Estimating the Spontaneous Mutation Rate of Loss of Sex in the Human Pathogenic Fungus *Cryptococcus neoformans*

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ABSTRACT

Few events have evolutionary consequences as pervasive as changes in reproductive behavior. Among those changes, the loss of the ability to undergo sexual reproduction is probably the most profound. However, little is known about the rate of loss of sex. Here I describe an experimental system using the fungus *Cryptococcus neoformans* and provide the first empirical estimate of the spontaneous mutation rate of loss of sex in fungi. Two critical steps in sexual reproduction in *C. neoformans* were examined: mating and filamentation. Mating, the fusion of cells of opposite sexes, is a universal first step in eukaryotic sexual reproduction. In contrast, filamentation, a prerequisite process preceding meiosis and sexual spore development, is restricted to *C. neoformans* and a few other fungal species. After ~600 mitotic divisions under favorable asexual growth conditions, mean abilities for mating and filamentation decreased significantly by >67 and 24%, respectively. Similarly, though statistically not significant, the mean vegetative growth rates also decreased and among the mutation accumulation lines, the vegetative growth rates were negatively correlated to the mating ability. The estimated mutation rates to decreases in mating ability and filamentation were in excess of 0.0172 and 0.0036, respectively. The results show that *C. neoformans* can be a highly attractive model for analyses of reproductive system evolution in fungi.

CHANGES in reproductive systems have multiple biological effects and are of significance in understanding many biological phenomena, including the origin and maintenance of sex, population structure, mutation accumulation and extinction in small populations, and life history variation in natural populations (*e.g.*, Bell 1982; Charlesworth and Charlesworth 1998). However, the rates of such changes are little investigated. In the fungal kingdom, closely related species often exhibit diverse reproductive systems, from highly sexual to completely asexual reproduction (Raper 1966; Hawksworth *et al.* 1995; Geiser *et al.* 1996; Taylor *et al.* 1999). Such phylogenetic distributions suggest frequent reproductive system changes in fungi. In addition, there is anecdotal evidence that fungi may lose the ability for sexual reproduction in cultures in laboratories. However, the genomic mutation rate for those changes has not been empirically estimated in any fungus. The objective of this study is to provide an empirical estimate of the spontaneous mutation rate of loss of sexual reproduction, using the human pathogenic fungus *Cryptococcus neoformans* as a model system.

C. neoformans is an encapsulated haploid basidiomycetous yeast (Casadevall and Perfect 1998). It is ubiquitous in the environment and can cause significant morbidity and mortality especially in immunocompromised hosts (Casadevall and Perfect 1998). Its mating system is controlled by one locus with two alternative alleles, **a** and α (Kwon-Chung 1976). Under suitable conditions, unicellular yeasts with opposite mating types can mate to form dikaryotic or diploid cells (Kwon-Chung 1976; Sia *et al.* 2000). These mated cells can grow in filamentous form (*i.e.*, hyphae) with meiosis occurring at the terminal cell (basidium) of the hyphae. It should be noted that filamentation is necessary but not sufficient for proper meiosis and basidiospore formation in *C. neoformans*. On media conducive for mating and filamentation, the filamentous phase can be maintained indefinitely and sexual basidiospores may be produced continuously (Xu *et al.* 2000). Basidiospores can germinate to form vegetative yeast cells to complete the sexual life cycle. For a graphic representation of the complete life cycle of *C. neoformans*, please see a recent review by Alspaugh *et al.* (2000). Current evidence indicates that mating and filamentation are regulated by multiple, potentially interconnected signal transduction pathways, with many of the genes still unknown and under intense investigation (*e.g.*, see a review by Lengeler *et al.* 2000a).

Mating in fungi has traditionally been considered a qualitative trait. Two strains were either capable or not capable of mating to form stable diploids or heterokaryons. However, it is increasingly recognized that mating ability is a quantitative trait. For example, it is not uncommon to find that strains from nature differ in their abilities to mate with those of compatible mating types (*e.g.*, Xu 1995; Xu *et al.* 1996; Yan *et al.* 2002). In addi-

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tion, molecular genetic analyses have repeatedly demonstrated that strains containing certain gene deletions show reduced ability to mate or reproduce sexually (Lengeler *et al.* 2000a). One method for quantifying mating ability is to use strains containing different auxotrophic markers, thus facilitating the screening of mated, prototrophic products on minimum medium. In *C. neoformans*, several auxotrophic mutants of both mating types **a** and α with isogenic backgrounds are available for genetic experiments (Moore and Edman 1993). These strains are ideal for quantifying mating abilities among strains in laboratory evolution experiments. In this study, the relative number of mated cells in a cell mixture is used as a measure of mating ability. Similarly, the proportion of mated cells capable of forming hyphae provides a quantitative estimate for filamentation.

The genomic mutation rate is most commonly estimated by using mutation accumulation experiments (*e.g.*, Mukai 1964; Drake *et al.* 1998; Zeyl and de Visser 2001). In these experiments, spontaneous mutations are allowed to accumulate in replicate lines in the absence of selection for the trait under investigation. Two typical assumptions underlie these experiments. The first is that in the absence of selection, deleterious mutations will continuously accumulate in the trait not under selection, leading to a lower fitness for that trait. The second is that divergence among replicate lines in trait values should increase over time. The relative rate of decrease and divergence among lines in trait values allow estimation of various mutational parameters, including the genomic mutation rate, the average mutational effect, and the mutational heritability (Lynch and Walsh 1998). Like bacteria, fungi are ideal for mutation accumulation experiments as they can be clonally propagated, manipulated, and stored efficiently in the laboratory.

Despite its importance, to my knowledge, no direct estimates of genomic mutation rates for loss of sex have as yet been reported. Here, I performed a mutation accumulation experiment in *C. neoformans* under laboratory conditions to provide the first estimations in fungi for mutation rates, mutational effects, and mutational heritabilities for two traits, mating and filamentation. In addition, vegetative growth rates of the derived clones are measured to determine the relationships among these traits in the mutation accumulation lines.

MATERIALS AND METHODS

Mutation accumulation experiments: The strains used in this study were JEC50 (*MAT*α *ade2*) and MCC3 (*MAT***a** *ura5*). These two strains were haploid and isogenic except for the indicated genotypes (Sia *et al.* 2000). Mutation accumulation (MA) lines were grown and maintained on the rich medium YEPD agar (1% yeast extract, 2% Bacto-peptone, 2% dextrose, and 2% Bacto-agar in distilled water) at 25°. A single colony (the founder colony) was picked and streaked onto YPD agar to establish eight independent lines for each strain (Figure 1). The 16 MA lines were maintained by repeating the picking-streaking-incubating procedure (called a growth cycle) of a single colony for each line every 3 days. By the end of the third day, each colony increased from 1 cell to $\sim 1 \times 10^6$ cells. This cell count was obtained by cutting agar pieces containing entire colonies and resuspending them individually in 1 ml sterile water through vigorous vortexing. The cell suspensions were then diluted and spread-plated on YEPD plates for viable cell counts. The growth of 1 cell to ~1 million represented ~20 mitotic divisions (±1 cell division). These 16 MA lines were grown under identical conditions for 30 growth cycles, equivalent to ~600 mitotic divisions. To ensure that picked colonies descended from a single cell, a small cluster of cells ($\sim 10^5$) from a progenitor colony was resuspended in 1 ml sterile water by vigorous vortexing, and 1 μl of the suspension was streaked onto agar medium. Streaked single cells were confirmed by microscopy and marked for future transfers after 3 days growth. Cells from generations 0, 100, 200, 400, and 600 (abbreviated G0, G100, G200, G400, and G600, respectively) were stored in glycerol (18%) in a −80° freezer. This protocol intensified genetic drift by forcing each line through a bottleneck of one random cell in each growth cycle. Because the abilities for mating and filamentation were not selected, mutations affecting these two traits were expected to accumulate, with independently maintained lines accumulating different numbers and types of mutation.

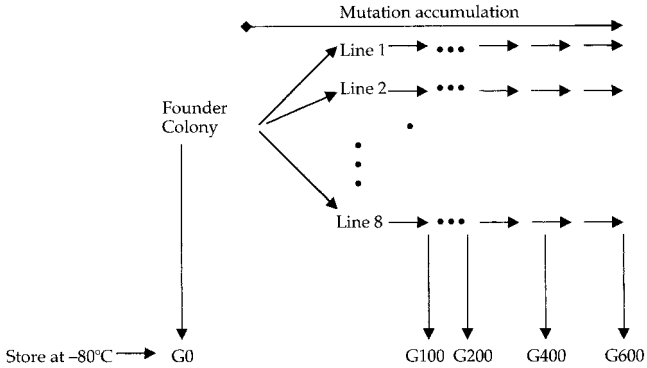

Figure 1.—Representation of mutation accumulation phase of the experiment in *Cryptococcus neoformans*. Eight lines were established and maintained for each of two strains, JEC50 and MCC3.

Quantification and analyses of mating and filamentation: To assess the abilities of mating and filamentation, cells from all stored cultures were first grown on synthetic medium (SD) containing 0.17% (w/v) Difco yeast nitrogen base with ammonium sulfate and amino acids, 2% dextrose, and 2% Bacto-agar. After 4 days of growth, cells were collected and resuspended in 200 μl of sterile distilled H_2O. Cell density for each culture was determined and adjusted to 5×10^4 cells/μl. For each mating, 4 μl of the transferred clones was mixed with an equal volume of the founder clone of the opposite mating type. Specifically, the same suspension of the founder clone for strain JEC50 (G0) was mixed with an equal volume of each evolved clone from strain MCC3 (*i.e.*, cells from G0, G100, G200, G400, and G600 for all eight MA lines). Similarly, the same suspension of the founder clone of strain MCC3 was used to quantify mating and filamentation of all evolved clones from strain JEC50. After mixing by repeated pipetting, half of each mating mixture (4 μl) was plated on SSΔN agar (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% sucrose, and 2% Bacto-agar), a medium that selects for mated, prototrophic cells. Four microliters of each individ-

ual culture was plated as a negative control to monitor if any evolved lines reversed from auxotrophic to prototrophic during the transfer events.

After incubating the mating mixtures at 25° for 24 hr, each mating mixture was cut out of the agar medium, placed in 1 ml of sterile distilled H_2O, washed into the H_2O by vigorous vortexing, and diluted (10–100 times). Aliquots of the original and diluted cells were plated onto SDΔN agar (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 2% dextrose, and 2% agar). Eight replicate pairings were performed between the parental starting clones (those at G0). Three replicates were performed for each of the other 64 pairings.

All plates were incubated at 25° for 5 days. Two colony types of mated cells were found and counted: those with hyphae and those without any hyphae. Neither parental strains nor the 64 derived clones grew independently on SDΔN agar, confirming that none of the 16 MA lines reverted to prototroph. For each mating, the total number of mated cells and the proportion of the mated cells not producing hyphae were obtained. PCR amplification using mating-type-specific primers of the STE20 gene (Lengeler *et al.* 2000b; Yan *et al.* 2002) confirmed that both colony types were heterozygous at the mating type locus, a result consistent with their prototrophic growth patterns on SDΔN medium.

Vegetative growth: Because individual colonies were randomly marked during transfers, asexual reproduction for all MA lines was therefore not directly selected. In this protocol, the ability to multiply on YEPD agar medium was maintained; however, spontaneous mutations could accumulate for vegetative fitness for the evolved clones. To determine if vegetative fitness of the evolved clones changed compared to the original clones, I used two methods. The first measured the doubling time of individual clones in YEPD broth (1% yeast extract, 2% Bacto-peptone, 2% dextrose in distilled water), following traditional protocols. The second measured colony sizes on YEPD plates (Zeyl and Bell 1997). In the second method, cell suspension and streaking were prepared the same way as described above for colony transfers. Five well-separated cells were randomly marked for later measurement. Cells were incubated for 72 hr and diameters of individual colonies were measured under microscope using an ocular scale. Both experiments were performed at 25°, the temperature used for mutation accumulation and mating and filamentation assays.

Data analyses: The means and standard deviations of vegetative growth rate and the abilities of mating and filamentation were calculated for the 2 starting and the 64 evolved clones. These data were then used to estimate the genome-wide mutation rates ($\hat{U}$), the average effects per mutation ($\hat{a}$), and the mutational heritability (h_m^2). These estimates were done for each of the two traits examined here. Since the two strains of *C. neoformans* examined here are haploid, estimation procedures followed those for haploids as described by Lynch and Walsh (1998). Because phenotypic changes in mutation accumulation lines were joint products of mutation rate and the effect per mutation, with a given phenotypic change, mutation rate and mutational effects would be in inverse relationship. Current procedures provide estimates for minimum mutation rate ($\hat{U}_{min}$) and maximum mutational effects ($\hat{a}_{max}$; Lynch and Walsh 1998).

Briefly, for haploid organisms, the minimum mutation rate ($\hat{U}_{min}$) and the maximum mutational effects ($\hat{a}_{max}$) were calculated as

$$\hat{U}_{min} = (\Delta M)^2/[\Delta V(1 + C_{\Delta M})(1 + C_{\Delta V})]$$

and

$$\hat{a}_{max} = \Delta V/[\Delta M\,(1 + C_{\Delta M})],$$

where ΔM and ΔV denote estimates of the rates of change of mean and variance, and $C_{\Delta M}$ and $C_{\Delta V}$ are squared coefficients of sampling variance (ratios of sampling variance to squared estimates) of ΔM and ΔV, respectively. The mutational heritability (h_m^2) was obtained as mutational variance scaled by environmental variance. These functions have been used extensively in mutation accumulation studies in bacteria, plants, and animals (*e.g.*, see a summary in Lynch and Walsh 1998).

To examine the potential variation in estimates of mutational parameters between strains, calculations were made separately for the two strains. Two-level nested ANOVA was used to assess divergence and heterogeneity for MA lines between the two strain backgrounds and among replicate MA lines within each strain background. Student's *t*-test was used to compare phenotypes between evolved and ancestral clones. Pearson correlation analyses were performed among mating, filamentation, and vegetative growth to determine the relationships of mutations affecting these three traits. These statistical tests followed the procedures in Sokal and Rohlf (1981).

RESULTS

Reduced abilities for mating among derived clones: The relative ability of mating for each of the 16 MA lines is presented in Figure 2 for clones from strain JEC50 and Figure 3 for clones from strain MCC3. For mating between the two starting clones, a total of 16,290 prototrophic colonies were recovered from the eight replicates, yielding a mean mating success rate of 2.036% (16,290/800,000), with a range 1.817–2.254% and a standard deviation of 0.108% (slightly >5% of the mean). Similarly, standard deviations among replicates were low for each of the other 64 pairings, typically between 5 and 10% of their mean. For easy comparison, the mating ability between the two starting clones was scaled to 1 and other mating combinations were adjusted accordingly (Figures 2 and 3).

After 30 growth cycles (~600 mitotic divisions), though none completely lost the ability for mating, all 16 MA lines showed reduced mating ability (Table 1; Figures 2 and 3). There were quantitative differences between the MA lines of the two original strains in the losses of mating (Table 1), with lines from strain MCC3 showing slightly more declines than those from strain JEC50. However, at G600, while significant heterogeneities in mating ability were observed among the 8 replicate lines within each of the two strains [$F_{(14,32)} = 282.08$, $P < 0.001$], the difference between the two strain backgrounds was statistically not significant [$F_{(1,14)} = 1.235$, $P > 0.05$] (Table 2).

The patterns of decline differed among replicate lines for the two strains (Figures 2 and 3). For example, MA line 1 from JEC50 showed a small decrease in mating ability while the other seven lines from JEC50 all showed significant decreases at G600. All eight lines from MCC3 showed significant reductions at G600. Overall, the rates of reduction in mating ability were greater during the first 200 mitotic generations than during the last 400 generations, regardless of strain background. Coupled

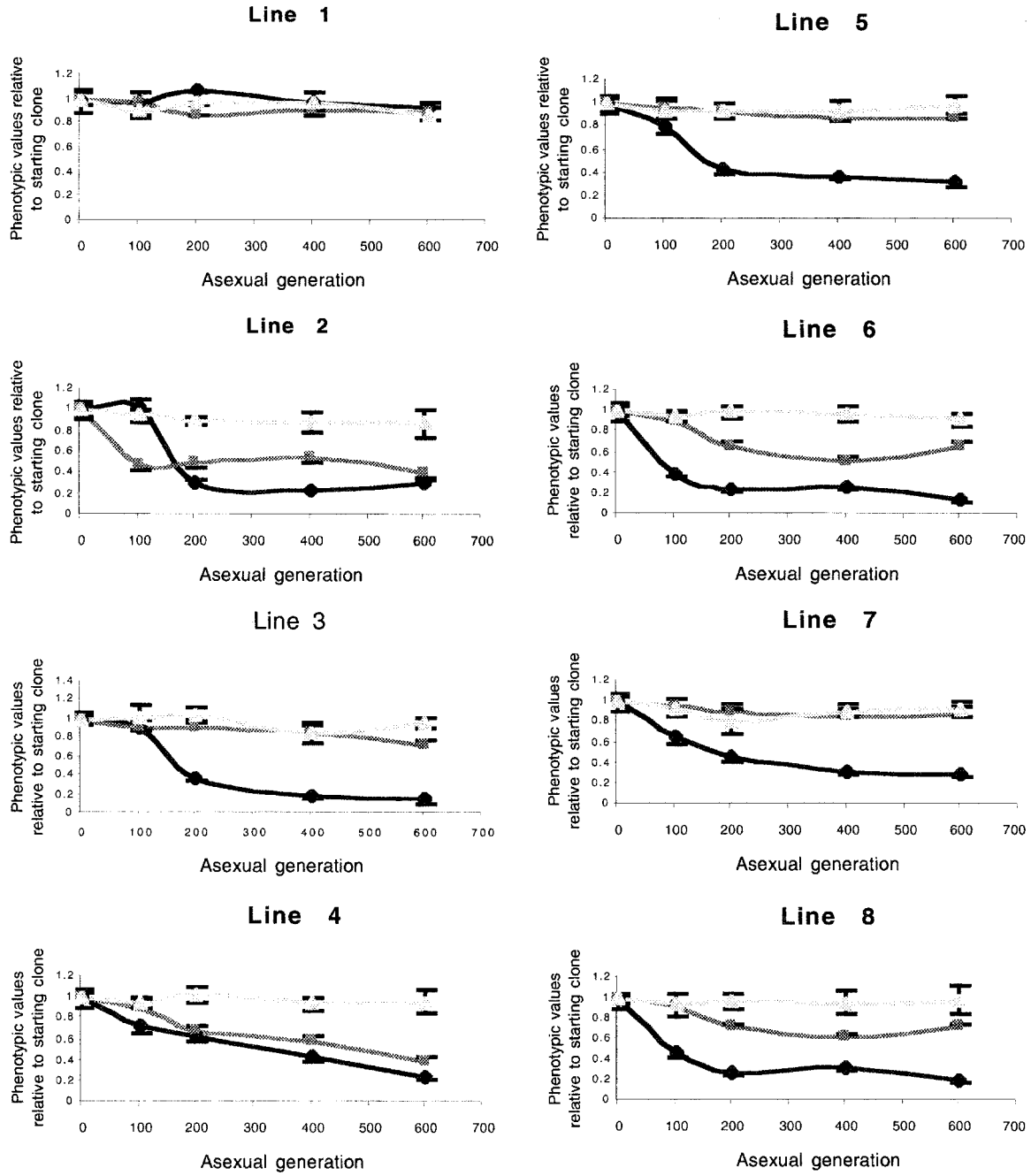

FIGURE 2.—Relative phenotypic values of three traits for each of the eight MA lines from strain JEC50. The *x*-axis represents the number of asexual generations and the *y*-axis represents phenotypic values relative to the starting clone. Phenotypic values of all three traits for the starting clone were scaled to 1. Lines with triangles represent relative vegetative growth rates of the derived clones. Lines with squares represent relative filamentation abilities of the derived clones. Lines with diamonds represent relative mating abilities of the derived clones. Standard deviation is shown for each data point.

with this pattern of decline, the among-line variance for each strain increased more rapidly in the first 200 generations than in the last 400 generations (Figures 4 and 5). On the basis of the rate of decrease and divergence among lines, estimates of minimum mutation rate ($\hat{U}_{\min}$), maximum mutational effect ($\hat{a}_{\max}$), and mutational heritability (h_m^2) to the loss of mating ability were calculated for each strain background and are presented in Table 1.

Estimates for the three mutational parameters for mating differed between the two strain backgrounds. The $\hat{U}_{\min}$ for mating was 0.0172 as estimated from the eight MA lines derived from JEC50 and 0.0772 for those derived from MCC3, a difference of over fivefold. Consequently, the mutational effects differed by over fivefold, but in the reverse direction (Table 1). The mutational heritabilities for mating were 0.009 for MCC3 and 0.0293 for JEC50, respectively.

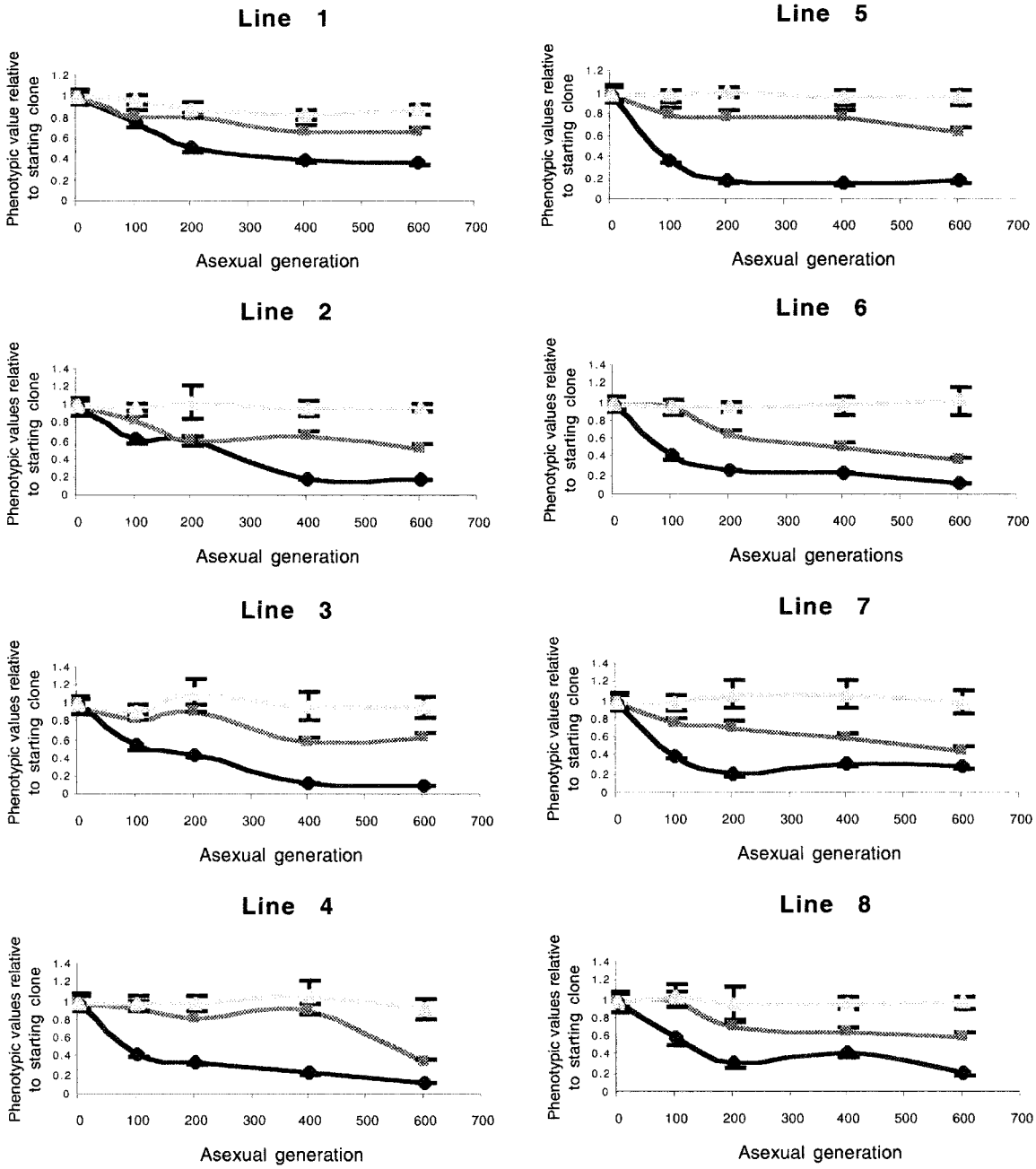

FIGURE 3.—Relative phenotypic values of three traits for each of the eight MA lines from strain MCC3. The *x*-axis represents the number of asexual generations and the *y*-axis represents phenotypic values relative to the starting clone. Phenotypic values of all three traits for the starting clone were scaled to 1. Lines with triangles represent relative vegetative growth rates of the derived clones. Lines with squares represent relative filamentation abilities of the derived clones. Lines with diamonds represent relative mating abilities of the derived clones. Standard deviation is shown for each data point.

Loss of filamentation among derived clones: The relative ability of mated cells to form filaments for each of the 16 MA lines is presented in Figure 2 for clones from strain JEC50 and Figure 3 for clones from strain MCC3. Among the eight replicates for the two starting clones, 5.69% (SD = ±0.21%) of the mated cells were unable to form filaments. Standard deviations of filamentation ability among replicates were typically low for mated cells from each of the other 64 pairings, typically <10% of their mean. Similar to the analysis of mating ability, filamentation ability between the two starting clones was scaled to 1 and other mating combinations were adjusted accordingly (Figures 2 and 3).

After 30 growth cycles, although none completely lost the ability for filamentation, all 16 MA lines showed reduced ability to form filaments (Table 1; Figures 2 and 3). There were quantitative differences between the MA lines of the two original strains in the loss of

TABLE 1

Decreases in mating and filamentation in mutation accumulation lines of *Cryptococcus neoformans* over 600 asexual generations

Trait measures[a]	JEC50 (*MAT*α *ade*−)	MCC3 (*MAT***a** *ura*−)
Mating		
Mean decrease (range)	0.6761 (0.0751–0.8636)	0.7879 (0.6062–0.8764)
$\hat{U}_{min}$	0.0172	0.0772
$\hat{a}_{max}$	0.0604	0.0110
h_m^2	0.0293	0.0090
Filamentation		
Mean loss (range)	0.2488 (0.0456–0.5431)	0.4045 (0.2674–0.5795)
$\hat{U}_{min}$	0.0036	0.0232
$\hat{a}_{max}$	0.0948	0.0519
h_m^2	0.0343	0.0108

The mutational parameters $\hat{U}_{min}$, $\hat{a}_{max}$, and h_m^2 are defined in MATERIALS AND METHODS. The maximum decrease of mating ability was 1 when no mated cells were observed, and the maximum loss of filamentation was 1 when none of the mated cells were able to form hyphae.

[a] Please refer to MATERIALS AND METHODS for calculations.

filamentation (Table 1), with lines from strain MCC3 showing greater declines than those from strain JEC50. However, at G600, while significant heterogeneities in filamentation ability were observed among the 8 replicate lines within each of the two strains [$F_{(14,32)} = 27.34$, $P < 0.001$], the difference between the two strain backgrounds was statistically not significant [$F_{(1,14)} = 2.854$, $P > 0.05$] (Table 2).

Similar to the declines in mating ability, the patterns and rates of decline in filamentation ability differed among replicate lines for each of the two strains (Figures 2 and 3). For example, at G600, MA lines 1, 5, and 7 from JEC50 showed small, insignificant decreases in filamentation ability while the other five lines from JEC50 showed significant decreases. In contrast, all eight lines from MCC3 showed significant reductions at G600. In contrast to the patterns of mating, the rates of reduction in filamentation were more uniform across the 600 mitotic generations (Figures 2 and 3). In addition, the among-line divergences were more uniform (Figures 4 and 5). Estimates of $\hat{U}_{min}$, $\hat{a}_{max}$, and h_m^2 to the loss of filamentation were calculated for each strain background and are presented in Table 1.

Overall, the MA lines from MCC3 had a larger decrease in filamentation ability than those from JEC50. As a result, estimates for the three mutational parameters for filamentation differed between the two strain backgrounds. The $\hat{U}_{min}$ for filamentation was 0.0036 as estimated from the eight MA lines derived from JEC50 and 0.0232 for those from MCC3, a greater than sixfold difference. The mutational effects differed by less than twofold, in the reverse direction (Table 1). The muta-

TABLE 2

Two-level nested ANOVA for losses of mating and filamentation in experimental populations of *C. neoformans*

Trait	Source of variation	d.f.	Sum of squares	Mean Square	F_s
Mating	Between JEC50 and MCC3 strain background	1	0.1445	0.1445	1.235 (NS)
	Among MA lines within a strain background	14	1.4939	0.1067	282.08*
	Among repeats within a MA line	32	0.0121	0.000378	
	Total	47	1.6506		
Filamentation	Between JEC50 and MCC3 strain background	1	0.2374	0.2374	2.854 (NS)
	Among MA lines within a strain background	14	1.1646	0.0831	27.34*
	Among repeats within a MA line	32	0.0973	0.00304	
	Total	47	1.4994		

Only data from G600 were used in these analyses. NS, statistically not significant; *, statistically highly significant ($P < 0.001$).

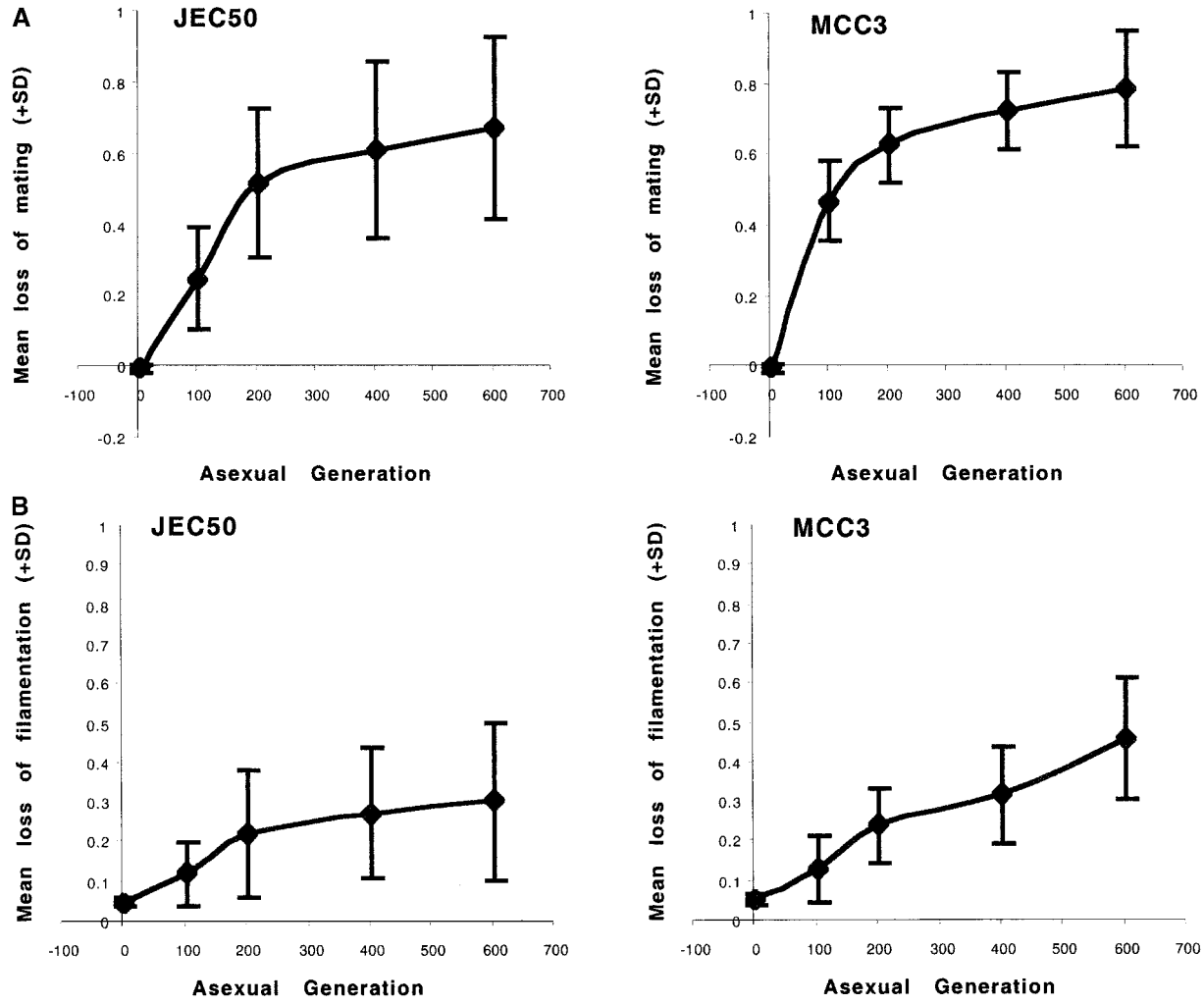

FIGURE 4.—Mean loss and the among-line standard deviations in mating (A) and filamentation (B) over 600 mitotic generations for each of the two strains. The *x*-axis shows the number of asexual generations, while the *y*-axis represents the mean and standard deviation of losses of mating or filamentation. For each data point, the standard deviation was among eight MA lines. The maximum losses of both mating and filamentation were 1 when no mated cells were found or when no mated cells formed hyphal filaments, respectively.

tional heritability for filamentation was 0.0108 for MCC3 and 0.0343 for JEC50, respectively.

Vegetative growth rate: Vegetative growth rates were measured by two methods (see MATERIALS AND METHODS) for the 2 starting clones and the 64 derived clones. Overall, the two methods yielded identical patterns; therefore, only colony size data are presented here. Similar to the analyses performed for mating and filamentation, growth rates of the starting clones were scaled to 1 and the results for all 16 MA lines are presented in Figures 2 and 3. No overall significant reduction of vegetative growth rate was observed for any of the derived clones. At G600, the 8 MA clones from JEC50 had a mean growth rate of 0.939, with a standard deviation of 0.059, not significantly different from the starting clone [mean (±SD) = 1 (±0.054)]. Similarly, at G600, the 8 derived clones from strain MCC3 had a mean growth rate of 0.973 (SD = 0.069), not significantly different from the starting clone of MCC3 [mean (±SD) = 1 (±0.057)]. Because of the lack of statistically significant differences, mutational parameters were not estimated for vegetative growth rate. However, correlation analyses among filamentation, mating, and vegetative growth rate were performed, as described below for clones from G600.

Correlation analyses among mating, filamentation, and vegetative growth rate: Because clones from different time points of the same MA line were not independent, only data from G600 were analyzed for potential correlation between traits. Only the mean value of each clone was plotted for each trait. The results of the analyses are presented in Figure 5. For the three pairwise analyses, two showed statistically significant correlations. Mating and filamentation were positively correlated (Pearson correlation coefficient $R = 0.4709$, $P = 0.0496$). In contrast, mating and vegetative growth rate

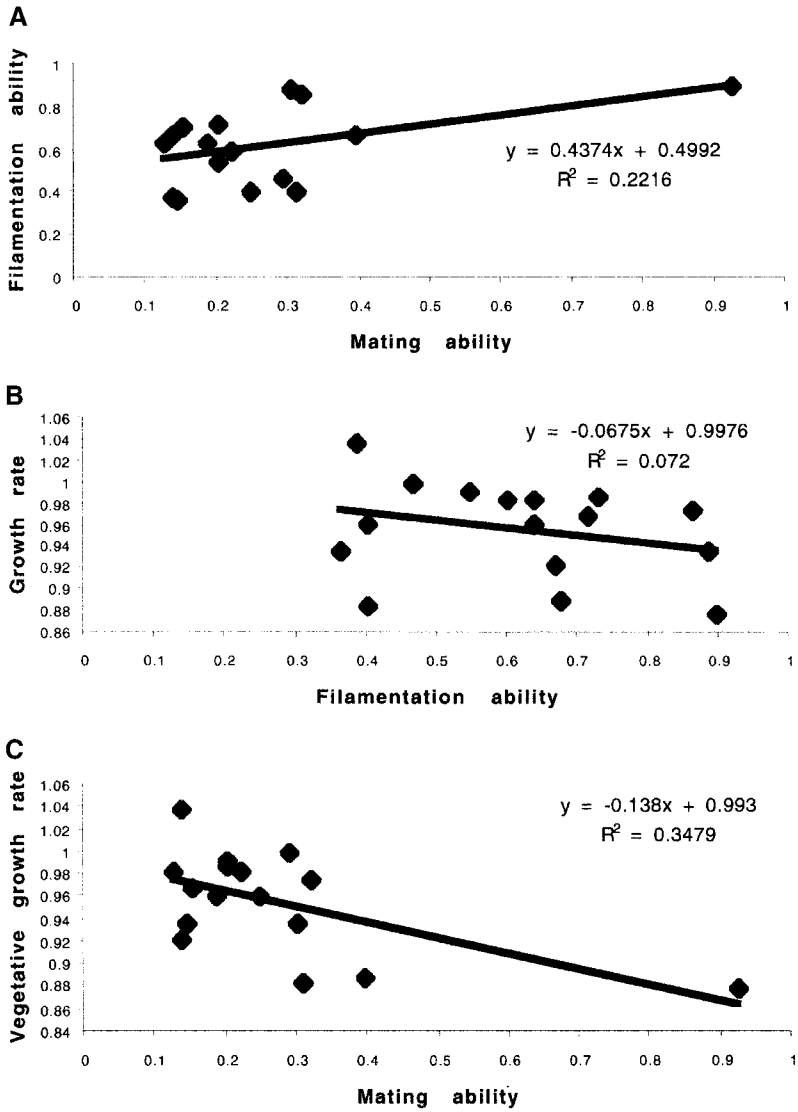

FIGURE 5.—Graphic representation of correlations among three traits in experimental populations of *C. neoformans*. For each of the 16 mutation accumulation lines, only data from G600 were used. (A) Positive correlation between mating and filamentation (Pearson correlation coefficient $R = 0.4709$; $P = 0.0496$). (B) Negative, but statistically insignificant correlation between filamentation and vegetative growth rate ($R = -0.2711$; $P = 0.3099$). (C) Negative correlation between mating and vegetative growth rate ($R = -0.5899$; $P = 0.0162$).

were negatively correlated ($R = -0.5899$, $P = 0.0162$). Filamentation and growth rate were negatively correlated ($R = -0.2711$), but this correlation was statistically not significant ($P = 0.3099$). However, as seen in Figure 5, A and C, 1 data point (from MA line 1 of JEC50) was very different from the other 15 points. If data were reanalyzed without data from JEC50-line 1, no statistically significant correlation was found in any of the three pairwise analyses. It should be emphasized that there were significant heterogeneities among MA lines for both traits in both strain backgrounds, with or without data from JEC50-line 1 (Table 2, data not shown).

DISCUSSION

In this study, I performed MA experiments and investigated mutational parameters for mating and filamentation in the human pathogenic yeast *C. neoformans*. Though none of the eight MA lines from each of the two original strains completely lost the ability to undergo mating and filamentation, their abilities for both traits were significantly reduced at G600. Because there was no significant loss of vegetative growth rates among the evolved clones, the results here suggested that reductions in mating and filamentation were specific for mating and filamentation, with little effect on vegetative growth rate.

Mutational parameters: The estimates of mutation rates ($\hat{U}_{\min}$) of 0.0172–0.0772 for mating and 0.0036–0.0232 for filamentation were similar to those determined for a variety of traits in microbes, plants, and animals (MUKAI 1964; KEIGHTLEY and CABALLERO 1997; DRAKE *et al.* 1998; LYNCH and WALSH 1998; VASSILIEVA and LYNCH 1999; ZEYL and DE VISSER 2001). For example, $\hat{U}_{\min}$ were 0.02–0.6 for egg-adult viability in Drosophila (MUKAI 1964; DRAKE *et al.* 1998), 0.0024–0.054 for fitness traits in *Arabidopsis thaliana* (SCHULTZ *et al.* 1999), and 0.003–0.060 for various life-history traits in the model nematode *Caenorhabditis elegans* (KEIGHTLEY and CABALLERO 1997; VASSILIEVA and LYNCH 1999). Com-

pared to those in Drosophila and *C. elegans*, the ranges of estimates were slightly narrower for the two traits examined here in *C. neoformans*. The mechanisms for such differences are not known at present. However, they might reflect the genetic backgrounds of strains. In this study, the two strains JEC50 and MCC3 were isogenic except at the three indicated loci. It is entirely possible that strains of other genetic backgrounds might provide estimates outside the ranges as determined here but closer to the ranges determined for other traits in other species.

Despite their isogenic backgrounds, quantitative differences between strains JEC50 and MCC3 were observed in estimates of mutational parameters for both mating and filamentation. The exact mechanisms for these differences are unknown at present. However, there are a few possibilities. First, the two auxotrophic loci (*ade* and *ura*) could have contributed to the difference. Because *ade* and *ura* are different auxotrophic markers, they could have exerted different pressures on cellular metabolisms even on nutrient-rich medium, thereby indirectly influencing mutational patterns. Second, the mutational target sites might differ between mating types **a** and α. At present, the exact boundaries of genes located within the mating-type locus are not known and are under intense investigation. Third, the mutational differences between these two strains might represent random effects and not differences in the genetic backgrounds of the two strains. As shown in Table 2, MA lines from the two strains did not differ in overall rates of loss of mating and filamentation at G600. In contrast, significant heterogeneity among MA lines was observed for both traits within each of the two strain backgrounds (Table 2). These results are consistent with the third hypothesis that the differences in estimates of mutational parameters between the two strains were due not to genetic differences between the two starting clones but likely to stochastic differences due to random mutations.

Sexual *vs.* vegetative fitness in MA lines: Because our MA protocol involved extensive genetic drift, mutations influencing both sexual and vegetative fitness were expected to accumulate, with most mutations having deleterious effects (see also Kibota and Lynch 1996; Zeyl and de Visser 2001). This protocol was different from batch cultures where increases in vegetative fitness among asexual evolving lines were observed (*e.g.*, see Travisano *et al.* 1995). Similar to previous MA experiments in microorganisms (*e.g.*, Kibota and Lynch 1996; Zeyl and de Visser 2001), I observed overall reduced vegetative growth rates for clonal MA lines at G600. However, unlike previous studies, the reductions observed here were not statistically significant for the MA lines. Furthermore, no significant divergence in vegetative growth rates was observed among these MA lines. For example, standard deviations among MA lines at G600 were comparable to those within each of the two original clones (see above). Several factors could have contributed to the different results. The first is intrinsic differences among the species with regard to intracolony competition and selection. Though intercolony competition was eliminated through our protocol, occasional beneficial mutations could arise during colonial growth. These mutants could be more numerous within a given colony and therefore more likely to be transferred to the next growth cycle. It is possible that during MA there was a higher level of intracolony competition in *C. neoformans* than in *Escherichia coli* and *Saccharomyces cerevisiae* as studied by Kibota and Lynch (1996) and Zeyl and de Visser (2001), respectively.

The second possibility for differences among studies could be due to differences in the amount of prior exposure to the MA culture conditions that the starting cells experienced (*e.g.*, medium, temperature, etc.). The longer the exposure, the more likely the starting cells obtained optimal adaptation to those conditions. Single-colony transfers as performed in these studies were more likely for better-adapted cultures to show greater losses of fitness. While the amounts of exposures to the respective culture conditions for each of the strains used were almost impossible to obtain, the laboratory strains of the model bacterial species *E. coli* used by Kibota and Lynch (1996) and the model yeast *S. cerevisiae* used by Zeyl and de Visser (2001) were probably more adapted to their respective MA conditions than were those of *C. neoformans*.

We observed a negative correlation between sexual and vegetative fitness among evolved clones. This result suggests that, for a given evolved clone, the greater decline in sexual fitness was generally associated with slower decline in vegetative fitness. This result was consistent with the hypothesis of a trade-off between sexual and vegetative fitness. Such a trade-off could exist both before and during the MA experiment. Alternatively, adaptations of strains to the MA conditions might have pleiotropic effects on mating and filamentation. Trade-offs due to genes with pleiotropic effects have been reported among life history traits in a variety of species (*e.g.*, see Bell and Koufopanou 1986; Scheiner *et al.* 1989). The negative correlation as observed here was similar to that reported by Da Silva and Bell (1992) in their selection experiment using the unicellular alga, *Chlamydomonas reinhardtii*. However, unlike their results, the loss of sexual function observed here was not accompanied by increases in vegetative fitness. Although not statistically significant, the mean vegetative fitness declined in the MA lines in both strain backgrounds (Table 1, Figure 5). It is also possible that the accumulated mutations might exert deleterious or beneficial effects on other traits (*e.g.*, virulence in a mouse model). Further experiments are needed to test for other potential pleiotropic effects of the accumulated mutations.

Relationship between losses of mating and filamentation among MA lines: The positive correlation between

the losses of mating and filamentation suggests that some of the accumulated mutations might have had deleterious effects on both traits, a result consistent with our current knowledge about the pathways regulating mating and filamentation in *C. neoformans* and other fungi (Lengeler *et al.* 2000a). Interestingly, as seen in Table 1 and Figures 2–4, the rate of decrease was greater for mating than for filamentation. Two factors might have contributed to such a difference between the two traits. The first factor is that more genes might be involved in mating than in filamentation, and therefore there might be more targets for mutations. Mating in *C. neoformans* involves interactions between two haploid cells and is a multistep process, including pheromone production, secretion, and sensing; cell-cell recognition; conjugation-tube formation; and cell fusion. In contrast, filamentation includes the initiation and maintenance of hyphal cells after successful mating.

A second factor that may have contributed to the differences in the rates of decline in mating and filamentation is that those differences might reflect different patterns of expression of mutations affecting the two traits. Because unmated cells in *C. neoformans* are haploid, both dominant and recessive deleterious mutations could have been expressed in the haploid phase to negatively affect mating. In contrast, filamentation involves interactions between the two nuclei within a single cell, and recessive mutations in one nucleus could be masked by normal alleles in the other nucleus. Under this second hypothesis, the loss of filamentation observed here might reflect only the effects of dominant or partially dominant mutations.

Relevance to natural populations: It should be emphasized that the mutation accumulation experiments reported here were performed under laboratory conditions unlikely to be found in natural environments. Therefore, the mutational estimates might not correspond exactly to those in nature. In their natural habitats, most microorganisms, including *C. neoformans*, likely experience fluctuations in temperature and nutrient levels. And currently, there are no effective methods for estimating generation times, mating success rates, and other fitness traits in natural or clinical environments. However, for several reasons, the observations of rapid declines in mating and filamentation in laboratory populations of *C. neoformans* are relevant to natural populations of fungi.

First, environmental or clinical strains of *C. neoformans* unable to mate and reproduce sexually have been reported by many investigators (*e.g.*, Casadevall and Perfect 1998; Lengeler *et al.* 2000b). For example, Lengeler *et al.* (2000b) recently described a clinical strain from Tanzania that had completely lost its ability for mating and filamentation. Interestingly, this strain grew well both *in vitro* on media and *in vivo* to cause systemic cryptococcal infection in an experimental mouse model (Lengeler *et al.* 2000b). More recently, Yan *et al.* (2002) found that among 358 strains of *C. neoformans*, only 111 successfully mated with either one of two standard tester strains, despite repeated crossing attempts. Molecular surveys indicated that all 358 strains contained at least one allele for genes examined within the mating-type locus (Yan *et al.* 2002).

Second, genetic mutations deleterious to sexual reproduction have been reported in natural isolates from various other fungal species capable of sexual reproduction. These species include the commercial button mushroom *Agaricus bisporus* (Xu 1995), the plant pathogen *Gibberella fujikuroi* species complex (Leslie and Klein 1996), the model filamentous fungus *Neurospora crassa*, and other filamentous fungi (Perkins 1997).

Third, current literature indicates that the sexual states of ~20% of the >70,000 fungal species described so far remain to be identified (Hawksworth *et al.* 1995). These species are collectively called Fungi Imperfecti or Deuteromycota. Undoubtedly, some species in the Division Deuteromycota may in fact be capable of sexual reproduction but are so classified due only to our inability to observe and experimentally induce the processes of sexual reproduction in the laboratory. Indeed, *C. neoformans* was included in the Division Deuteromycota from its discovery in the nineteenth century until the early 1970s, when its capacity for sexual reproduction was finally discovered (Kwon-Chung 1976; Casadevall and Perfect 1998). However, for some species, *e.g.*, the human pathogenic yeast *Candida albicans*, the complete sexual reproductive cycle has yet to be observed (Hull and Johnson 1999; Magee and Magee 2000). Interestingly, recent genetic analyses of natural populations of some of these apparently asexual species have shown that random associations of alleles within and between loci are common (Taylor *et al.* 1999; Xu *et al.* 1999). Such random associations suggest that recombination has played a significant role in the recent past in populations of these asexual species.

Last, the patterns of decrease observed here (Figures 2 and 3) suggest that genetic mutations with quantitative effects contributed to the loss of mating and filamentation in *C. neoformans*. The seemingly wide distribution of mutations with quantitative effects on sexual reproduction suggests a potential alternative approach to our understanding of sexual reproduction in fungi. This alternative, quantitative genetic approach should complement current research efforts that focus on gene disruption experiments. This alternative approach could have practical implications as well. For example, there is a concerted effort to induce sexual reproduction in the human pathogenic yeast *C. albicans*. Genetic analyses of populations of *C. albicans* have detected evidence of recombination (*e.g.*, Xu *et al.* 1999). Recent evidence from genetic and genomic studies also suggests that the *C. albicans* genome contains virtually all the genes necessary for mating and sexual reproduction (Hull and Johnson 1999; Magee and Magee 2000; Tzung *et*

al. 2001). However, a complete sexual reproduction cycle has not been found or induced. The results from this study suggest that the failure to undergo sexual reproduction in *C. albicans* could reflect in mutations with subtle, quantitative effects. Further molecular analysis of mutations accumulated in *C. neoformans* and other species may help elucidate quantitative genetic loci and potential environmental factors influencing sexual reproduction in fungi.

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