80 66.56 5.00 45.67 57.22 55.63 44.75 42.11 65.67 3.60 10.90
60.44 56.13 47.33 5.00 35.22 17.80 22.11 46.70 3.10 39.20 26.30
39.44 3.30 20.60 50.00 41.63 62.60 3.60 58.89 14.80 10.90 24.44
12.40 43.90 38.67 61.89 5.10 62.20 11.30 50.56 11.50 1.20 11.60
21.00 6.00 6.00 2.50 57.10 36.00 17.10 18.30 47.44 5.70 60.89
21.67 18.10 52.33 46.63 61.44 11.10 10.90 6.80 8.10 4.40 7.60
50.78 33.90 4.30 10.60 14.30 6.10 3.60

*trait08 64.80 15.10 68.70 67.90 41.70 50.60 66.80 53.70 71.60 48.00
41.50 46.40 45.30 19.80 20.70 54.10 78.80 2.20 61.60 115.00 65.20
27.50 73.30 52.40 4.40 69.70 8.80 58.80 19.50 63.40 3.80 53.00
50.10 39.70 6.00 40.90 5.10 4.30 57.00 7.00 44.00 4.60 15.30
60.60 44.30 60.50 42.40 18.20 11.20 43.10 18.60 59.90 29.90 16.50
49.00 35.90 24.80 45.00 45.90 8.20 26.90 59.20 8.80 7.90 18.20
4.20 36.70 6.20 35.20 22.60 1.00 33.70 43.50 36.00 45.10 5.70
3.40 18.10 4.00 51.30 50.10 52.40 7.00 59.40 5.10 47.50 43.90
2.50 63.70 7.80 1.80 45.50 2.90 9.50 30.00 72.50 57.80 23.00
2.40 54.00 41.70 14.80 51.10 27.10 28.30 54.70 48.60 35.60 51.80
56.70 59.30 58.70 4.90 5.50 6.10 5.00 53.40 48.30 4.30 5.90
49.40 4.80 49.60 12.20 49.50 59.80 49.70 12.80 54.50 61.70 52.10
58.20 57.90 55.40 38.78 11.29 10.50 2.43 10.38 9.67 57.50 56.00
3.56 52.50 7.88 1.40 10.88 11.43 19.83 50.14 43.00 11.00 6.11
2.00 22.43 26.50 46.71 2.20 16.63 20.13 1.67 22.38 57.33 19.78
54.00 20.67 16.44 38.14 11.78 2.70 7.20 35.29 56.43 1.78 22.63
18.44 2.30 54.60 4.56 16.50 2.70 51.29 57.89 4.70 10.29 19.11
53.22 30.71 53.56 27.60 9.60 29.38 41.63 4.50 13.60 65.14 5.00
2.30 18.30 3.70 4.10 6.67 1.20 2.60 64.63 3.44 3.50 23.30
60.50 65.44 2.50 29.88 34.11 36.63 32.38 33.78 43.86 3.60 12.00
40.14 39.00 41.56 3.60 36.50 12.70 20.78 44.20 2.50 30.40 16.30
41.29 2.90 21.20 48.89 49.25 67.80 1.70 58.67 14.50 5.50 22.33
7.50 43.20 37.11 62.60 4.00 63.60 10.00 55.00 9.20
1.20 13.40 10.60 5.70 6.00 1.20 58.80 5.78 17.30 17.20 48.78
3.40 57.33 16.40 14.10 51.67 44.44 60.30 11.20 10.50 5.30 4.40
2.40 2.60 52.56 15.60 2.30 6.20 12.60 5.50 2.30

*trait16 43.00 6.90 54.00 63.10 38.80 35.80 68.20 51.40 55.50 40.40
21.20 32.90 46.10 13.70 17.00 32.70 65.10 2.20 57.60 54.40 55.70
26.30 57.90 31.90 4.10 48.00 8.00 49.60 20.20 52.00 2.30 44.60
47.30 36.20 2.70 27.80 2.80 3.50 55.90 2.40 47.70 4.30 15.80
51.80 37.90 51.50 41.10 11.40 11.60 35.00 18.00 45.80 24.60 11.00
47.50 24.20 12.60 29.70 36.10 8.50 24.60 51.20 7.00 7.90 15.90

2.10 29.60 5.50 28.00 22.50 1.00 31.70 40.70 14.50 44.50 3.00
2.00 19.10 2.60 46.50 36.10 43.40 7.00 52.00 2.10 29.00 24.70
2.00 54.70 7.20 1.50 33.90 2.30 10.50 29.75 68.20 38.90 18.50
2.10 40.30 38.50 8.40 45.50 23.50 21.40 46.70 43.20 14.20 49.50
48.20 52.40 63.80 3.80 2.60 3.70 2.40 51.00 47.60 2.40 1.90
39.70 4.60 46.50 11.10 53.50 52.20 37.80 5.90 51.40 51.70 49.80
28.40 55.50 55.20 37.33 8.00 9.33 2.43 10.63 4.44 51.00 51.17
2.50 40.33 5.38 1.40 10.25 10.57 20.50 30.17 39.63 10.89 5.40
1.70 22.75 11.13 46.67 1.50 15.63 7.78 1.67 10.20 51.00 20.00
45.25 22.22 17.11 33.50 9.67 2.20 9.40 32.14 48.22 1.33 18.00
20.11 2.20 55.29 2.50 7.56 2.50 42.75 57.44 2.40 10.00 7.00
45.13 25.56 45.13 20.10 9.00 12.56 41.22 4.40 13.50 48.38 5.00
1.90 5.70 2.30 3.50 4.10 1.20 2.70 61.56 2.50 2.10 21.00
56.00 43.89 1.90 33.13 32.78 36.78 34.11 37.44 37.50 2.40 11.60
19.80 38.89 42.67 1.80 31.56 6.40 19.80 45.80 1.60 35.30 3.60
28.70 1.90 17.60 37.22 40.75 54.56 1.60 50.67 16.10 2.70 20.33
2.40 20.90 10.40 53.70 1.10 54.70 10.20 42.11 4.10 1.10 12.70
2.90 2.80 5.80 1.20 35.60 2.56 12.80 18.80 32.00 3.20 53.44
5.10 8.30 37.90 46.78 52.44 13.70 11.60 2.60 3.00 1.50 2.30
44.89 7.30 1.90 3.50 11.90 5.70 2.10

*trait32 12.10 2.40 20.40 24.50 15.90 6.00 31.10 20.10 18.60 25.40
6.00 11.50 37.30 6.60 9.60 8.20 23.50 2.00 41.90 21.70 19.20
21.90 16.30 13.70 4.00 16.50 9.20 18.80 18.40 24.20 1.50 34.80
25.00 33.60 1.50 13.20 1.50 2.30 19.40 1.90 34.80 2.70 11.30
18.20 13.20 19.60 20.80 5.10 12.10 21.80 11.50 18.10 17.30 4.90
15.20 9.50 5.00 10.90 24.40 4.00 21.80 21.70 7.40 6.90 13.60
1.60 12.20 2.90 21.80 20.60 1.00 14.10 24.10 7.80 13.60 1.90
1.50 14.50 2.20 5.80 4.50 5.00 6.50 16.20 1.90 8.70 6.90
2.00 24.60 2.30 1.50 8.90 2.30 6.70 21.71 53.30 8.80 17.80
1.90 15.60 21.80 7.30 20.50 21.30 20.30 18.80 13.80 6.90 41.20
13.00 14.20 55.70 1.50 1.30 2.20 1.90 18.20 16.60 2.10 1.70
11.10 1.50 15.40 6.50 20.00 14.60 12.60 2.40 23.20 11.30 24.30
5.50 48.10 14.20 13.17 4.63 6.25 2.00 5.50 2.67 28.38 35.83
2.33 8.67 2.25 1.30 10.25 8.33 20.29 9.43 13.33 11.20 4.20
1.30 19.00 5.56 18.11 1.30 10.63 3.89 1.00 4.75 15.75 11.67
14.30 20.89 12.56 13.00 8.78 2.20 6.50 20.50 28.78 1.22 11.50
19.67 1.60 41.25 1.70 3.30 2.30 21.00 46.38 2.20 10.11 4.50
23.63 19.56 23.63 9.40 9.50 7.00 29.11 2.80 11.50 12.50 5.00
1.50 4.50 1.50 2.10 3.80 1.30 2.50 43.44 2.00 1.90 12.60
15.40 20.60 1.30 19.88 20.78 17.44 20.22 32.78 12.40 1.80 5.70
6.90 32.22 19.10 1.20 31.11 6.00 11.10 25.40 1.40 20.40 2.90
11.78 1.70 12.00 12.78 38.25 14.80 1.30 17.00 13.60 1.60 23.50
2.20 7.80 6.50 39.50 1.00 36.40 9.30 16.89 3.80 1.00 12.90

2.40 2.50 3.70 1.00 11.60 2.33 8.40 17.00 16.88 1.80 14.40
2.50 6.90 17.20 22.11 12.44 8.00 9.30 2.30 3.00 1.30 1.40
19.70 3.00 1.60 2.30 9.80 3.90 1.90

*trait64 1.20 2.40 4.70 4.90 4.90 2.40 5.10 3.30 6.00 7.70
1.90 2.90 16.20 2.70 3.90 4.00 3.90 1.30 27.10 6.20
5.70 12.60 5.30 5.30 3.20 5.70 6.50 11.40 17.90 12.50
1.00 12.20 4.90 6.00 1.10 5.50 1.00 1.30 6.30 1.60
12.00 1.60 7.20 5.30 5.30 5.40 1.10 2.80 10.30 6.80
5.70 4.30 9.00 2.60 8.30 4.00 3.00 5.80 7.50 2.20
10.30 5.90 7.30 4.60 9.50 1.00 4.70 1.20 11.90 9.30
1.00 7.50 10.30 5.00 7.20 1.30 1.40 14.40 2.00 11.90
6.10 14.10 6.60 3.40 1.50 3.40 5.90 1.80 4.90 1.70
1.20 3.60 1.80 4.10 19.22 29.30 4.60 10.40 1.70 6.10
4.90 3.60 4.90 13.10 13.60 10.30 5.50 2.90 20.80 4.50
5.20 28.40 1.30 1.00 1.70 1.70 6.80 6.60 1.40 1.40
5.70 1.40 11.40 6.00 7.70 5.10 6.30 1.50 7.00 4.70
10.90 2.40 41.00 5.50 5.71 2.10 4.22 1.57 5.88 2.22
8.00 9.88 2.13 3.38 1.13 1.22 5.30 2.43 18.57 4.29
5.88 10.33 2.11 1.30 13.33 3.56 8.78 1.30 5.11 1.67
1.00 2.89 7.33 6.00 5.20 11.25 7.30 4.89 5.00 1.20
4.00 7.75 11.11 1.11 4.78 17.22 1.10 13.75 1.30 1.67
1.80 8.67 16.22 2.20 10.10 2.33 7.33 6.30 7.50 4.60
6.20 2.44 8.00 2.50 4.10 3.70 4.70 1.20 2.30 1.10
1.70 2.30 1.10 1.70 17.90 1.80 1.30 8.90 5.80 5.00
1.20 9.33 5.20 5.67 14.90 10.78 3.40 1.70 3.70 2.50
6.60 6.60 1.10 16.70 1.80 3.10 6.30 1.40 7.20 2.10
3.20 1.20 5.50 4.78 9.22 5.20 1.30 5.67 14.70 1.50
15.50 2.30 3.90 3.30 18.20 1.00 18.00 10.10 6.78 2.80
1.00 2.30 1.80 2.10 2.20 1.00 4.10 1.89 3.30 12.50
6.30 1.70 6.30 2.10 3.20 4.50 8.33 6.10 11.80 10.20
2.10 2.00 1.60 1.40 15.60 2.00 1.40 1.70 7.80 2.30
1.00

Table A 4 To understand whether a single gene or multiple genes is associated with MIC of progeny at different fluconazole concentrations, we categorized MICs into different classes 2, 4, 8, 16, 32 64, and 128 and then a single class of MIC was subdivided into a pair of subclass in which one subclass is greater than and the other is less than or equal to that level of MIC. Number of progeny whose MIC fall into two different subclasses was counted separately. For a single genetic marker, we summed number of progeny whose segregating marker scores with "1" and "0" independently. Since each variable has two levels, hypothesis tests were performed on 2x2 contingency tables in order to decide whether or not relationship between MIC and genetic marker is present. Hypothesis tests on contingency tables are based on a statistic test Chi-square. Last two columns represent the significant association between MIC and genetic classes at $\alpha = 0.01^{**}$ and 0.05^{*}.

Table A 4 Summary of Association between MIC and Genetic Classes for A Single Marker

Marker	MIC<=	0(a)	1(b)	MIC>	0©	1(d)	X^2	P<=0.05) p<=0.01)
Marker1	2	14	6	2	149	110	1.19	
	4	25	15	4	138	101	0.32	
	8	38	31	8	125	85	0.42	
	16	55	40	16	108	76	0.02	
	32	119	82	32	44	34	0.18	
	64	148	100	64	15	16	1.45	
	128	163	116	128	0	0		
Marker2	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	11	9	2	173	86	1.15	
	4	22	18	4	162	77	2.49	
	8	39	30	8	145	65	3.63	
	16	52	43	16	132	52	8.07 *	**
	32	132	69	32	52	26	0.02	
	64	161	87	64	23	8	1.06	
	128	184	95	128	0	0		
Marker3	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	11	9	2	87	139	2.09	
	4	19	20	4	79	128	1.53	
	8	28	34	8	70	114	0.98	
	16	37	48	16	61	100	0.74	
	32	70	105	32	28	43	0.01	
	64	84	133	64	14	15	0.98	
	128	98	148	128	0	0		
Marker4	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	18	2	2	202	57	1.61	
	4	32	8	4	188	51	0.04	
	8	53	16	8	167	43	0.23	
	16	75	20	16	145	39	0.00	
	32	161	40	32	59	19	0.67	
	64	198	50	64	22	9	1.30	
	128	220	59	128	0	0		
Marerk5	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	19	1	2	215	44	1.97	
	4	34	6	4	200	39	0.04	
	8	53	16	8	181	26	4.53 *	
	16	77	18	16	157	27	0.85	
	32	173	28	32	61	17	2.57	
	64	211	37	64	23	8	2.41	

	128	234	45	128	0	0				
Marker6	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	13	7	2	117	142	2.93			
	4	23	17	4	107	132	2.23			
	8	35	34	8	95	115	0.63			
	16	50	45	16	80	104	2.11			
	32	100	101	32	30	48	2.88			
	64	116	132	64	14	17	0.03			
	128	130	149	128	0	0				
Marker7	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	10	10	2	158	101	0.94			
	4	22	10	4	146	93	0.70			
	8	37	32	8	131	79	1.66			
	16	53	42	16	115	69	1.18			
	32	121	80	32	47	31	0.00			
	64	150	98	64	18	13	0.07			
	128	168	111	128	0	0				
Marker8	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	20	0	2	243	16	1.31			
	4	39	1	4	224	15	0.90			
	8	63	6	8	200	10	1.49			
	16	88	7	16	175	9	0.71			
	32	191	10	32	72	6	0.77			
	64	235	13	64	28	3	1.00			
	128	263	16	128	0	0				
Marker9	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	3	15	2	96	160	3.16			
	4	6	32	4	93	143	7.91	*		**
	8	12	55	8	87	120	12.76	*		**
	16	20	73	16	79	102	13.05	*		**
	32	53	145	32	46	30	27.12	*		**
	64	73	77	64	22	9	5.12	*		
	128	99	175	128	0	0				
Marker10	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	14	4	2	205	51	0.06			
	4	24	14	4	195	41	7.73	*		**
	8	42	25	8	177	80	0.93			
	16	63	30	16	156	25	13.03	*		**
	32	157	41	32	62	14	0.18			
	64	193	50	64	26	5	0.34			
	128	219	55	128	0	0				

Marker11	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	14	4	2	233	23	3.32	
	4	29	9	4	218	18	9.50 *	**
	8	66	11	8	191	16	2.80	
	16	81	12	16	166	15	1.47	
	32	179	19	32	68	8	0.05	
	64	218	25	64	29	2	0.46	
	128	247	27	128	0	0		
Marker12	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	16	2	2	186	70	2.29	
	4	30	8	4	172	64	0.62	
	8	51	16	8	151	56	0.26	
	16	71	22	16	131	50	0.50	
	32	147	51	32	55	21	0.10	
	64	180	63	64	22	9	0.14	
	128	202	72	128	0	0		
Marker13	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	5	13	2	35	221	2.68	
	4	10	28	4	30	206	4.86 *	
	8	13	54	8	27	180	1.64	
	16	17	76	16	23	158	1.53	
	32	31	167	32	9	67	0.64	
	64	36	207	64	4	27	0.08	
	128	40	234	128	0	0		
Marker14	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	8	10	2	94	162	0.43	
	4	17	21	4	85	151	1.06	
	8	30	37	8	72	135	2.16	
	16	39	54	16	63	118	1.34	
	32	69	129	32	33	43	1.73	
	64	88	155	64	14	17	0.94	
	128	102	172	128	0	0		
Marker15	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	2	16	2	24	232	0.06	
	4	3	35	4	23	213	0.13	
	8	5	62	8	21	186	0.42	
	16	8	85	16	18	163	0.13	
	32	15	183	32	11	65	3.04	
	64	22	221	64	4	27	0.47	
	128	26	248	128	0	0		
Marker16	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		

	2	15	3	2	238	18	2.21			
	4	31	7	4	222	14	7.21	*		**
	8	58	9	8	195	12	4.17	*		
	16	82	11	16	171	10	3.45			
	32	179	19	32	74	2	3.76			
	64	223	20	64	30	1	0.97			
	128	253	21	128	0	0				
Marker17	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	5	13	2	46	210	1.07			
	4	13	25	4	38	198	7.09	*		**
	8	21	46	8	30	177	9.49	*		**
	16	24	69	16	27	154	4.81	*		
	32	37	161	32	14	62	0.00			
	64	45	198	64	6	25	0.01			
	128	51	223	128	0	0				
marker18	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	14	5	2	213	44	1.02			
	4	31	8	4	196	41	0.24			
	8	50	20	8	177	29	7.52	*		**
	16	69	26	16	158	23	9.17	*		**
	32	165	35	32	62	14	0.03			
	64	205	41	64	22	8	1.83			
	128	227	49	128	0	0				
Marker19	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	2	17	2	70	187	2.56			
	4	7	32	4	65	172	1.56			
	8	18	52	8	54	152	0.01			
	16	27	68	16	45	136	0.41			
	32	53	147	32	19	57	0.06			
	64	67	179	64	5	25	1.55			
	128	72	204	128	0	0				
Marker20	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	5	13	2	130	127	3.50			
	4	13	26	4	123	114	4.62	*		
	8	13	41	8	107	99	13.37	*		**
	16	38	57	16	98	83	4.99	*		
	32	99	101	32	37	39	0.01			
	64	120	126	64	16	14	0.22			
	128	136	140	128	0	0				
Marker21	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	14	5	2	151	106	1.64			

		4	27	12	4	138	99	1.69
		8	44	26	8	121	85	0.37
		16	63	32	16	102	79	2.57
		32	118	82	32	47	29	0.19
		64	145	101	64	20	10	0.66
		128	165	111	128	0	0	
Marker22	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**	
		2	17	2	2	209	48	0.79
		4	34	5	4	192	45	0.86
		8	56	14	8	170	36	0.22
		16	75	20	16	151	30	0.84
		32	166	34	32	60	16	0.61
		64	203	43	64	23	7	0.62
		128	226	50	128	0	0	
Marker23	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**	
		2	12	7	2	184	73	0.61
		4	25	14	4	171	66	1.05
		8	46	24	8	150	56	1.28
		16	61	34	16	135	46	3.26
		32	134	66	32	62	14	5.69*
		64	171	75	64	25	5	2.48
		128	196	80	128	0	0	
Marker24	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**	
		2	5	13	2	83	152	0.42
		4	9	23	4	79	142	0.72
		8	19	38	8	69	127	0.07
		16	30	52	16	58	113	0.17
		32	64	116	32	24	49	0.16
		64	80	146	64	8	19	0.35
		128	88	165	128	0	0	
Marker25	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**	
		2	9	8	2	130	96	0.14
		4	18	12	4	121	92	0.11
		8	30	25	8	109	79	0.20
		16	43	35	16	96	69	0.20
		32	100	72	32	39	32	0.21
		64	124	92	64	15	12	0.03
		128	139	104	128	0	0	
Marker26	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**	
		2	12	5	2	154	72	0.04
		4	20	10	4	146	67	0.04

	8	31	24	8	135	53	4.69	*
	16	48	30	16	118	47	2.44	
	32	120	52	32	46	25	0.58	
	64	150	66	64	16	11	1.15	
	128	166	77	128	0	0		
Marker27	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	9	12	2	132	115	0.87	
	4	21	19	4	120	108	0.00	
	8	34	34	8	107	93	0.25	
	16	45	47	16	96	80	0.77	
	32	99	97	32	42	30	1.29	
	64	125	116	64	16	11	0.53	
	128	141	127	128	0	0		
Marker28	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	11	10	2	162	84	1.54	
	4	25	15	4	148	79	0.11	
	8	89	29	8	134	65	2.32	
	16	54	38	16	119	56	2.29	
	32	124	72	32	49	22	0.76	
	64	154	86	64	19	8	0.41	
	128	173	94	128	0	0		
Marker29	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	9	12	2	104	142	0.00	
	4	16	24	4	97	130	0.10	
	8	25	43	8	88	111	1.15	
	16	35	57	16	78	97	1.05	
	32	83	113	32	30	41	0.00	
	64	100	140	64	13	14	0.42	
	128	113	154	128	0	0		
Marker30	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	16	5	2	166	80	0.68	
	4	27	13	4	155	72	0.01	
	8	42	26	8	140	59	1.72	
	16	60	32	16	122	53	0.56	
	32	131	65	32	51	20	0.60	
	64	162	78	64	20	7	0.48	
	128	182	85	128	0	0		
Marker31	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**		
	2	6	15	2	64	183	0.07	
	4	13	27	4	57	171	0.99	
	8	19	50	8	51	148	0.10	

		16	25	68		16	45	130	0.04	
		32	51	146		32	19	62	0.18	
		64	63	178		64	7	20	0.00	
		128	70	198		128	0	0		
Marker32	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	10	11		2	169	77	3.89	*
		4	23	17		4	156	71	1.94	
		8	42	26		8	137	62	1.15	
		16	55	37		16	124	51	3.35	
		32	123	73		32	56	15	6.13	*
		64	158	82		64	21	6	1.57	
		128	179	88		128	0	0		
Marker33	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	10	7		2	105	113	0.72	
		4	19	13		4	96	107	1.62	
		8	34	22		8	81	98	4.08	*
		16	46	32		16	69	88	4.71	*
		32	86	83		32	29	37	0.92	
		64	105	105		64	10	15	0.89	
		128	115	120		128	0	0		
Marker34	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	8	8		2	69	159	2.70	
		4	14	19		4	63	148	2.09	
		8	25	31		8	52	136	5.76	*
		16	29	50		16	48	117	1.44	
		32	61	111		32	16	56	4.12	*
		64	68	147		64	9	20	0.00	
		128	77	167		128	0	0		
Marker35	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	0	18		2	61	166	6.44	*	
		4	0	33		4	61	151	12.64	*	**
		8	3	54		8	58	130	15.32	*	**
		16	9	72		16	52	112	12.30	*	**
		32	34	142		32	27	42	10.41	*	**
		64	45	174		64	16	10	20.88	*	**
		128	61	184		128	0	0			
Marker36	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**				
		2	7	12		2	66	188	1.06		
		4	15	24		4	58	176	3.19		
		8	26	42		8	47	158	6.11	*	
		16	35	60		16	38	140	7.59	*	**

		32	56	142		32	17	58	0.88	
		64	68	175		64	5	25	1.75	
		128	73	200		128	0	0		
Marker37	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	0	17		2	0	213		
		4	0	32		4	0	198		
		8	0	55		8	0	175		
		16	0	77		16	0	153		
		32	0	169		32	0	61		
		64	0	206		64	0	24		
		128	0	230		128	0	0		
Marker38	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	20	1		2	245	8	0.16	
		4	38	3		4	227	6	2.47	
		8	63	5		8	202	4	4.71	*
		16	88	5		16	177	4	1.94	
		32	191	8		32	74	1	1.24	
		64	235	9		64	30	0	1.14	
		128	265	9		128	0	0		
Marker39	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	5	15		2	83	180	0.37	
		4	9	31		4	79	164	1.61	
		8	15	56		8	73	139	4.40	*
		16	21	77		16	67	118	6.54	*
		32	67	139		32	21	56	0.72	
		64	80	172		64	8	23	0.45	
		128	88	195		128	0	0		
Marker40	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	4	16		2	82	181	1.10	
		4	12	28		4	74	169	0.00	
		8	20	51		8	66	146	0.22	
		16	31	67		16	55	130	0.11	
		32	60	146		32	26	51	0.57	
		64	76	176		64	10	21	0.06	
		128	66	197		128	0	0		
Marker41	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
		2	3	17		2	54	209	0.35	
		4	9	31		4	48	195	0.16	
		8	17	54		8	40	172	0.85	
		16	22	76		16	35	150	0.50	
		32	32	174		32	25	52	9.99	*

**

	64	45	207	64	12	19	7.46	*		**
	128	57	226	128	0	0				
Marker42	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	6	14	2	64	199	0.32			
	4	12	28	4	58	185	0.69			
	8	21	50	8	49	163	1.19			
	16	30	68	16	40	145	2.78			
	32	50	156	32	20	57	0.09			
	64	60	192	64	10	21	1.06			
	128	70	213	128	0	0				
Marker43	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	8	12	2	109	138	0.13			
	4	17	20	4	100	130	0.08			
	8	29	35	8	88	115	0.08			
	16	38	52	16	79	98	0.14			
	32	85	110	32	32	40	0.02			
	64	109	131	64	8	19	2.46			
	128	117	150	128	0	0				
Marker44	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	9	12	2	112	151	0.00			
	4	15	26	4	106	137	0.71			
	8	25	46	8	96	117	2.12			
	16	35	63	16	86	100	2.91			
	32	90	117	32	31	46	0.24			
	64	107	146	64	14	17	0.09			
	128	121	163	128	0	0				
Marker45	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	8	12	2	90	142	0.01			
	4	13	26	4	85	128	0.60			
	8	24	44	8	74	110	0.51			
	16	35	58	16	63	96	0.10			
	32	65	119	32	33	35	3.64			
	64	85	141	64	13	13	1.51			
	128	98	154	128	0	0				
Marker46	**MIC<=**	**0**	**1**	**MIC>**	**0**	**1**				
	2	15	6	2	227	36	3.42			
	4	30	11	4	212	31	5.51	*		
	8	48	23	8	194	19	23.28	*		**
	16	73	25	16	169	17	13.65	*		**
	32	174	33	32	68	9	0.81			
	64	215	38	64	27	4	0.10			

	128	242	42		128	0	0			
Marker47	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
	2	8	13		2	100	163	0.00		
	4	14	27		4	94	149	0.31		
	8	27	44		8	81	132	0.00		
	16	34	64		16	74	176	0.85		
	32	73	134		32	35	42	2.47		
	64	93	160		64	15	16	1.58		
	128	108	176		128	0	0			
Marker48	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
	2	11	10		2	146	117	0.08		
	4	24	17		4	133	110	0.21		
	8	44	27		8	113	100	1.71		
	16	58	40		16	99	87	0.92		
	32	115	92		32	42	35	0.02		
	64	143	110		64	14	17	1.44		
	128	157	127		128	0	0			
Marker49	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
	2	13	8		2	222	41	6.90	*	**
	4	27	14		4	208	35	9.58	*	**
	8	51	20		8	184	29	7.90	*	**
	16	69	29		16	166	20	15.96	*	**
	32	166	41		32	69	8	3.49		
	64	209	44		64	26	5	0.03		
	128	235	49		128	0	0			
Marker50	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
	2	13	8		2	119	130	1.54		
	4	23	18		4	109	120	1.01		
	8	34	36		8	98	102	0.00		
	16	46	51		16	86	87	0.13		
	32	93	103		32	39	35	0.59		
	64	117	123		64	15	15	0.02		
	128	132	138		128	0	0			
Marker51	**MIC<=**	**0**	**1**		**MIC>**	**0**	**1**			
	2	5	16		2	68	192	0.06		
	4	13	28		4	60	180	0.82		
	8	21	50		8	52	158	0.64		
	16	27	71		16	46	137	0.19		
	32	52	154		32	21	54	0.22		
	64	67	184		64	6	24	0.62		
	128	73	208		128	0	0			

Table A 5 Chi-square test of LAC and URA5 genes that were associated with MIC at different fluconazole concentrations. Last two columns represent the significant association between MIC and genetic classes at $\alpha = 0.01^{**}$ and 0.05^{*}.

Table A 5 Chi-square test of LAC and URA5 genes that were associated with MIC at different fluconazole concentrations

Gene	MIC<=	1	2	1/2	MIC>	1	1	1/2	X^2	p	
LAC	2	5	5	11	2	46	40	177	1.994	0.369	
	4	10	7	24	4	41	38	164	1.574	0.4551	
	8	21	12	38	8	30	33	150	9.482	0.009	**
	16	29	16	52	16	22	29	136	15.26	0.001	**
	32	35	33	138	32	16	12	50	0.477	0.788	
	64	44	40	169	64	7	5	19	0.542	0.762	
	128	51	45	188	128	0	0	0			
URA5											
	2	5	6	8	2	32	56	162	4.43	0.109	
	4	10	13	16	4	27	49	154	10.33	0.005	**
	8	14	29	25	8	23	33	145	28.32	0.0001	**
	16	15	39	40	16	22	23	130	31.57	0.0001	**
	32	28	47	120	32	9	15	50	0.837	0.657	
	64	33	47	152	64	4	7	18	0.238	0.888	
	128	37	62	170	128	0	0	0			