

Investigating How Ploidy and Stress-induced Ploidy Change Affects Fitness in *Candida albicans*

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Abstract

Candida albicans is a human pathogen that is lethal in 40-50% of systemic infections. Fluconazole (FLC) is the most commonly prescribed drug used to treat these infections. However, resistance to FLC is common and of the clinical isolates found to be FLC-resistant, 50% are aneuploid (having an abnormal number of chromosomes). To investigate differences between the adaptability of different ploidy states, isolates of two non-resistant diploid and two non-resistant tetraploid strains were grown up in 96-well plates with and without FLC. The resulting growth curves were similar between diploids and tetraploids in both approximate growth rate and final OD. After growing and passaging the plates for four consecutive growth cycles, the ploidy states of each of the isolates were measured using flow cytometry. A small number of diploid isolates changed ploidy, while a much larger number of tetraploid isolates changed ploidy and tetraploid isolates showed a much wider range of ploidies than diploids, with most changes being a decrease in ploidy. Tetraploid isolates usually decreased their growth rate when compared to their parents; few diploids showed this phenotype. Thus tetraploid strains exposed to FLC produced more variability in ploidy than diploids, and while YPAD growth rate decreases were observed with ploidy change, they were not linearly associated with the degree of ploidy change.

Introduction

Candida albicans is the most prevalent human fungal pathogen. *C. albicans* infections can be oral, vaginal, or systemic. Humans with weakened immune systems are at greater risk for infection and systemic infections have a relatively high rate of morbidity (40-50%). Fluconazole is the most common drug used to treat yeast infections. The drug targets an essential enzyme in the pathway in the ergosterol biosynthetic pathway, required for the generation of a component of the fungal plasma membrane (Uppuluri, 2013). After prolonged exposure to FLC, *C. albicans* have shown increased resistance to the drug, resulting in ineffective treatment (Uppuluri, 2013).

Recent work has shown that 40-50% of the FLC-resistant strains are aneuploid (having an abnormal number of chromosomes) with some of these aneuploidies conferring drug resistance (Selmecki, 2006). Recently, fluconazole-resistant tetraploid isolates were recovered from patients, indicating that the diploid state is not the only ploidy state that exists in nature and suggesting that ploidy shifts can increase fitness under drug stress. In a study conducted on the distantly related yeast *Saccharomyces cerevisiae*, ploidy levels were linked directly to the expression of proteins in the cell, suggesting that aneuploidy in cells can increase fitness under specific stresses (Pavelka, 2010). By studying stress-induced ploidy changes, we will better understand mechanisms that lead to increase FLC resistance. This understanding could help with the development of drugs targeting these pathways and thus could lead to more effective treatment of *C. albicans* infections.

In this study, four strains were used – two non-resistant diploids and two non-resistant tetraploids. For each strain, 48 isolates were grown at 30°C in both YPAD and YPAD with 10µg/ml FLC. After this evolution assay, ploidy and growth rates of parents (no FLC exposure) and evolved isolates (FLC-exposed) were compared. The evolved diploid isolates averaged a 35% increase in max growth rate from their parents when grown in drug (YPAD + FLC), while FLC-exposed tetraploid isolates averaged a 90% increase in growth rates. In YPAD without drug, the diploid isolate’s max growth rate was not statistically different while the tetraploid isolates grew significantly worse than their parents in YPAD. The genomic changes that occurred during the initial FLC exposure affected the subsequent max growth rate of the evolved tetraploids isolates more than the diploids.

Evolution experiment procedure

To investigate how *C. albicans* ploidy changes after prolonged FLC exposure, multiple isolates were grown in the presence of the drug for approximately one week (see schematic in Figure 1). Every 48 hours, a subset of cells was diluted into fresh media. All of the isolates transferred were diluted by the same factor. This process was repeated for four passages. Cell growth was monitored using a plate reader for the purpose of deciding a dilution factor.

Experimental Procedure

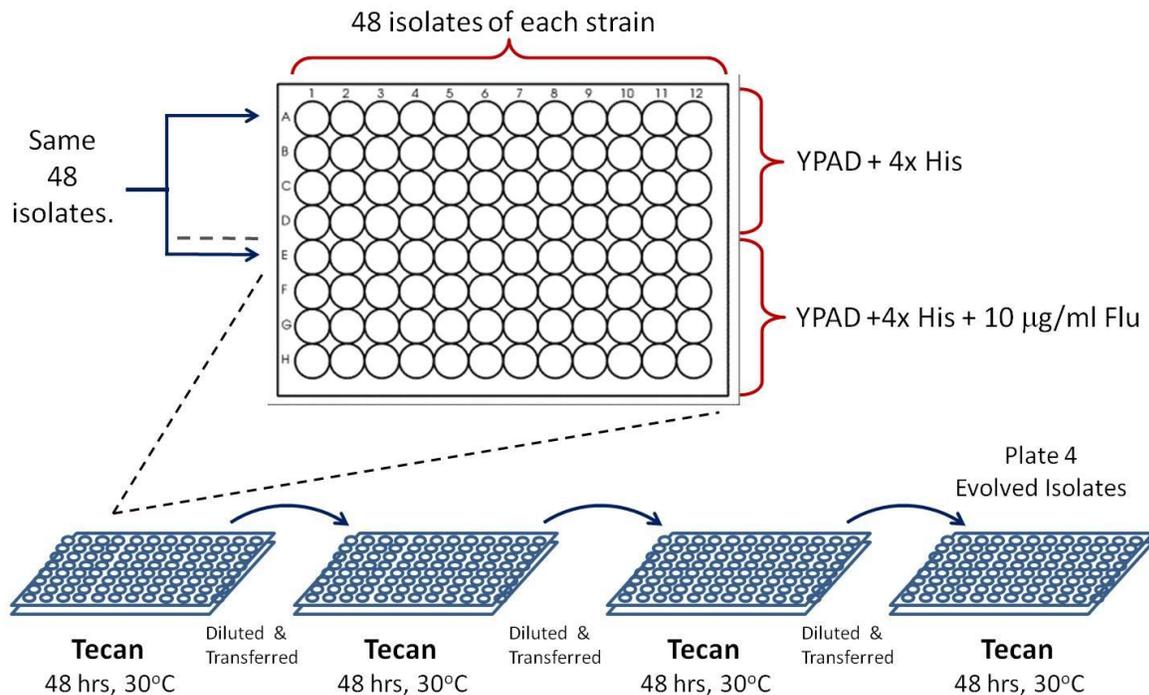


Figure 1

The experiment was performed using two diploid and two tetraploid strains. FLC was added to half the wells at a concentration of 10 µg/ml. Cell growth was monitored using optical density measurements in a Tecan plate reader.

Analysis of ploidy change during four passage FLC exposure

The role of ploidy in adaption and the development of FLC resistance is not well understood. To observe how growth in the presence of FLC affected genome size, flow cytometry was used to measure the ploidy states of each isolate after passage two (Approx. 100 hours in FLC) and after passage four (Approx. 200 of hours in FLC). The parental ploidies (both in diploid and tetraploid) were the same when they were grown up in the absence of environmental stresses (YPAD without drug). In the presence of FLC, the passage two isolates (~100 h of growth in FLC) for both the diploids and tetraploids showed more variability in ploidy. The passage two isolates that started as diploids ranged from 1.9 to 2.5N with an average ploidy change of 0.15 (in reference to 2N). Thus, in diploids, most ploidy changes were small increases in DNA content. In contrast, for the tetraploid isolate the ploidies of passage two isolates ranged from 2 to 4.2N, with an average ploidy change of -0.73 (in reference to 4N). Similar results were seen at passage four: diploid passages had an average ploidy change of 0.17 (in reference to 2N); tetraploid passages had an average ploidy change of 0.93 (in reference to 4N). Although passage two and passage four isolates had similar ranges in ploidy for both starting strains, many individual isolates did not maintain the same ploidy between the passages, indicating that the isolates were not genetically stable. Tetraploids showed greater variability than diploids in ploidy state after exposure to FLC in both passages 2 and 4 and tetraploid isolates also underwent more ploidy changes between passages 2 and 4 than did the diploid isolates.

Nearly all this ploidy change seen in tetraploids was due to loss of ploidy. The diploid isolates remained close to 2N and usually exhibited an increase in ploidy. A notable (and exciting) exception is that two isolates in the diploid lineage significantly reduced ploidy to 1.2 and 1.6 (Figure 2). This suggests they were becoming haploid, a process recently detected in the Berman lab. Interestingly, this was seen for diploid isolates but not with tetraploid isolates.

Tetraploids show greater variability than diploids in ploidy state after exposure to FLC

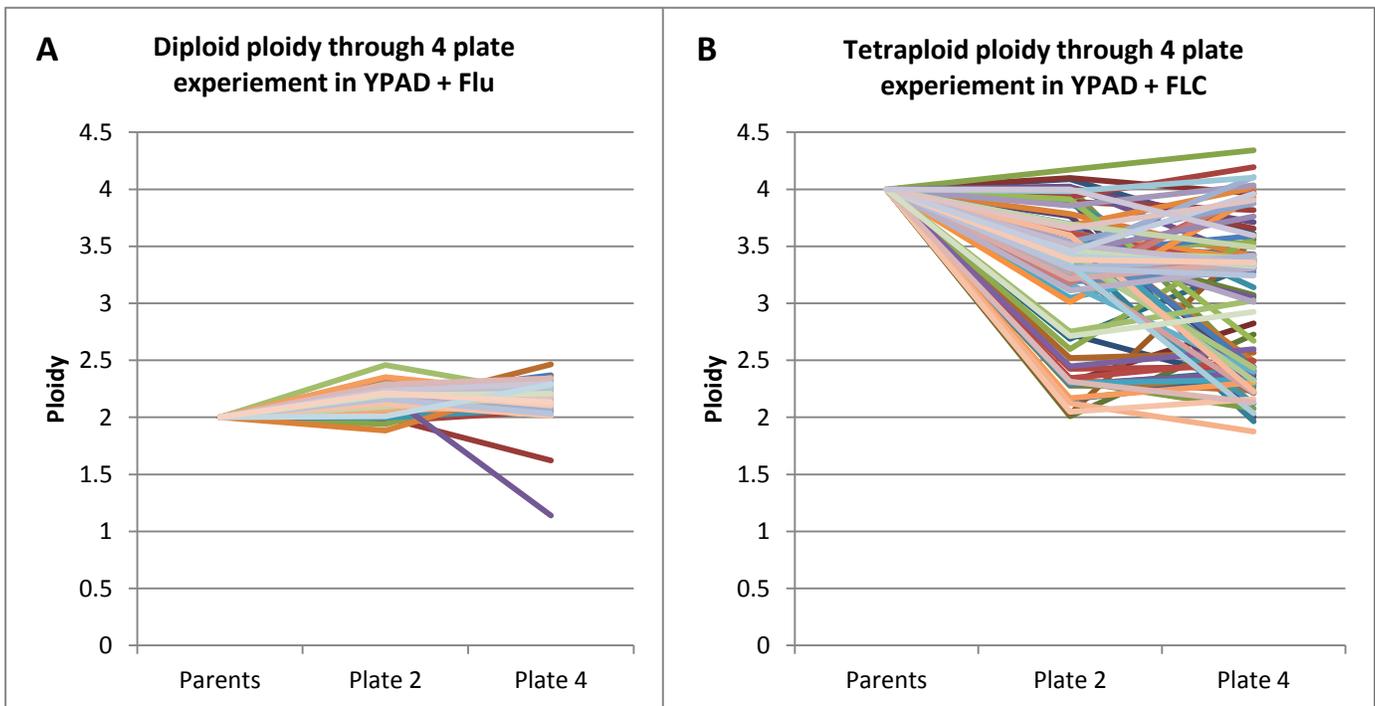


Figure 2

The ploidy of each isolate was measured after passage two and after passage four of the evolution experiment depicted in Figure 1. The graph depicts how each isolate's ploidy changed throughout the four passages.

Determination of a growth metric

To determine a metric best suited to measure fitness of parents and evolved isolates, maximum growth rate and maximum optical density (OD) were quantified to compare two characteristics of cell growth: how fast the cells are able to replicate (max growth rate), and how efficiently the cells are able to use resources in their environment (max OD). Three trials were run and then averaged to ensure that we accounted for variability between replicates. These measurements were plotted against each other for each isolate to observe their correlation. In YPAD without drug the max growth rate and max OD had a correlation coefficient of 0.7756 and in YPAD + FLC they had a correlation of 0.8372 (Figure 3). Due to the strong correlation observed in both environments, further comparisons between parents and isolates were done using the maximum growth rate metric.

Max Growth Rate and Max OD Correlation

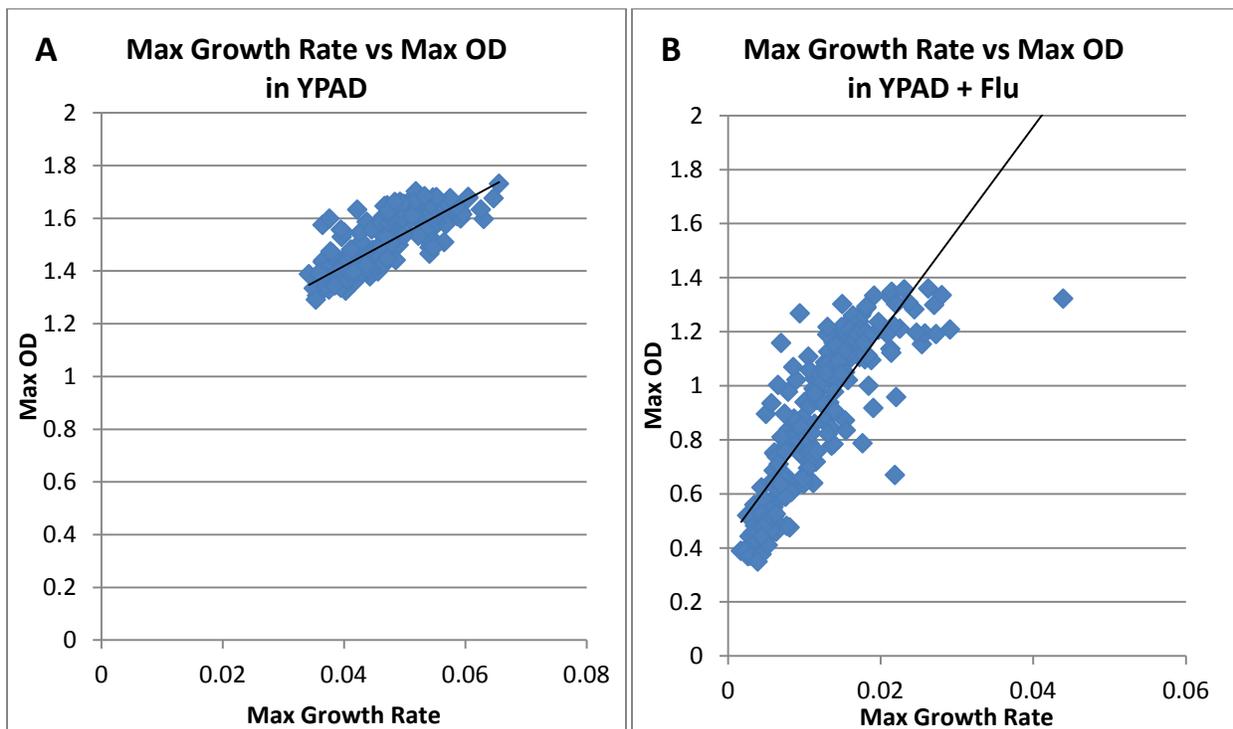


Figure 3

These graphs show the different growth metrics plotted against each other of 160 diploid and tetraploid isolates in two different environments.

Growth comparisons between parents and evolved isolates

To test for increased FLC tolerance in the evolved isolates, each evolved isolate from passage 4 (~200 hours FLC exposure) and their parents from passage 1 (no FLC exposure) were re-grown in YPAD (no drug) and YPAD with 10 μ g/ml FLC. In YPAD the diploid isolates did not show a significant difference in max growth rate from their parents (Figure 4A boxes 1 and 2), while in YPAD+FLC, they had a significantly higher max growth rate than their parents (see Figure 4A boxes 3 and 4). This suggests that the FLC-induced genotypic changes that occurred in the diploid isolates were advantageous for FLC resistance, but did not affect isolate fitness in the absence of drug stress.

In YPAD+FLC the tetraploid isolates had a significantly higher max growth rate than the diploid parents (see Figure 4B boxes 3 and 4). In both diploids and in tetraploids, the evolved isolates in YPAD+FLC had a greater max growth rate than their parents in YPAD+FLC, indicating that increased drug resistance had occurred during the 200 hours of exposure to FLC. In the absence of drug, tetraploid isolates had a significantly lower max growth rate than their parents (see Figure 4B boxes 1 and 2), suggesting the FLC-induced genotypic changes in the tetraploid isolates reduced their fitness in the absence of drug stress. In both environments diploid parents had a greater max growth rate than the tetraploid parents and the diploid isolates had a greater max growth rate than the tetraploid isolates.

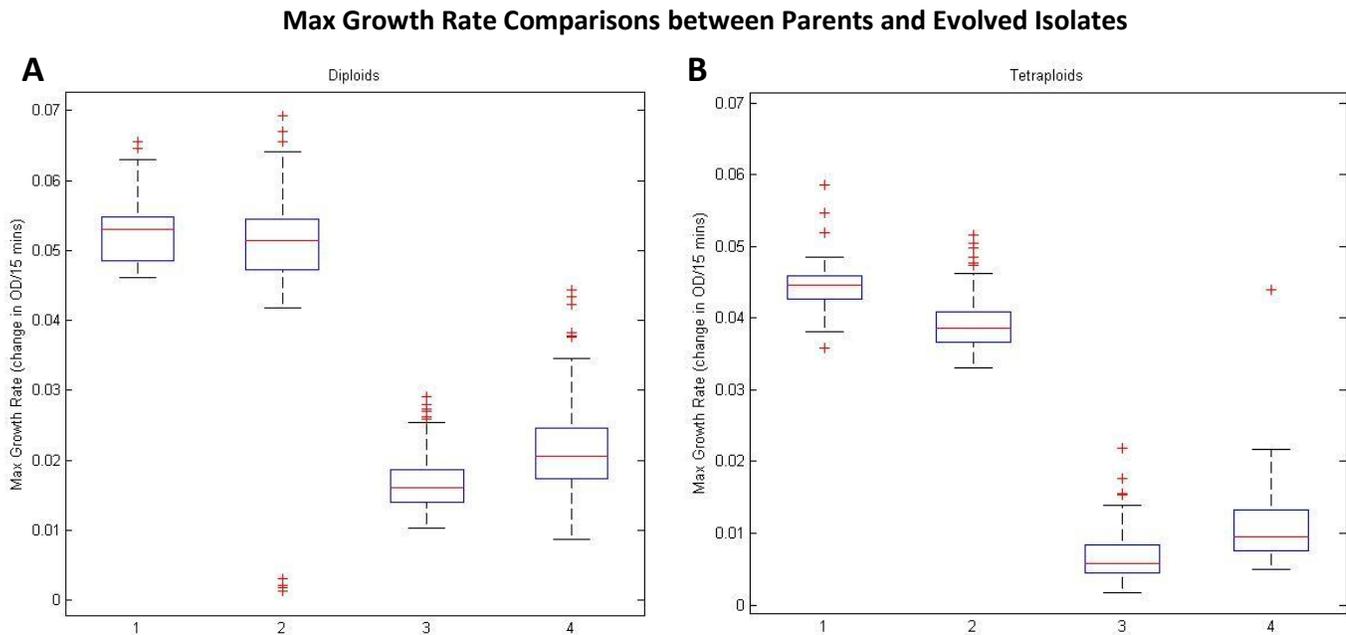


Figure 4

Box 1: Box and whisker plot where the red line is the median, the box is the 25-75% range, the whiskers are the range in which a data point is not considered an outlier, and the red + marks are outliers. Parents in no drug, Box 2: Isolates in no drug, Box 3: Parents in FLC, Box 4: Isolates in FLC

Comparisons between genotypic and phenotypic change

To examine the relationship between the genotype and phenotypic change that occurred during the 200 hours of FLC exposure, the growth rates of isolates and their parents were determined by growth in FLC. The change in max growth rate was then calculated by subtracting the parental max growth rate in FLC from that of its evolved isolate. These values were plotted against the isolate's final ploidy state in passage four. 78% of the diploid and 84% of the tetraploid isolates increased in max growth rate (showing positive x values). This indicates that during the 200 hours of FLC exposure, adaptations were made that resulted in the cells having increased resistance to the drug. In contrast, 22% of the diploid and 16% of the tetraploid isolates decreased in max growth rate (showing negative x values), indicating the isolates grew worse than their parents in FLC. There did not appear to be a specific ploidy state associated with a significant increase in max growth rate in FLC. These results suggest that DNA content level and growth in FLC do not correlate in a linear manner.

Phenotypic Change and Genotype Have Little Correlation

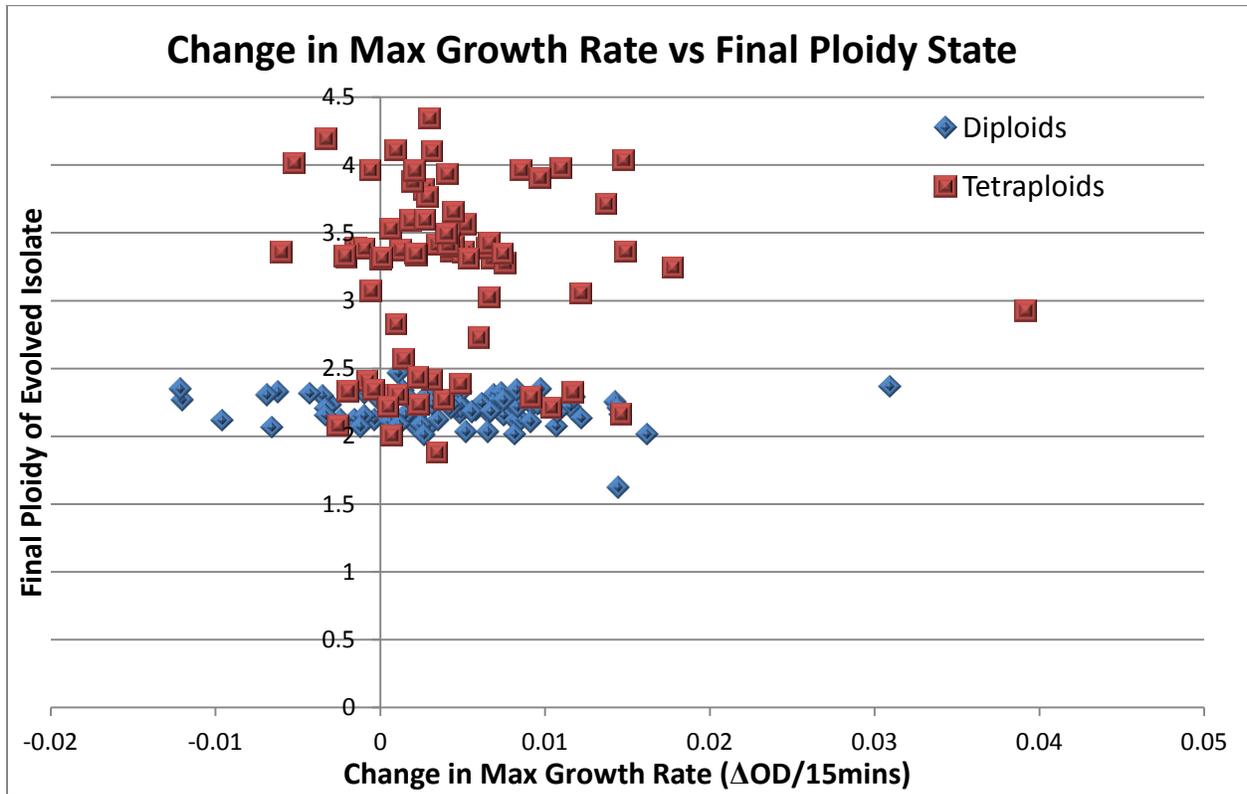


Figure 5

The change in max growth rate was found by subtracting the parent max growth rate in FLC from the evolved isolate max growth rate in FLC. The red points are isolates that were originally tetraploid, and the blue points are isolates that were originally diploid. (Isolates with multiple ploidy states were left out because the phenotypic testing for subpopulations within isolates was beyond the scope of this paper.)

Discussion

In conclusion, these results suggest that tetraploids are less fit than diploids in the presence of FLC, but tetraploids evolve faster with more genomic change. In addition, ploidy change and drug resistance were found to not have a linear correlation. Non-linear correlation has been seen in a Selmecki study, formation of isochromosome 5L was directly related to azole resistance (Selmecki 2006). Furthermore, strains close to diploid but carrying extra chromosomes (3N) do not have an obvious fitness cost and do provide an advantage in drug. This ploidy state was seen frequently in isolates that were originally tetraploid.

References

Pavelka, J. (2010, Nov 11). Aneuploidy confers quantitative proteome changes and phenotypic variation in budding yeast. *Nature*, 468,331-336.

Selmecki, A. (2006). Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science*, 313, 367-369.

Uppuri, P. (2013). Synergistic Effect of Calcineurin Inhibitors and Fluconazole against *Candida albicans* Biofilms. *American Society for Microbiology*, 1-2.