Unisexual Reproduction Reverses Muller's Ratchet

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ABSTRACT *Cryptococcus neoformans* is a pathogenic basidiomycetous fungus that engages in outcrossing, inbreeding, and selfing forms of unisexual reproduction as well as canonical sexual reproduction between opposite mating types. Long thought to be clonal, >99% of sampled environmental and clinical isolates of *C. neoformans* are MAT α , limiting the frequency of opposite mating-type sexual reproduction. Sexual reproduction allows eukaryotic organisms to exchange genetic information and shuffle their genomes to avoid the irreversible accumulation of deleterious changes that occur in asexual populations, known as Muller's ratchet. We tested whether unisexual reproduction, which dispenses with the requirement for an opposite mating-type partner, is able to purge the genome of deleterious mutations. We report that the unisexual cycle can restore mutant strains of *C. neoformans* to wild-type genotype and phenotype, including prototrophy and growth rate. Furthermore, the unisexual cycle allows attenuated strains to purge deleterious mutations and produce progeny that are returned to wild-type virulence. Our results show that unisexual populations of *C. neoformans* are able to avoid Muller's ratchet and loss of fitness through a unisexual reproduction cycle involving α - α cell fusion, nuclear fusion, and meiosis. Similar types of unisexual reproduction may operate in other pathogenic and saprobic eukaryotic taxa.

THE observation that most eukaryotic species retain the ability to reproduce sexually is puzzling when considering the costs of sex. The first is the “two-fold cost of sex”: females must produce males, which do not themselves produce offspring (Weismann 1887; Maynard Smith 1978). Sexual reproduction thus entails a twofold disadvantage in the number of offspring per generation when compared to asexual reproduction. Asexual mothers produce twice as many reproducing daughters in each generation because sexual mothers also produce males. The second type of twofold cost associated with sex is with the random reassortment of a diploid genome during meiosis to produce haploid gametes and offspring that inherit only 50% of each parental genome (Williams 1975; Maynard Smith 1978).

The ubiquity of sexual reproduction among eukaryotes suggests that the advantages of such a reproductive strategy must significantly outweigh the disadvantages over both the short and the long term. In the short term, sexual reproduction generates novel allele combinations and increased genetic diversity, of particular importance during “Red Queen” genetic

arms races or changing environments (Spassky *et al.* 1958; Dobzhansky *et al.* 1959; Spiess 1959; Van Valen 1973, 1974). In the long term, sexual organisms are able to avoid the accumulation of deleterious mutations known as Muller's ratchet (Muller 1932, 1964; Felsenstein 1974). Every lineage in an asexually growing population will eventually suffer a harmful mutation, and selection will not be sufficient to maintain the fittest genome (Kondrashov 1982). Theoretical modeling of asexually dividing or exclusively selfing populations predicts a continual loss of fitness and eventual extinction (Haigh 1978; Heller and Smith 1978; Pamilo *et al.* 1987; Stephan *et al.* 1993; Loewe and Cutter 2008; Loewe and Lamatsch 2008). Because mutations occur in every lineage, in these theoretical models selection is not sufficient to remove harmful mutations from the population, and the deleterious mutations accumulate in the genome, reducing fitness. Sexual reproduction allows recombination to shuffle the genomes of individuals, providing an opportunity to purge deleterious mutations from offspring, thereby restoring fitness and avoiding Muller's ratchet.

The basidiomycetous fungus *Cryptococcus neoformans* and its sibling species *Cryptococcus gattii* are the most common causes of fungal meningoencephalitis, a fatal disease if untreated. *C. neoformans* is a worldwide pathogen that most commonly infects immunocompromised individuals (Hull and Heitman 2002). Unlike most basidiomycetes, which have tetrapolar mating systems and up to thousands of mating

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doi: 10.1534/genetics.114.170472

Manuscript received July 30, 2014; accepted for publication September 2, 2014; published Early Online September 11, 2014.

Supporting information is available online at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170472/-DC1>.

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types, *C. neoformans* employs a bipolar mating system, with just two mating types, α and **a**. *C. neoformans* grows as a budding yeast in culture and during infection of the host. During the sexual cycle, however, it undergoes a filamentous, dimorphic transition. Under nutrient-limiting conditions, α and **a** cells secrete pheromones that are sensed by pheromone receptors and trigger cell–cell fusion. However, unlike *Saccharomyces cerevisiae*, the nuclei remain separate, producing a dikaryon that grows filamentously as a hypha. The tips of the filaments differentiate into basidia where nuclear fusion and meiosis occur. Haploid basidiospores are then produced by multiple rounds of mitosis, forming long chains (Kwon-Chung 1976). The spores produced by the sexual cycle are thought to be the infectious agent, easily dispersed as aerosols and inhaled into the host's lungs (Hull and Heitman 2002; Botts *et al.* 2009; Giles *et al.* 2009; Velagapudi *et al.* 2009; Botts and Hull 2010).

While the laboratory-defined sexual cycle has long been known, the environmental and clinical population consists predominantly of *MAT* α cells (Kwon-Chung and Bennett 1978). Extensive sampling of *C. neoformans* populations revealed >99% of cells are *MAT* α (Lengeler *et al.* 2000; Litvintseva *et al.* 2003, 2005; Heitman 2010). The preponderance of *MAT* α cells raises the question of how frequently *C. neoformans* undergoes sexual reproduction in natural populations. Homothallic fungi can overcome self-incompatibility through four general mechanisms: (1) mating-type switching, wherein only one allele is expressed from the active *MAT* locus while additional silent *MAT* cassettes are present and enable mating-type switching through gene conversion of the active *MAT* locus; (2) the fusion and expression of both *MAT* loci, as in *Sordaria macrospora*, a filamentous ascomycete, containing tandem copies of the *MAT* α and *MAT***a** loci; (3) the presence of both mating types in a single genome, as in *Aspergillus nidulans*, at unlinked loci, allowing expression of both mating-type loci in its genome and self-fertility; and (4) unisexual reproduction, which is unique among homothallic mechanisms in that it can dispense with the necessity of an opposite mating type (Lindegren and Lindegren 1943, 1944; Hawthorne 1963a,b; Oshima and Takano 1971; Hicks *et al.* 1977, 1979; Pöggeler *et al.* 1997; Lin *et al.* 2005; Debuchy and Turgeon 2006; Paoletti *et al.* 2007; Gioti *et al.* 2012). *C. neoformans* undergoes just such a novel homothallic unisexual sexual cycle, overcoming the relative paucity of *MAT***a** cells (Lin *et al.* 2005). During α – α unisexual reproduction, diploidization occurs either through cell fusion between cells of the same mating type or endoreplication, followed by the formation of hyphae, meiosis in the basidia, and production of spores.

The population of *C. neoformans* is globally distributed and thought to be large, limiting the impact of genetic drift and slowing Muller's ratchet. The population of *C. neoformans* is highly structured, however, and we do not yet understand the population history. There are two capsular agglutination serotypes in the species complex currently identified as *C. neoformans* (Belay *et al.* 1996). Interserotype, interspecies hybrids are found in both clinical and environmental samples, but spores produced

by these hybrids rarely germinate (Lengeler *et al.* 2001). In addition, there are multiple molecular types in each serotype, comprising both cosmopolitan and geographically isolated types. There appears to be little if any genetic exchange between molecular types, and there is significant population structure even within some of the defined molecular types (Campbell *et al.* 2005; Bui *et al.* 2008; Litvintseva and Mitchell 2012). *C. neoformans* may not be one large, global, interbreeding population but numerous, local, genetically isolated populations with a variety of population sizes. If *C. neoformans* represents many smaller isolated populations, the effects of genetic drift and Muller's ratchet will be much larger in some and possibly many of these populations. Unisexual reproduction may be of particular importance to populations with few or no alleles of *MAT***a** and that are genetically isolated from other populations.

We show that unisexual reproduction between strains that carry deleterious mutations generates progeny that have returned to the wild-type genotype. Unisexual reproduction allows *C. neoformans* to escape from Muller's ratchet and produce phenotypically and genotypically fit offspring. In addition to restoring growth rate, unisexual is also able to produce virulent *F*₁ progeny from avirulent parents.

Materials and Methods

Strain construction

The parental strain for all assays in this study is the well-characterized self-fertile strain XL280 α whose genome sequence is known (Lin *et al.* 2005; Ni *et al.* 2013). Genomic DNA was extracted using the CTAB method described previously (Pitkin *et al.* 1996). Split marker transformation methods were adapted from Fu *et al.* (2006). Primers were designed to the flanking regions of ORFs and complementary M13 primer sequences were added (Supporting Information, Table S2). Selectable markers were amplified from plasmids pJAF1 for neomycin (NEO) and pAIF3 for nourseothricin (NAT), and homologous sequences were amplified from XL280 α genomic DNA, and products were separated on a 1% agarose gel and extracted using a Qiagen PCR purification kit (Qiagen; catalog no. 28106). Homologous and selectable marker PCR products were combined using an overlap PCR. Two microliters of PCR product was analyzed on a 1% agarose gel, and the remainder of the 5' and 3' homologous split PCR products were combined and purified using a Qiagen PCR purification kit.

Mutant strains (Table S1) were isolated by transforming XL280 α using a biolistic transformation method (Toffaletti *et al.* 1993) (Table S2). Twenty-five-milliliter XL280 α cultures were grown overnight in YPD medium, pelleted, and resuspended in 5 ml of YPD. Approximately 300 μ l of the cell suspension was spread on each YPD plate with 1 M sorbitol. Plates were allowed to dry. DNA samples were prepared by mixing 250 μ g of gold microcarrier beads (0.6 μ m Bio-Rad no. 165–2262) with 1–4 μ g of PCR product, 10 μ l of 2.5 M CaCl₂, and 2 μ l of 1 M spermidine. This was incubated for

5 min at room temperature, centrifuged, washed with 0.5 ml of 100% ethanol, and then resuspended in 12 μ l of 100% ethanol. The solution was placed on macrocarrier disks (Bio-Rad no. 165–2335) to be used for biolistic transformation. The plates were then bombarded with the DNA-bound gold microcarrier beads in a Bio-Rad biolistic transformation apparatus at a helium gas pressure of $\sim$ 1350 psi under a vacuum of 29 in. of Hg. The plates were then incubated at 30° (except for the *cna1* and *cnb1* transformants, which were incubated at 24°) for 24 hr before transferring to selective media to isolate transformants.

Unisexual mating

For each mating assay, two independent matings were performed. Cells of each mutant strain were grown overnight in 5 ml of YPD at 24°. Cells were pelleted, washed, and resuspended in sterile H₂O. Equal volumes of each strain were mixed, and the mixture was incubated on V8 medium in the dark at 24° (Lin *et al.* 2005). After 2 days, cells were sampled and plated on selective media to obtain the diploid mating products KCR241 and KCR242. After isolation of single colonies on selective media, the diploids were validated by PCR and FACS analysis. The diploid isolates were sporulated by incubation on V8 medium in the dark at 24°. Approximately 50 spores from the hyphae growing at the periphery were isolated by micromanipulation with a Zeiss joystick equipped microdissection microscope and germinated on YPD. After germination, 50 colonies were scored for NAT^r, NEO^r, auxotrophy (*lys*[−] or *ura*[−]), and temperature sensitivity. The phenotypes of the progeny were validated by PCR (Figure S2 and Figure S3; see Table S2 for primers).

Phenotypic characterization

Strains were grown to obtain single colonies. Single colonies were cultured in YPD liquid medium overnight at 24° and washed with sterile H₂O and diluted to an OD₆₀₀ of 0.4. Each strain was fivefold serially diluted, spotted onto media, and incubated for 48 hr.

Growth kinetics during competitive co-culture

Cells were grown overnight at 24°, washed twice with dH₂O, and counted with a hemocytometer. Cells were diluted to a density of 20 cells/ μ l and recounted with a hemocytometer. One hundred microliters of each test strain was mixed with an equal quantity of the control strain. One hundred microliters ($\sim$ 1000 cells of each strain) of the mixture was added to fresh YPD medium and incubated at 30° with shaking while the rest of the mixture was diluted 10-fold and plated on YPD and selective media to validate that the starting cultures contained a 1:1 mixture of the marked drug-resistant strain and the unmarked drug-sensitive strain. The cultures were diluted, and after incubation for 24 hr, single colonies were isolated and tested for dominant selectable markers. The experiments were performed in triplicate, and data were plotted with Excel 2011.

Virulence assays

Strains were grown overnight in 5 ml of YPD at 24°. The cells were pelleted by centrifugation, washed with Dulbecco's

Figure 1 Independent assortment of physically linked and unlinked loci during unisexual reproduction. Two independent matings of each cross were dissected, and 50 spores from each were characterized by scoring the segregation of the integrated dominant selectable markers. Independent matings produced consistent ratios of progeny genotypes and are presented together. The independently assorting markers *cna1Δ::NEO* and *ura5Δ::NAT* do not vary from the expected 1:1 independent assortment ratio (χ^2 goodness-of-fit test, $P > 0.93$). The physically linked markers *lys1Δ::NEO* and *cnb1Δ::NAT* on chromosome 4 are genetically unlinked and recombined to assort at the expected 1:1 ratio (χ^2 goodness-of-fit test, $P > 0.90$).

phosphate buffered saline (DPBS) (Mediatech catalog no. 21-031-CV), repelleted, and resuspended in DPBS. Cells were counted with a hemocytometer and resuspended in sterile PBS at 2.5×10^7 cell/ml. *Galleria mellonella* in the final instar larval stage (Vanderhorst, Inc., St. Marys, Ohio) were stored in the dark and used within 7 days from the day of receipt. Ten randomly chosen healthy larvae were used per cohort. A Hamilton syringe was used to inject 4- μ l aliquots of the inoculum (1×10^5 cells) into the hemocoel of each larvae via the last left proleg. After injection, the larvae were incubated in plastic containers with wood shavings. Larvae showing signs of severe morbidity, such as changes in body color and no response to touch, were sacrificed by cold treatment at -20° . The number of surviving larvae was scored daily. The experiment was repeated with similar result as those presented in Figure 3.

Seven- to 8-week-old female A/JCr mice (Jackson Laboratory, 16–20 g weight) were used in this study. For infection, strains were cultured in YPD liquid medium overnight at 24° and washed twice with sterile DPBS. Cells were counted with a hemocytometer and resuspended in sterile PBS at 2×10^6 cells/ml. Dilutions of the cells were plated onto YPD and incubated at 24° for 48 hr to determine CFU. Groups of five mice per strain were anesthetized with pentobarbital (Lundbeck Inc., Deerfield, IL) and inoculated with an inocula of 1×10^5 cells of *C. neoformans* in 50 μ l via intranasal instillation. Survival was monitored daily, and moribund mice were sacrificed with CO₂.

To determine fungal tissue burden, the lungs, spleens, and brains of mice sacrificed ($n = 3$ for each strain) on day 112 post-infection were dissected within 1 hr of sacrifice. Organs were transferred to a 15-ml Falcon tube filled with 2 ml PBS and homogenized for 10 sec at $13,600 \times$ g/min (Power Gen 500, Fisher Scientific). Tissue homogenates were serially

Figure 2 Unisexual reproduction of growth-impaired parents produces wild-type progeny. (A) F₁ progeny from the mating of the *cna1Δ::NEO* and *ura5Δ::NAT* parental strains were characterized using the dominant resistance markers and tested under stress conditions restrictive for the parental strains (37° for *cna1*, SD-ura for *ura5*). Only those progeny that inherited the wild-type *CNA1* and *URA5* alleles and were returned to the wild-type genotype were able to grow under restrictive conditions. (B) Progeny that purged parental mutations through recombination were able to grow under stressful conditions restrictive to the parental strains. (C) Parental strains were grown in competition with a wild-type strain, XL280, in liquid YPD medium at 30°. For F₁ progeny without a selectable marker, an XL280 derivative with a neutral integrated NEO drug-resistance cassette was used for the competition. Each competition was performed in triplicate, and error bars indicate the standard deviation between biological replicates. The mutant parental strains had lower fitness when competing with wild-type cells. The wild-type F₁ progeny produced by unisexual reproduction did not have this defect and were equally competitive in co-culture with the wild-type strain XL280 from which the mutant parents were derived.

diluted, and 100 μ l was plated onto YPD plates. The plates were incubated at 24° for 48–72 hr to determine CFU per gram of organ or tissue. The recovered strains were validated using dominant selective markers.

Ethics statement

Murine studies conducted in the Division of Laboratory Animal Resources facilities at Duke University Medical Center (DUMC) were conducted in full compliance with the guidelines of the DUMC Institutional Animal Care and Use Committee and in compliance with the U.S. Animal Welfare Act. The vertebrate experiments were reviewed and approved by the DUMC Institutional Animal Care and Use Committee under protocol no. A113-09-04.

Results

Unisexual reproduction can purge mutations from congenic populations

To model the type of deleterious mutations that accumulate in asexual populations, we deleted genes necessary for wild-type

fitness in the well-characterized self-fertile strain XL280, which undergoes robust unisexual reproduction (Ni *et al.* 2013). We performed crosses between MATα strains derived from XL280 with the calcineurin A (*CNA1*) or uracil biosynthesis (*URA5*) genes deleted with dominant selectable markers (*cna1Δ::NEO* and *ura5Δ::NAT*) to test whether unisexual reproduction between mutants is capable of independently assorting chromosomes to produce wild-type offspring. These mutations are relatively benign under standard laboratory culture conditions but allowed for rapid genotyping and phenotyping of progeny. The *CNA1* gene is located on chromosome 10 while the *URA5* gene is located on chromosome 7. Thus, the *CNA1* and *URA5* loci are expected to assort independently during sexual reproduction.

The two mutant strains were mixed and incubated on mating (V8) medium for 2 days. Diploid mating products were selected using the dominant markers, validated by PCR and FACS analysis (Figure S1), and incubated on V8 media for 7 days to yield spores that were dissected and germinated on YPD medium. Fifty spore germinated isolates each from two independent replicates (100 total) were transferred to

YPD + NAT and YPD + G418 containing media to assess the genotype of the progeny (Figure 1). The phenotypes conferred by the *cna1* (temperature-sensitive growth) and *ura5* (uracil auxotrophy) mutations were also scored and were congruent with segregation of the dominant selectable drug-resistance marker integrated to replace the gene. The genotypes of the progeny did not deviate from the expected 1:1:1:1 ratio (χ^2 test for independence = 0.05, $P > 0.82$). Thus, unisexual mating results in cell and nuclear fusion and meiosis, leading to independent assortment of chromosomes in the haploid progeny. This independent assortment during unisexual reproduction produces wild-type offspring from mutant parents, providing an opportunity to purge deleterious mutations from lineages of *C. neoformans*.

We next assayed whether unisexual reproduction also allowed deleterious mutations harbored on the same chromosome to be purged by meiotic recombination. We crossed *MAT α* strains in which the *CNB1* or *LYS1* gene had been deleted with a dominant selectable marker. Both *CNB1* and *LYS1* are located on chromosome 4, *CNB1* from bp 80,833 to 81,737 and *LYS1* from bp 1,740,795 to 1,742,510 (contig NC_006686) in the JEC21 genome (XL280 and JEC21 are congenic and share 100% nucleotide identity throughout 81% of the genome) (Loftus *et al.* 2005; Stajich *et al.* 2012; Ni *et al.* 2013). After unisexual α - α mating and selection, diploids were recovered and validated by PCR and FACS and then incubated on V8 medium to promote sporulation. Spores were dissected and germinated on the surface of YPD solid medium. The genotypes of the progeny were determined by scoring drug resistance conferred by the selectable markers and phenotypes (temperature sensitivity and lysine auxotrophy) conferred by the *cnb1* and *lys1* mutations (Figure 1).

Although the *CNB1* and *LYS1* genes are physically linked on chromosome 4, the distance between the two loci is great enough that the genes are expected to be genetically unlinked. The genotypes among the unisexual progeny did not deviate from the expected 1:1:1:1 ratio (χ^2 test for independence = 0.13, $P > 0.71$), indicating that the two loci segregate independently and reflect a high frequency of recombination. Chromosomes recombine during α - α unisexual reproduction, just as during α - α bisexual reproduction, and this recombination is able to purge the genome of physically linked, deleterious mutations to produce wild-type progeny.

Unisexual reproduction can produce progeny of increased fitness

We next tested whether the purging of the genome by unisexual reproduction resulted in increased fitness and the amelioration of the growth defects associated with the parental mutations. *lys1 Δ* and *ura5 Δ* are auxotrophic mutants while *cna1 Δ* and *cnb1 Δ* are calcineurin mutants whose growth is sensitive to elevated temperature or stress (Edman and Kwon-Chung 1990; Nakamura *et al.* 1993; Mendoza *et al.* 1994; Hemenway *et al.* 1995; Odom *et al.* 1997). Parental strains from the independently assorting cross *ura5 Δ* $\times$ *cna1 Δ* and the four classes of progeny were grown under permissive and selective

Figure 3 Unisexual reproduction restores virulence in *G. mellonella*. (A) 1×10^5 cells of *C. neoformans* were injected into the hemocoel of *G. mellonella* larvae in cohorts of 10. Both the *cna1 Δ ::NEO* and *ura5 Δ ::NAT* parental strains exhibited reduced virulence while the *CNA1 URA5* wild-type progeny from their cross were returned to wild-type virulence (see Table 1 for *P*-values). (B) The *lys1 Δ ::NEO* and *cnb1 Δ ::NAT* parental strains exhibited significantly reduced virulence in *G. mellonella* larvae compared to the control strain XL280 and the wild-type progeny (*LYS1 CNB1*) of a unisexual cross between the mutant parental strains (see Table 1 for *P*-values).

conditions (Figure 2A). The wild-type progeny that lack both dominant markers demonstrated restored growth under permissive conditions and were viable under conditions restrictive to the parental strains. These *URA5 CNA1* strains generated by unisexual reproduction display the same phenotype as the wild-type control strain, XL280. Unisexual progeny with either parental genotype, *ura5 Δ ::NAT CNA1* or *URA5 cna1 Δ ::NEO*, are unable to grow under restrictive conditions, showing that the genetic changes facilitated by chromosome reassortment are essential to the improvement in fitness. The double-mutant progeny *ura5 Δ ::NAT cna1 Δ ::NEO* showed reduced growth even under permissive conditions (YPD at 30°) and were unable to grow under either of the restrictive conditions (SD-*ura* or YPD at 37°), and thus their fitness is reduced compared to either parental isolate.

We tested the progeny from the unisexual cross of physically linked deleterious mutations under similar permissive and restrictive conditions (Figure 2B). The *LYS1 CNB1* wild-type recombinant strains generated through unisexual reproduction display the same phenotype as the control strain XL280 and were able to grow under either restrictive condition. Unisexual

Table 1 Unisexual reproduction restores virulence in a *Galleria* model

Strain	XL280			Parent 1			Parent 2		
	Log rank			Log rank			Log rank		
	χ^2	P	One-way ANOVA	Strain	χ^2	P	Strain	χ^2	P
<i>cna1Δ::NEO URA5</i> (KCR237)	17.31	$P < 0.0001$	$P < 0.05$						
<i>CNA1 ura5Δ::NAT</i> (MN138.11)	19.17	$P < 0.0001$	$P < 0.01$						
<i>CNA1 URA5</i> (KCR244.1)	1.21	$P > 0.26$	$P > 0.05$	<i>cna1Δ::NEO URA5</i> (KCR237)	16.90	$P < 0.0001$	<i>CNA1 ura5Δ::NAT</i> (MN138.11)	19.29	$P < 0.0001$
									$P < 0.05$
<i>CNA1 URA5</i> (KCR244.1)	0.93	$P > 0.33$	$P > 0.05$	<i>cna1Δ::NEO URA5</i> (KCR237)	18.08	$P < 0.0001$	<i>CNA1 ura5Δ::NAT</i> (MN138.11)	18.83	$P < 0.0001$
									$P < 0.05$
<i>cnb1Δ::NAT LYS1</i> (KCR236)	19.42	$P < 0.0001$	$P < 0.001$						
<i>CNB1 lys1Δ::NEO</i> (KCR238)	18.46	$P < 0.0001$	$P < 0.001$	<i>cnb1Δ::NAT LYS1</i> (KCR236)	16.61	$P < 0.0001$	<i>CNB1 lys1Δ::NEO</i> (KCR238)	14.38	$P < 0.001$
<i>CNB1 LYS1</i> (KCR248.1)	3.81	$P > 0.05$	$P > 0.05$						$P < 0.001$
				<i>cnb1Δ::NAT LYS1</i> (KCR236)	17.13	$P < 0.0001$	<i>CNB1 lys1Δ::NEO</i> (KCR238)	15.12	$P < 0.001$
<i>CNB1 LYS1</i> (KCR248.2)	2.60	$P > 0.10$	$P > 0.05$						$P < 0.001$

The virulence of mutant parental strains was compared to the strain from which they were derived, XL280, using the ANOVA and log-rank test statistics. The wild-type F_1 progeny's virulence was also compared to the XL280 control strain and to both mutant parental strains. Mutant parental strains were significantly less virulent than XL280 while the virulence of wild-type F_1 progeny was indistinguishable from the virulence of XL280. In addition, the F_1 progeny lacking the parental deleterious mutations displayed increased virulence compared to either mutant parental strain.

progeny with either mutant parental genotype were still unable to grow under the respective restrictive conditions, and the double-mutant progeny *lys1Δ::NEO cnb1Δ::NAT* showed reduced growth even under permissive conditions (YPD at 30°) and no growth under either restrictive condition (SD–lys, YPD at 37°). Together, these results show that *C. neoformans* is able to undergo unisexual reproduction to purge deleterious mutations via independent chromosome assortment and recombination to restore fitness equivalent to the wild-type parental genotype.

The rates of growth of the mutant parental strains are reduced, even under permissive conditions when compared to the wild-type strain. We quantified this defect by competitive growth experiments in liquid YPD medium at 30°. Cultures were seeded with two strains in a 1:1 mixture, and then, following a period of competitive growth, the ratio of the two strains was determined by scoring the number of drug-resistant and drug-sensitive cells in the mixed culture. The parental strains (*ura5Δ::NAT CNA1* and *URA5 cna1Δ::NEO*) grew more slowly than the XL280 strain from which they were derived (Figure 2C). Wild-type F_1 progeny of unisexual reproduction between *ura5Δ::NAT CNA1* and *URA5 cna1Δ::NEO* strains had restored competitive growth rates equivalent to XL280. Recombinant wild-type progeny of unisexual reproduction showed similar restoration of growth rates when compared to either mutant parental strain (Figure 2C). The selective advantage of producing strains of greater fitness, even under permissive conditions, would confer significant benefits to unisexually reproducing organisms over asexual competitors. Unisexual reproduction produced progeny of greater fitness under both ideal and stressful growth conditions.

Unisexual reproduction between attenuated strains restores the virulence of progeny

Recent evidence has suggested that unisexual reproduction and recombination play an important role in generating virulent strains of *Cryptococcus* and other eukaryotic pathogens, including both fungi and parasites (Jenni *et al.* 1986; Gaunt *et al.* 2003; Fraser *et al.* 2005; Akopyants *et al.* 2009; Byrnes *et al.* 2010, 2011; Wendte *et al.* 2010; Minot *et al.* 2012; Inbar *et al.* 2013; Voelz *et al.* 2013; Billmyre *et al.* 2014). We tested whether parental strains with mutations that compromise virulence are able to undergo unisexual reproduction to produce virulent progeny. Studies of *C. neoformans* have shown that calcineurin is required for virulence in mammalian models (Odom *et al.* 1997; Cruz *et al.* 2000; Fox *et al.* 2001). We injected inocula containing *C. neoformans* into the *G. mellonella* larvae to assess the virulence of parental and progeny strains. We found that both parental strains with a calcineurin or an auxotrophic mutation exhibited attenuated virulence in *G. mellonella* when compared to the wild-type parental strain XL280 (Figure 3, A and B). The progeny of unisexual reproduction (*MATα ura5Δ::NAT CNA1* × *MATα URA5 cna1Δ::NEO* and *MATα lys1Δ::NEO CNB1* × *MATα LYS1 cnb1Δ::NAT*) were indistinguishable in virulence from the XL280 starting strain and

were significantly more virulent than either mutant parental strain (Figure 3, A and B; Table 1).

The restoration of virulence in progeny produced by unisexual reproduction of avirulent parents was further assessed in a murine inhalation model. Cohorts of animals were infected through nasal instillation, and both virulence and organ colonization were assessed. While the parental calcineurin A or B and *ura5* or *lys1* auxotrophic mutants had attenuated virulence in *G. mellonella*, all four mutants were avirulent in the murine model (Figure 4, A and B). Unisexual wild-type progeny from the cross of either pair of parental mutant avirulent strains showed fully restored virulence (Table 2).

To further characterize the virulence of the progeny strains, we assessed the *in vivo* proliferation in the murine host by assaying the colonization of internal organs. We performed a fungal burden analysis of lung, spleen, and brain tissues after sacrifice or on day 112 post-infection for attenuated strains (Figure 4C). The *cna1Δ* and *cnb1Δ* mutant parental strains were absent from all host tissues, suggesting that they are unable to survive at mammalian body temperature as a consequence of their temperature- and stress-sensitive phenotypes and were cleared rapidly by the host. The *ura5Δ* and *lys1Δ* auxotrophic parental strains were not present in either the spleen or brain tissue but were present in the lungs, at the site of infection, in lower abundance compared to wild-type strain XL280. These auxotrophic mutants were thus able to survive in the host but did not cross into the central nervous system or disseminate from the lungs to other organs, both important for virulence after inhalation of spores or yeast cells. In contrast, all of the wild-type progeny showed abundant CFUs in the lungs, similar to wild-type XL280. The wild-type progeny also yielded robust numbers of CFUs from the brain and spleen, sites that the parental strains were unable to colonize. These findings show that avirulent parents, defective in invading the central nervous system and other organs, can undergo unisexual reproduction to produce fully virulent progeny, capable of invading other tissues after inhalation, the most common route of infection.

Discussion

We show in this study that unisexual reproduction is able to purge the genome of deleterious mutations to produce progeny with higher fitness and virulence. This may be counterintuitive, as unisexual reproduction can be conflated with exclusively selfing populations. We show that when closely related cells of the same mating-type but subtly different genotypes undergo unisexual reproduction, this generates increased genetic diversity and produces new phenotypes not exhibited by either parent.

Quantifying the threat of genome degradation and mutational burden from Muller's ratchet is difficult, requiring mathematical modeling or computer simulations, which are sensitive to changes in population or mutational parameters (Stephan and Kim 2002; Loewe 2006). However, population genomic studies have shown that most asexual lineages are

Figure 4 Unisexual reproduction between avirulent strains can produce offspring that are restored to virulence in a murine inhalation model. (A and B) Cells were grown in liquid YPD medium overnight at 25° and washed three times with PBS. Groups of five mice per strain were anesthetized and inoculated with 1×10^5 cells via intranasal instillation. Survival was monitored daily. All mutant parental strains (*lys1*, *ura5*, *cna1*, and *cnb1*) were avirulent when compared with the XL280 reference strain. The F₁ progeny purged of mutations by unisexual reproduction had significantly increased virulence compared to the mutant parental strains and were indistinguishable from the wild-type reference strain XL280 (see Table 2 for *P*-values). (C) After sacrifice for imminent mortality or at the end of experiment on day 112 post-infection, the lungs, spleen, and brain were removed and homogenized, and serial dilutions were plated for tissue burden cultures. The height of each column depicts the mean number of CFUs for three animals, and the standard deviation of three biological replicates are indicated by error bars. The wild-type progeny were present in the lungs and at the site of infection and were able to efficiently invade both the central nervous system and the spleen. The mutant parental strains were either entirely absent from the tissue (*cna1Δ::NEO* and *cnb1Δ::NAT*) or present in reduced numbers only in the lungs (*ura5Δ::NAT* and *lys1Δ::NEO*).

young and that linkage between mutations or polymorphisms increases the likelihood of fixation for deleterious alleles (Hill and Robertson 1966; Lynch and Gabriel 1990; Tucker *et al.* 2013). Disadvantages such as these raise serious questions about the persistence and long-term success of many eukaryotic pathogens thought to be clonal or asexual.

Table 2 Unisexual reproduction restores virulence in a murine model

Strain	XL280		Parent 1			Parent 2		
	χ^2	<i>P</i>	Strain	χ^2	<i>P</i>	Strain	χ^2	<i>P</i>
(KCR237)	9.7	<i>P</i> < 0.01						
<i>CNA1 ura5Δ::NAT</i> (MN138.11)	9.7	<i>P</i> < 0.01						
<i>CNA1 URA5</i> (KCR244.1)	0.25	<i>P</i> > 0.60	<i>cna1Δ::NEO URA5</i> (KCR237)	9.7	<i>P</i> < 0.01	<i>CNA1 ura5Δ::NAT</i> (MN138.11)	9.7	<i>P</i> < 0.01
<i>CNA1 URA5</i> (KCR244.1)	0.18	<i>P</i> > 0.67	<i>cna1Δ::NEO URA5</i> (KCR237)	9.7	<i>P</i> < 0.01	<i>CNA1 ura5Δ::NAT</i> (MN138.11)	8.1	<i>P</i> < 0.01
<i>cnb1Δ::NAT LYS1</i> (KCR236)	11.6	<i>P</i> < 0.001						
<i>CNB1 lys1Δ::NEO</i> (KCR238)	11.6	<i>P</i> < 0.001						
<i>CNB1 LYS1</i> (KCR248.1)	0.00	<i>P</i> > 0.97	<i>cnb1Δ::NAT LYS1</i> (KCR236)	9.7	<i>P</i> < 0.01	<i>CNB1 lys1Δ::NEO</i> (KCR238)	9.7	<i>P</i> < 0.01
<i>CNB1 LYS1</i> (KCR248.2)	0.58	<i>P</i> > 0.44	<i>cnb1Δ::NAT LYS1</i> (KCR236)	9.7	<i>P</i> < 0.01	<i>CNB1 lys1Δ::NEO</i> (KCR238)	9.7	<i>P</i> < 0.01

The virulence of mutant parental strains was compared to the virulence of a control strain from which they were derived, XL280, using the log-rank test statistic. The virulence of wild-type *F*₁ progeny was compared to the XL280 control strain and to both mutant parental strains. Mutant parental strains were significantly less virulent than XL280 while the virulence of wild-type *F*₁ progeny was indistinguishable from the virulence of XL280. In addition, the *F*₁ progeny lacking the parental deleterious mutations displayed increased virulence compared to either mutant parental strain.

C. neoformans is an example of just such a pathogen. Despite a long-recognized laboratory α - α sexual cycle, an overwhelming number of environmental and clinical isolates are of just one mating type, *MAT* α (Kwon-Chung and Bennett 1978; Halliday *et al.* 1999). In many pathogenic fungi, including *C. neoformans*, the population is largely clonal, yet these organisms maintain their ability to undergo both bisexual and unisexual reproduction (Litvintseva *et al.* 2003, 2005). In particular, clinical isolates of *C. neoformans* often are derived from large clonal populations of environmental isolates that show evidence of recombination, despite the paucity of *MAT* α isolates in some sampled populations. Previous studies have found evidence of both independent assortment and recombination during α - α mating (Lin *et al.* 2005). In this study, we extend these findings and show that, despite the low potential for outcrossing between cells of opposite mating type, *C. neoformans* can undergo unisexual mating and purge deleterious mutations from the genome. This study provides further evidence into the ways via which unisexual mating can provide evolutionary benefits similar to bisexual mating. Unisexual reproduction not only instigates recombination but also is able to remove deleterious mutations from the genome during α - α mating. This raises the intriguing possibility that at least some *C. neoformans* populations are able to maintain fitness via unisexual recombination despite a lack of *MAT* α cells. Unisexual reproduction can contribute to genetic diversity and potentially generate more infectious strains that are able to expand clonally to become a cosmopolitan clinical strain.

Muller's ratchet is expected to gradually decrease the fitness and virulence of pathogens. Unisexual *Cryptococcus* species are at least 40 million years old and have maintained, and in some cases increased in, virulence (James *et al.* 2006; Findley *et al.* 2009; D'Souza *et al.* 2011). The low frequency of *MAT* α in the population suggests that unisexual reproduction may be critical to sustaining virulence in the face of the inexorable accumulation of deleterious mutations over time. In our study, unisexual reproduction produced virulent progeny from avirulent parental strains. This laboratory validation supports the growing observational evidence that unisexual reproduction plays a role in producing virulent strains from avirulent or less virulent

parents in nature (Fraser *et al.* 2005; Byrnes *et al.* 2010, 2011). In outbreaks in the Pacific Northwest of the United States and Canada, population genomic data suggest that sexual reproduction occurred prior to clonal expansion of hypervirulent strains (Fraser *et al.* 2005; Billmyre *et al.* 2014). Together, this suggests that sexual reproduction may play an important role in producing and maintaining virulent pathogens.

Transitions between heterothallism (requiring outcrossing) and homothallism, including unisexual reproduction (which can involve outcrossing, inbreeding, or selfing), are common in the fungal kingdom, including among pathogenic fungi (Yun *et al.* 1999; Debuchy and Turgeon 2006; Gioti *et al.* 2012). Our study indicates that transitions to unisexual reproduction from bisexual reproduction may not inevitably lead to Muller's ratchet and an evolutionary dead end, as previously hypothesized (Bell 1982; Charlesworth *et al.* 1993; Lynch *et al.* 1995; Paland and Lynch 2006; Gioti *et al.* 2013). In addition, many pathogenic eukaryotes are thought to have clonal population structures, but recent work has uncovered evidence of cryptic sexual or unisexual cycles (Gräser *et al.* 1996; Peever *et al.* 1999; Berbee *et al.* 2003; Heitman 2006; Morgan *et al.* 2007; Heitman 2010; Li *et al.* 2010; Farrer *et al.* 2011; Stewart *et al.* 2013; Roach *et al.* 2014). Human pathogens of significant public health concern, such as those from the *Leishmania*, *Trypanosoma*, and *Toxoplasma* genera, show signatures of recombination and unisexual cycles (Jenni *et al.* 1986; Gaunt *et al.* 2003; Akopyants *et al.* 2009; Wendte *et al.* 2010; Minot *et al.* 2012). We have shown that unisexual reproduction can purge the genome of deleterious mutations and increase the virulence of a eukaryotic pathogen. Further research is needed to determine the full range of roles that unisexual reproduction plays in maintaining pathogenic eukaryotic genomes and their fitness and virulence.

Acknowledgments

We thank Marianna Feretzaki and Anna Averette for technical assistance, Daniel Kornitzer for insightful discussions at the 2013 Federation of European Biochemical Societies Human Fungal Pathogens Meeting that stimulated these investiga-

tions, and Thomas Petes and Harmit Malik for critical reading and thoughtful discussion of the manuscript. These studies were supported by National Institutes of Health/National Institute of Allergy and Infectious Diseases R37 MERIT Award AI39115-17 to Joseph Heitman. Kevin Roach is a supported fellow of the Tri-Institutional (Duke, University of North Carolina at Chapel Hill, and North Carolina State University) Molecular Mycology and Pathogenesis Training Program (MMPTP) supported by NIH/NIAID T32 award AI52080-11.

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Communicating editor: A. P. Mitchell

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Supporting Information

<http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170472/-/DC1>

Unisexual Reproduction Reverses Muller's Ratchet

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A **B**

Figure S1 PCR and FACS validation of diploid unisexual mating products. A) PCR amplification of DNA from diploids produced by unisexual mating, with primers specific to both wild-type and deletions of *CNA1*, *URA5*, *CNB1* and *LYS1*. **B)** FACS analysis of diploids products of unisexual reproduction. The XL280 parental strain is a 1N control and MN142.3 is a diploid control.

Figure S2 PCR validation of offspring of unisexual reproduction. A) PCR amplification of DNA of unisexual offspring with primers specific to both wild-type and deletions of *CNA1* and *URA5*. Individual PCR reactions were carried out with primer pairs and 3 ul of all four reactions were mixed and loaded in a single lane.

Figure S3 PCR validation of offspring of unisexual reproduction. A) PCR amplification of DNA of unisexual offspring with primers specific to both wild-type and deletions of *CNB1* and *LYS1*. Individual PCR reactions were carried out with primer pairs and 3 μ l of all four reactions were mixed and loaded in a single lane.

Tables S1-S2

Available for download as Excel files at <http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.114.170472/-/DC1>

Table S1 Yeast strains. Yeast strains used in this study.

Table S2 Primers. Primers used in this study.

Supplemental References

1. Lee SC, Heitman J (2012) Function of *Cryptococcus neoformans* *KAR7* (*SEC66*) in karyogamy during unisexual and opposite-sex mating. *Eukaryotic Cell* 11: 783-794.