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Selective Advantages of a Parasexual Cycle for the Yeast *Candida albicans*

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ABSTRACT The yeast *Candida albicans* can mate. However, in the natural environment mating may generate progeny (fusants) fitter than clonal lineages too rarely to render mating biologically significant: *C. albicans* has never been observed to mate in its natural environment, the human host, and the population structure of the species is largely clonal. It seems incapable of meiosis, and most isolates are diploid and carry both mating-type-like (*MTL*) locus alleles, preventing mating. Only chromosome loss or localized loss of heterozygosity can generate mating-competent cells, and recombination of parental alleles is limited. To determine if mating is a biologically significant process, we investigated if mating is under selection. The ratio of nonsynonymous to synonymous mutations in mating genes and the frequency of mutations abolishing mating indicated that mating is under selection. The *MTL* locus is located on chromosome 5, and when we induced chromosome 5 loss in 10 clinical isolates, most of the resulting *MTL*-homozygotes could mate with each other, producing fusants. In laboratory culture, a novel environment favoring novel genotypes, some fusants grew faster than their parents, in which loss of heterozygosity had reduced growth rates, and also faster than their *MTL*-heterozygous ancestors—albeit often only after serial propagation. In a small number of experiments in which co-inoculation of an oral colonization model with *MTL*-homozygotes yielded small numbers of fusants, their numbers declined over time relative to those of the parents. Overall, our results indicate that mating generates genotypes superior to existing *MTL*-heterozygotes often enough to be under selection.

KEYWORDS *Candida albicans*; mating; parasexual cycle; cryptic sexual cycle

SEX is costly and disrupts well-adapted allele combinations. It can also be advantageous by speeding up adaptation or by purging deleterious mutations. It has been difficult to establish how, precisely, these and other benefits outweigh the cost of sex. Indeed, abandoning sex in favor of clonal reproduction can be advantageous—asexual species arise frequently. Their life spans are short, however, indicating that sex may be essential for the long-term survival of species (Otto and Lenormand 2002; Rice 2002).

Sexual cycles have not been observed for ~20% of fungal species (Carlile *et al.* 2001). Whether these species are truly asexual or merely restrict the frequency of sex—a strategy believed to maximize its benefits (Heitman 2006)—is difficult to determine. Genetic marker distributions in such species often suggest limited recombination (Carlile *et al.* 2001). However, clonal reproduction will initially copy the genetic marker distributions that were generated by sex, and new mutations and genetic drift will only slowly erase evidence of past recombination (Schmid *et al.* 2004; Cox *et al.* 2013). With rare exceptions, fungi without observable sexual cycles are derived from recent sexual ancestors (Carlile *et al.* 2001; Schmid *et al.* 2004; Butler 2007). Thus genetic marker distributions may reflect limited ongoing recombination or be an "echo" of recombination events that occurred in sexual ancestors.

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The yeast *Candida albicans*, a commensal colonizer and occasional opportunistic pathogen of humans (Odds 1988), can mate, but it is a convoluted process. Most isolates are diploid and carry both mating-type-like (*MTL*) locus alleles, preventing mating. Apparently incapable of meiosis, loss of an entire copy of chromosome 5 (chr 5), on which the *MTL* locus is located, or localized loss of heterozygosity (LOH), generate *MTL*-homozygotes, which can mate. To do so they must, in addition, undergo an epigenetic switch from the "white" phenotype to the mating-competent "opaque" phenotype (Miller and Johnson 2002; Alby *et al.* 2009; Heitman 2010; Xie *et al.* 2013). A small fraction of mating encounters generates fusants with genetic markers from both parents (Lockhart *et al.* 2003; Bennett *et al.* 2005). These subsequently return to diploidy by chromosome loss (Bennett and Johnson 2003) and, in the process, intrachromosomal recombination can generate true recombinants (Forche *et al.* 2008). Alternatively, mating can be induced between haploids, which spontaneously arise from diploids by concerted chromosome loss, resulting immediately in diploid progeny (Hickman *et al.* 2013).

However, mating has never been observed in the human host, which is likely to be the natural environment of strains found on humans (Edelmann *et al.* 2005; Jacobsen *et al.* 2008; Wrobel *et al.* 2008) and the predominant environment of the species as a whole (Skinner and Fletcher 1960; Odds 1988). That mating is very rare is also indicated by the largely clonal population structure of the species (Gräser *et al.* 1996; Tibayrenc 1997; Tavanti *et al.* 2004; Bougnoux *et al.* 2008).

The existing data are consistent with two hypotheses: The first is that, while rare, mating in the natural environment produces progeny (fusant) lineages that surpass clonal lineages in fitness often enough to render the process biologically significant. If so, mutations that have turned mating into a convoluted process may have been selected because, for *C. albicans*, the benefits of mating are maximized by restricting its frequency (Heitman 2006). Alternatively, it is possible that mating so rarely generates fusants that are superior to clonal lineages that the process is no longer biologically significant. In that case, the mutations that make mating convoluted would indicate progressive mutational decay of a process that is no longer protected by selection—indeed mutations rendering strains entirely incapable of mating are not uncommon (Legrand *et al.* 2004). To distinguish between these two hypotheses we have investigated whether mating is under selection in *C. albicans* and, if so, why.

Materials and Methods

Analyses of d_n/d_s

The d_n/d_s analyses for the *C. albicans* mating genes *MTLα1*, *MTLα2*, *MTL***a***1*, and *MTL***a***2* and their orthologs in other species were carried out using PAML (Yang 2007) as described previously (Zhang *et al.* 2010). Sequences were aligned and the M2 model in the codeml program from PAML v. 4.5 was used to model their evolution, allowing different d_n/d_s ratios for groups or species with full and cryptic sexual cycles in different parts of a phylogenetic tree based on ITS sequences (Supporting Information, Figure S1; the tree is consistent with relationships between these species established by other authors; Tavanti *et al.* 2005b; Wang *et al.* 2009; Butler 2010). The group *dn/ds* ratios reported in Results are those extracted from these models. We also used PAML to calculate d_n/d_s ratios from pairwise ortholog sequence comparisons between *C. albicans* and each of the three fully sexual species.

Table 1 Strains used for mating experiments: Clinical isolates

Strain	Ca3 type[a]	Geographical origin	Body site
AU35	C	New Zealand	Sputum
AU7	GPG A2	New Zealand	Urine
Au90	GPG A2	New Zealand	Skin wound
RIHO11	C	United States	Bloodstream
W43	GPG A2	New Zealand	Oral
FJ11	C	Fiji	Throat
HUN97	GPG A2	Britain	Bloodstream
OD8916	GPG A2	Britain	Oral
W17	C	New Zealand	Throat
YSU63	C	Malaysia	Urine

Source: Schmid *et al.* (1999).
[a] Based on Ca3 fingerprinting; GPG, general-purpose genotype, equivalent to major group A, which is subdivided in to subgroups A1 and A2 (Schmid *et al.* 1999)

Strains, culture conditions, and growth rate comparisons

All strains used are listed in Tables 1-3. The 10 strains used for mating experiments are listed in Table 1. They were chosen (Holland and Schmid 2005) from a collection of 266 infection-causing isolates from 12 geographical regions in 6 countries (Schmid *et al.* 1999) so that 5 represented the GPG group (equivalent to clade 1; Schmid *et al.* 1999; Tavanti *et al.* 2005a; Odds *et al.* 2007) and 5 the remainder of the species. The strains had not been extensively subcultured after their isolation from patients and were revived from glycerol stocks for these experiments. Twenty-three *MTL*-homozygous clinical isolates used for growth-rate determinations were WO-1 (Slutsky *et al.* 1987; Miller and Johnson 2002); L26, GC75, p37005, 19F, p87, 12C, p60002, p78048, p57072, p94015 (Wu *et al.* 2007); 85/005, AM2005/0377, T101, M97105, 81/139, SCS103353G, SCS103354N, AM2003/018, AM2003/0165, AM2002/087, RIHO9, 81/196 (Schmid *et al.* 1999; Odds *et al.* 2007).

Serial transfers and growth-rate determinations were carried out under two conditions: Either (i) in YPD (1% yeast extract, 2% Bacto-peptone (Bacto™, Becton Dickinson, Sparks, MD) and 2% glucose (Asia Pacific Specialty Chemical Limited, NSW, Australia)) at 37° or (ii) in a defined medium containing 0.17% yeast nitrogen base without

Table 2 Strains used for mating experiments: *MTL*-homozygous derivatives

MTL-homozygote	Genotype	Drug-resistant derivative	Genotype
AU35**a**	*MTL***a**	AU35**a**-pNZ4	*MPA*^{R}, *MTL***a**
AU7**a**	*MTL***a**	AU7**a**-pNZ4	*MPA*^{R}, *MTL***a**
AU90**a**	*MTL***a**	AU90**a**-pNZ4	*MPA*^{R}, *MTL***a**
RIHO11**a**	*MTL***a**	RIHO11**a**-pNZ4	*MPA*^{R}, *MTL***a**
W43**a**	*MTL***a**	W43**a**-pNZ4	*MPA*^{R}, *MTL***a**
FJ11α	*MTL*α	FJ11α-pNZ11	*NAT*^{R}, *MTL*α
HUN97α	*MTL*α	HUN97α-pNZ11	*NAT*^{R}, *MTL*α
OD8916α	*MTL*α	OD8916α-pNZ11	*NAT*^{R}, *MTL*α
W17α	*MTL*α	W17α-pNZ11	*NAT*^{R}, *MTL*α
		W17α-pNZ11.2	*NAT*^{R}, *MTL*α
YSU63α	*MTL*α	YSU63α-pNZ11	*NAT*^{R}, *MTL*α
3685[a]	*MTL*α	3685[a]	*His*$^-$, *Arg*$^-$, *MPA*^{r}, *MTL*α
3710[a]	*MTL***a**	3710[a]	*His*$^-$, *Arg*$^-$, *MPA*^{r}, *MTL***a**

[a] Source: Magee *et al.* (2002); *MTL*-homozygous derivatives of MPA-resistant strains.

amino acids and ammonium sulfate (Becton and Dickinson), 0.5% ammonium sulfate, 0.08% glucose, 0.2 M NaCl (Goddard *et al.* 2005) at 42°.

During serial transfers, cultures were grown overnight, to saturation. Then 3.0×10^6 cells (from YPD medium) or 2×10^5 cells (from defined medium) were transferred for further propagation. The number of generations was calculated from the increase in cell number. Growth rates of exponentially growing cultures were determined by measuring optical density at 600 nm (OD_{600}) at $\geq$ 8 time points. The growth rate was determined as the slope of the line of best fit in plots of $\ln(OD_{600})$ *vs.* time (r^2 values >0.99). Growth rates that were to be compared with each other were, as far as possible, determined in the same experiments. See File S1 for details.

Selection of MTL-homozygous derivatives

MTL-homozygous derivatives were identified by PCR amplification of *MTL***a** and *MTL*α alleles, either after inducing chr 5 loss on sorbose medium (Rustchenko *et al.* 1994) or from red sectors in colonies grown on YPD + phloxine B medium (Lockhart *et al.* 2002). See File S1 for details.

Resistance cassettes for selection of fusants

Strains to be used in mating experiments were transformed with a mycophenolic acid resistance (MPA^r) cassette or a nourseothricin resistance cassette (NAT^r), by lithium acetate heat shock (Beckerman *et al.* 2001) or electroporation (De Backer *et al.* 1999), to allow selection of fusants on the basis of resistance to both drugs.

The MPA-resistance vector, pNZ4 (Figure 1A), contained the *IMH3*^{r} gene under the control of a tetracycline-responsive promoter, the ORF encoding the tetracycline activator *tetR–ScHAP4–WH11* under the control of the *ENO1* promoter and two flanking sequences targeting its integration to a noncoding region of chr 7. It was constructed as follows: A fragment containing *pENO* plus *tetR–ScHAP4–WH11* was amplified by PCR from the plasmid pCAITHE5 (Nakayama *et al.* 2000) with primers ENOpf and tetRWH11pr (the sequences of all primers used are given in Table S1). The fragment was cloned into *Sma*I-linearized plasmid pBSKS (+) to produce plasmid pNZ1. The tetracycline-responsive promoter (*pTR*) was PCR amplified from plasmid p99RLU (Nakayama *et al.* 2000) with primers TRpf and TRpr. The *IMH3*^{r} ORF was amplified from plasmid p3408 (Beckerman *et al.* 2001) using primers IMH3pfatg and IMH3pr. Both fragments were purified using a PCR cleanup kit (Roche) and used as templates for recombinant PCR to combine the TR promoter and the *IMH3*^{r} ORF using primers TRpf, TRpIMHp, and IMH3pr. The recombinant PCR product was cloned into *Eco*RV-linearized plasmid pBSKS(+) to produce plasmid pNZ2. The PCR reactions were carried out as described in File S1. Plasmid pNZ1 was cut with *Bam*HI and treated with Klenow enzyme (Roche) to generate blunt ends. The cassette containing *pTR* and *IMH3*^{r} was then removed from pNZ2 with *Sma*I and cloned into linearized pNZ1 resulting in plasmid pNZ3. To direct the integration of the resistance cassette to a noncoding region of the *C. albicans* genome, two targeting sequences, TS1 and TS2 (bp 367394–368202 and 366038–367194, respectively, on chr 7 of strain SC5314 as per genome assembly 21; http://www.candidagenome.org/) were PCR amplified with primers TS1pf/TS1pr and TS2pf/TS2pr and cloned into plasmid pGemT-Easy (Promega). TS1 and TS2 were removed from pGemT using *Not*I and *Hin*dIII, respectively, and cloned into pNZ3 to obtain pNZ4.

The NAT-resistance vector pNZ11 (designed so that integration would occur in chr 1; Figure 1B) was constructed as follows: The cassette containing a *CaNAT*-resistance gene flanked by an *ACT1* promoter and the *ACT1* terminator was PCR amplified from plasmid pJK850 (Shen *et al.* 2005) using primers M13pr and Sacpf1. The PCR product was purified and then digested with both *Kpn*I and *Sac*I and inserted into *Kpn*I/*Sac*I-digested vector pBlueScript KS(+) to obtain pNZ11.

All constructs were verified by DNA sequencing. The sequences of the pNZ4 and pNZ11 cassettes were submitted to GenBank (accession nos. FJ804172.1 and FJ804173.1).

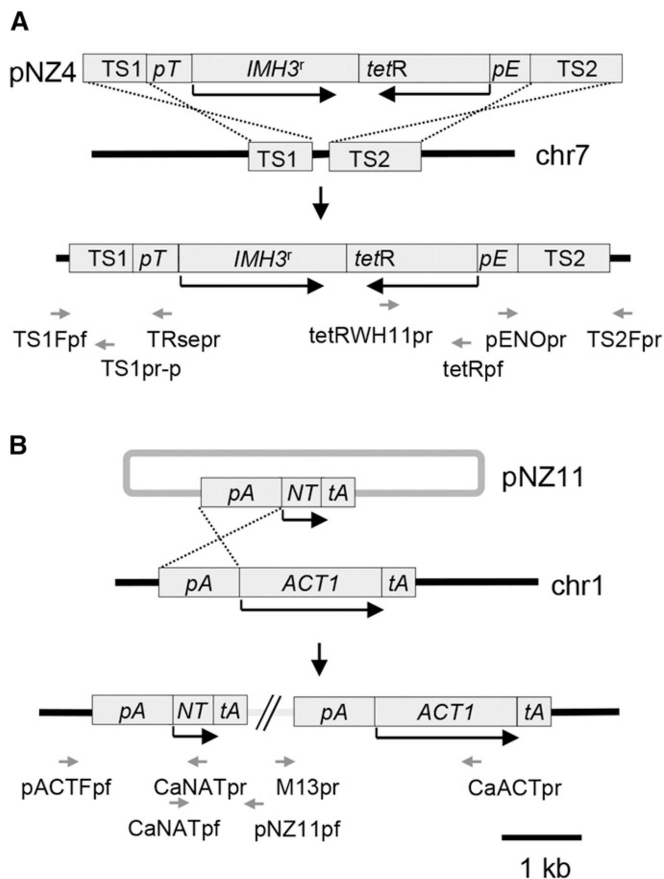

Figure 1 Resistance cassettes used to mark parental strains by integration following transformation. (A) The pNZ4 cassette contains: two noncoding sequences, TS1 and TS2, from the region of chr 7 to which integration of the cassette is targeted; the MPA resistance-conferring *IMH3* allele (*IMH3*^{r}) under the control of a tetracycline-responsive promoter (*pT*); and the gene encoding the tetracycline transactivator (*tet*R) under control of the *ENO1* promoter (*pE*). In the absence of tetracycline, the tetracycline activator induces the tetracycline-responsive promoter controlling *IMH3*^{r} expression thus conferring resistance to MPA. Integration occurs by a double crossover in TS1 and TS2. (B) Plasmid pNZ11 contains, in a pBlueScript KS(+) backbone (the latter not drawn to scale): the *CaNAT* ORF (*NT*) under control of the *ACT1* promoter (*pA*), followed by the *ACT1* terminator region (*tA*). It integrates by a single crossover in the *ACT1* promoter region on chr 1. Black arrows indicate ORFs, and grey arrows indicate the position of primers.

Mating in laboratory culture

The mating protocol (Figure 2) was a modification of the method of Legrand *et al.* (2004). In brief, both parents were grown as broad bands across YPD plates and replica plated at right angles to each other on a single YPD or spider medium (Bennett *et al.* 2005) plate. The cross-replicated plate was incubated for 7 days at room temperature. We did not find a growth medium in which both MPA and NAT were effective when *C. albicans* was plated at high cell density and therefore we sequentially exposed cells to each drug to select for fusants. We first replica plated from the mating plate onto YPD + NAT (200 μg/ml). After incubation at 30° for 2 days, all cells from areas where mating could have occurred (the intersections of the bands of cells) were scraped off the plate with a loop, suspended in 1 ml water, and 100 μl aliquots of this suspension were plated on 10 minimal medium plates containing MPA (5 μg/ml) and incubated at 30° for 5 days. Large colonies were patch plated on both minimal medium + MPA and on YPD + NAT plates. After incubation at 30° for 2 days, the patches from the YPD + NAT plates, whose counterparts on minimal medium + MPA also grew, were tested by PCR for the presence of both *MTL* alleles as described above. If both *MTL* alleles could be amplified, cells from the patches were streaked on YPD agar plates and the PCR assay was repeated for single colonies. These precautions were necessary because MPA selection has a high false-positive background; cells without the resistance marker can still grow, albeit more slowly. Only single colonies with both *MTL* alleles from the final YPD plates were considered as true fusants and stored at −80°. In some cases, PCR analysis to detect the *MPA*^{r} and *NAT*^{r} markers (using primer sets TS2Fpr/pENOpr and pACTFpf/CaNATpr respectively) was used to confirm fusants.

Mating of clinical isolates with universal mating tester strains was performed to validate the mating method. The *MTL***a** derivatives of unmarked clinical isolates were mated with the universal mating tester strain 3685 (*His*$^-$, *Arg*$^-$, *MPA*^{r}, *MTL*α), and the *MTL*α derivatives were mated with the tester strain 3710 (*His*$^-$, *Arg*$^-$, *MPA*^{r}, *MTL***a**) as described above; fusants were selected on minimal medium plus MPA as described by Magee *et al.* (2002).

Flow cytometry analysis of DNA content

Cells from glycerol stocks were patched on YPD agar and, after overnight incubation at 30°, the patch was used to inoculate liquid YPD medium to an initial OD_{600} of approximately 0.2. This culture was incubated at 30° with shaking (150 rpm) and cells were harvested when the OD_{600} was between 1 and 2. The cells were then fixed in 70% ethanol at 4° for 1 hr to 4 days. They were then treated for 1 hr with RNAse A (Sigma) (2 mg/ml in 50 mM Tris-HCl pH 8.0, 5 mM EDTA) and then for 1 hr with Pepsin (Sigma) (5 mg/ml in 55 mM HCl) and were subsequently washed with 50 mM Tris-HCl pH 7.5, 5 mM EDTA, and stained with 1 mM Sytox green (Invitrogen) in 50 mM Tris-HCl pH 7.5, 5 mM EDTA, as described by Hull *et al.* (2000). Flow cytometry analysis was performed on a BD FACSCalibur (BD Biosciences), using an excitation wavelength of 488 nm (15 mW argon-ion laser). Emission from Sytox green was measured using a 530/30-nm band pass filter. The sample was collected at a rate of 12 μl/min equating to a rate of >500 events per second. A minimum of 50,000 events was collected. See Figure S2 for examples. DNA content of a fusant relative to that of parents was calculated by first determining the median fluorescence in its M1 and M3 peaks, followed by dividing the fusant's M1 median by the sum of its parents' M1 medians, and the

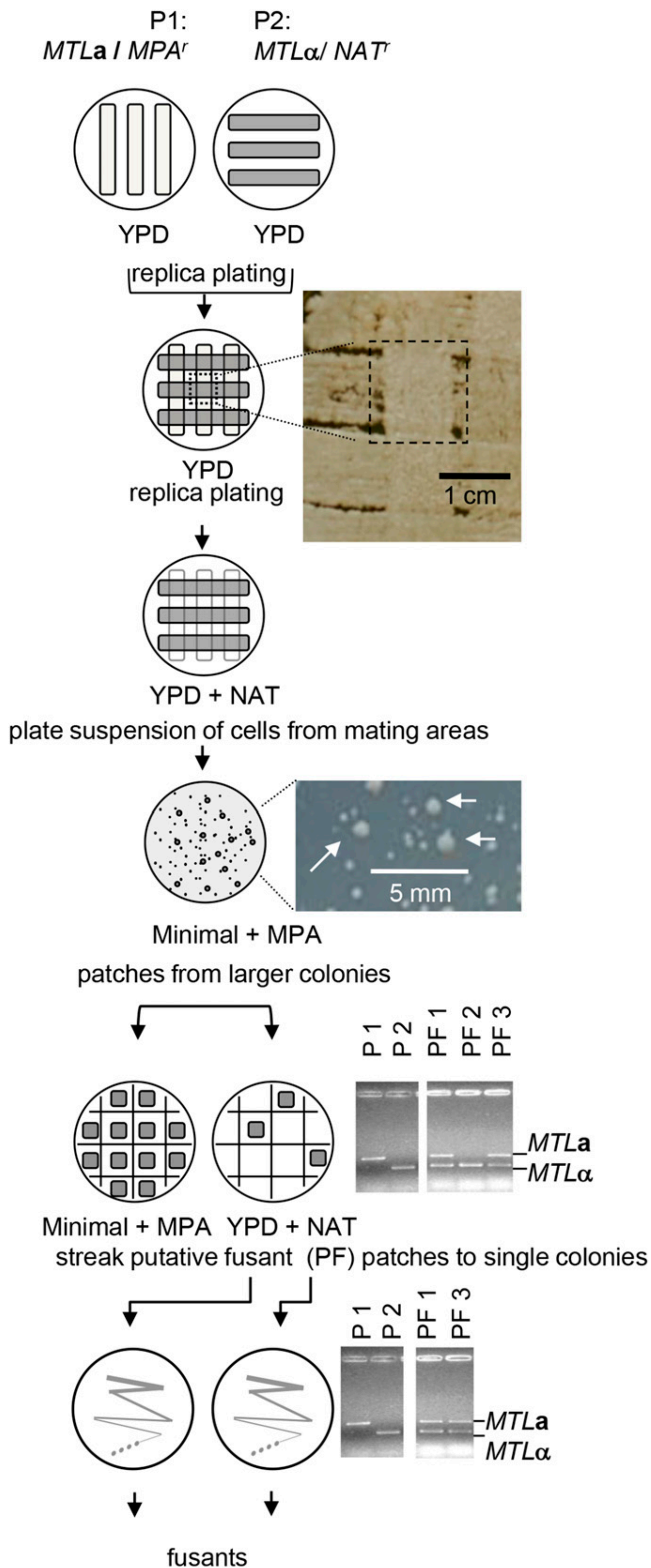

Figure 2 Overview of mating procedure and selection of fusants from two parent strains P1 and P2. Photographs are included to illustrate key steps, namely the area of mating on YPD plates, marked by a dashed rectangle, examples of large colonies, marked with arrows, on minimal medium plus MPA and gel images of *MTL* allele-based PCR products of parents and three putative fusants, PF1, PF2, and PF3. PF2 is an example of a putative fusant, which was not confirmed as a fusant by PCR (false positive).

fusant's M3 median by the sum of its parents' M3 medians, followed by averaging these two ratios.

Nuclear staining

Cells from patches grown on YPD plates at 37° overnight were fixed in 70% ethanol at 4° for at least 1 hr and stained with DAPI (4',6'-diamidino-2-phenylindole) dissolved in water (1 μg/ml) (Legrand *et al.* 2004). Cells were visualized using a DFC320 camera and a FM Olympus BX-51 microscope using a U-MWU2 filter set (excitation 330-385 nm, emission 420 nm, dichromatic mirror cut-off 400 nm) with a 100× Magna File objective (see Figure S3 for examples).

In vivo mating and competition

Details of these experiments are given in File S1. In brief, immunosuppressants (doxycycline and dexamethasone) were added to the drinking water of male Sprague-Dawley rats (~200 g in weight) and oral cavities were co-inoculated with mating-compatible pairs of *MTL*-homozygotes. Thereafter the tongues of rats were swabbed weekly with sterile swabs moistened in sterile saline. Swabs were vortex mixed in 1 ml sterile saline. Portions of the suspension (5 μl and 50 μl) were plated on YPD agar containing chloramphenicol to determine the total number of *C. albicans* cells present, and portions (100 μl) were plated on a double selection medium (DSM; 0.67% yeast nitrogen base with amino acids, 2% glucose, 1.5% bacteriological agar, 5 μg MPA/ml, 100 μg NAT/ml) selecting for fusants. Individual colonies were checked by PCR amplification of *MTL***a** or *MTLα* markers to determine the number of fusants and parents present.

Animal experiments were carried out in facilities approved by the New Zealand statutory body for animal experiments, ANZCCART (Animal Assurance no. A5608-01) in accordance with the ANZCCART Code of Practice for the Use of Veterinary and Human Medicines in Research, Testing and Teaching Organizations and the Animal Welfare Act 1999, New Zealand. Procedures were approved by the University of Otago Animal Ethics Committee (approval 60/2007).

Results

Mutations in mating genes indicate that mating is under selection

The *C. albicans MTL* locus contains four genes (*MTLα1*, *MTLα2*, *MTL***a***1*, *MTL***a***2*) that are required for mating. They are part of an evolutionarily conserved regulatory circuit, the primary function of which is the control of mating (Tsong *et al.* 2003; Butler *et al.* 2009). Expression of genes

Table 3 Fusants used in growth experiments

Fusant	Fusant
AU35**a** × HUN97α.1	AU90**a** × YSU63α.3
AU35**a** × OD8916α.1	RIHO11**a** × OD8916α.1
AU35**a** × OD8916α.2	RIHO11**a** × OD8916α.2
AU35**a** × W17α.1	RIHO11**a** × OD8916α.3
AU35**a** × W17α.2	RIHO11**a** × W17α.1
AU35**a** × W17α.3	RIHO11**a** × W17α.2
AU35**a** × YSU63α.1	RIHO11**a** × W17α.3
AU35**a** × YSU63α.2	RIHO11**a** × YSU63α.1
AU35**a** × YSU63α.3	W43**a** × OD8916α.1
AU7**a** × YSU63α.1	W43**a** × OD8916α.2
AU90**a** × HUN97α.1	W43**a** × OD8916α.3
AU90**a** × HUN97α.2	W43**a** × W17α.1
AU90**a** × OD8916α.1	W43**a** × W17α.2
AU90**a** × W17α.1	W43**a** × W17α.3
AU90**a** × W17α.2	W43**a** × YSU63α.1
AU90**a** × YSU63α.1	W43**a** × YSU63α.2
AU90**a** × YSU63α.2	W43**a** × YSU63α.3

All of the fusants are (*MPA*^{R}) (*NAT*^{R}) *MTL***a**/α. Names of fusants used under harsh conditions are underlined.

required for mating is costly, and mutations that abolish mating ability accumulate rapidly in the absence of selection for mating, as has been demonstrated in the yeast *Saccharomyces cerevisiae* (Lang *et al.* 2011). Mating competency also carries a cost in *C. albicans* (Ibrahim *et al.* 2005; Lockhart *et al.* 2005; Wu *et al.* 2007; see also below). The biological significance of the mating process should thus be deducible from an analysis of mutations in these genes.

A commonly used indicator of selection of gene function is the ratio of nonsynonymous to synonymous mutations (d_n/d_s) (Yang and Nielsen 2002; Spielman and Wilke 2015). If *MTLα1*, *MTLα2*, *MTL***a***1*, and *MTL***a***2* have retained their original function—mating—and if the ability to mate is under selection, then nonsynonymous mutations in these genes would tend to be eliminated by selection because many such mutations would interfere with mating. Selectively neutral synonymous mutations should be retained more often; as a result the ratio between the two (d_n/d_s) should be <1.0. If the genes have lost their function, nonsynonymous mutations will not be selected against and would be as likely to be retained as synonymous mutations ($d_n/d_s = 1$). Finally, it is conceivable that the genes are functional, but are in the process of acquiring a new primary function, different from mating. In this scenario they could still retain some marginal functionality in regard to mating even though mating ability itself is no longer under selection. If so, nonsynonymous, function-altering mutations may be favored by selection and be more likely to be retained than synonymous mutations ($d_n/d_s > 1$) (Yang and Nielsen 2002).

We calculated d_n/d_s for *MTLα1*, *MTLα2*, *MTL***a***1*, and *MTL***a***2* in *C. albicans* and their orthologs in six closely related yeasts. Two of these species, *Candida dubliniensis* and *C. tropicalis*, have limited (para) sexual abilities that resemble those of *C. albicans* (Pujol *et al.* 2004; Porman *et al.* 2011). One species, *C. orthopsilosis*, has a defective pheromone response and ineffective *MTL***a***1* splicing. The occurrence of *MTL*-homozygous and -heterozygous *C. orthopsilosis* strains suggest that it may, nevertheless, mate (Sai *et al.* 2011). Three species (*Pichia stipitis*, *C. lusitaniae*, and *Debaromyces hansenii*) are capable of sexual recombination resembling that of the fully sexual *S. cerevisiae*. Haploid cells are easily induced to mate and generate diploids, which then return to haploidy by meiosis or processes which lead to meiosis-like marker distributions (Breuer and Harms 2006; Reedy *et al.* 2009; Bajwa *et al.* 2010; Butler 2010).

Table 4 Ratio of nonsynonymous to synonymous mutations in mating genes

Gene[a]	d_n/d_s parasexual group[b]	d_n/d_s sexual group[b]	d_n/d_s *C. orthopsis*[b]	fraction of alignment used[c]
*MTL***a***1*	0.144	0.067	0.141	312/834
*MTL***a***2*[d] (i)	0.252	0.095	—	264/834
(ii)	0.077	0.030	0.058	276/450
MTLα1	0.057	0.023	0.188	432/663
MTLα2	0.099	—	—	555/618

[a] The sequences are from strains SC5314 (*C. albicans*), CD 36 (*C. dubliniensis*), 90–125 (*C. orthopsilosis*), MYA-3404 (*C. tropicalis*), CL143 (*C. lusitaniae*), CBS767 (*D. hansenii*), CBS 6054 (*P. stipitis*).

[b] The ratio of nonsynonymous to synonymous mutations was estimated using the M2 model in the codeml program from PAML v. 4.5. This model allows a different d_n/d_s ratio in different parts of the tree and is optimized using maximum likelihood. We fit three different ratios: one for the sub-tree including *C. tropicalis*, *C. albicans*, and *C. dubliniensis*; one for the sub-tree including *Pichia stipitis*, *C. lusitaniae*, and *D. hansenii*; and one for the branch leading to *C. ortholopsis*.

[c] Any deletions and insertions have to be removed from the alignment prior to the analysis.

[d] The *C. dubliniensis* and the *C. tropicalis* coding sequences were deduced from nonannotated genomic sequence provided by Gary Moran and Derek Sullivan and, since the gene contained an intron and parts of it are poorly conserved, the intron boundary was difficult to establish. To overcome this problem, the two species were either eliminated from the analysis (i), or the part of the alignment that could contain intron sequences in the two species, was removed prior to analysis (ii).

An analysis of the d_n/d_s ratios indicated that the *C. albicans* genes have retained their function in mating. First, even though d_n/d_s ratios were slightly higher in the parasexual than in the fully sexual group, they were well below 1.0 (ranges 0.099–0.252 *vs.* 0.023–0.188; Table 4), and the difference between the two groups was not statistically significant. We established this by simulating (with PAML; Yang 2007) the evolution of the aligned sequences, using two models, one in which d_n/d_s was allowed to differ between the parasexual group and other species and one in which d_n/d_s was constant. We found, using a likelihood test (Zhang *et al.* 2010), that allowing d_n/d_s to alter did not lead to models that fit the data significantly better than models with constant d_n/d_s. This analysis was carried out for each the three genes present in all species (*MTLα1*, *MTL***a***1*, *MTL***a***2*). *MTLα2* orthologs are absent in the fully sexual group (Butler *et al.* 2009) and for this gene we established that a model in which d_n/d_s was free to vary fits the data better than one in which d_n/d_s was set to 1.0—indicating

that the ratio was significantly <1.0. In addition, all nine d_n/d_s ratios for mutations distinguishing *C. albicans* *MTLα1*, *MTL***a***1*, and *MTL***a***2* from their orthologs in the three sexual species were <1.0 (range 0.15–0.56); *i.e.*, there was no indication that the *C. albicans* genes lost their function in mating as they and their orthologs in each of the fully sexual species diverged. These analyses were carried out on one reference genome per species, including the *C. albicans* SC5314 genome. We therefore cannot exclude that the results may not apply to the entire species. However, for one of the four genes, *MTL***a***1*, we found in GenBank the complete amino acid sequences it encodes in two other *C. albicans* strains, P94015 and P60002. These were identical to the sequence of the SC5314 protein.

Numerous mutations must occur to alter the d_n/d_s ratio of a gene significantly, but a single mutation can destroy mating ability. We therefore also assessed if mating is under selection by estimating the selection coefficient of mutations that prevent mating, *i.e.*, how detrimental such mutations are to survival. This is possible because the frequency (p) of a deleterious mutation in a population is determined by the balance between the rate at which mutations of this type arise (m), and the rate at which individuals carrying them are eliminated. The latter frequency is the selection coefficient s, which indicates by how much the probability of survival is reduced by a deleterious mutation ($s = m/p$) (Ridley 1996). The estimate is not entirely accurate because chance events (genetic drift) also influence the frequency of mutations in a population. However, genetic drift plays only a minor role in microbial species, given their large effective population sizes (Lynch and Conery 2003; Tsai *et al.* 2008; Charlesworth 2009).

We are aware of two naturally occurring mutations that abolish mating, one found in an analysis of 12 *MTL* homozygotes by Legrand *et al.* (2004) and one in the 10 strains tested in this study (see below; both render *MTLα1* dysfunctional). Thus our best estimate of the frequency of mutations that abolish mating (p) is 2/22, *i.e.*, 0.09 (binominal 95% confidence interval 0.011–0.292).

How frequently mutations that abolish mating arise can be estimated by multiplying (i) the genome-wide mutation rate [3.3×10^{-10}; point mutation rate determined in *S. cerevisiae* (Lynch *et al.* 2008); similar to the *C. albicans* rate (Gomez-Raja and Larriba 2013)] with (ii) the probability that any given mutation will affect function (0.107), (iii) the number of genes required for mating (25–80), and (iv) the average size of these genes (1539 bp; see File S1 for details on how the latter three estimates were derived). Based on the upper and lower estimate of the number of genes required for mating, the resulting estimates of m range from 1.7×10^{-6} to 5.5×10^{-6} per division. This results in a selection coefficient (s) between 5.9×10^{-6} and 4.9×10^{-4}. In other words, carrying a mutation that interferes with mating reduces the fitness of a strain by an estimated 0.0006–0.0490%.

These values for s are small, but nevertheless indicate that mating is under selection. Selection is not overwhelmed by genetic drift as long as selection coefficients are ≥4 times the inverse of effective population size (Charlesworth 2009) and our lowest estimate of s is greater than 4 times the inverse of typical effective population sizes of unicellular eukaryotic species (10^7–10^8; Lynch and Conery 2003; Tsai *et al.* 2008).

Mating of MTL-homozygotes derived from genetically distinct clinical isolates often yielded fusant offspring

To investigate why mating is under natural selection we wanted to assess directly the benefits of mating for naturally occurring strains, using 10 clinical isolates from our international collection (Table 1). None of the isolates had been extensively subcultured and they should thus represent naturally occurring genotypes. All isolates were *MTL*-heterozygous. We used sorbose selection (Magee and Magee 2000) to induce the loss of one copy of chr 5, and thus one *MTL* allele, to generate mating-competent *MTL*-homozygous derivatives. Most strains lost one of their *MTL* alleles much more frequently than the other, and for several we could recover only either *MTL*α or *MTL***a** derivatives (Table 5). We chose frequently recovered types of *MTL*-homozygous derivatives for further experiments.

A likely reason why mating is under selection would be that it can generate advantageous genotypes, not otherwise achievable through the frequent genome rearrangements in individual cells (Chibana *et al.* 2000; Forche *et al.* 2005; Rustchenko-Bulgac *et al.* 1990), by mating between the unrelated commensal strains which often cocolonize the same individual (Soll *et al.* 1991; Jacobsen *et al.* 2008). We therefore investigated the potential of genetically distinct *MTL*-homozygotes to generate fusants.

Only a small percentage of encounters between mating-compatible *MTL*-homozygous cells actually generate fusants (Lockhart *et al.* 2003; Bennett *et al.* 2005). We therefore marked five of our sorbose selection-derived *MTL***a** strains with a mycophenolic acid-resistance cassette (Beckerman *et al.* 2001) and five *MTL*α strains with a nourseothricin-resistance cassette (Shen *et al.* 2005) (Table 2), allowing selection of fusants after mating on the basis of dual drug resistance (see *Materials and Methods* for details).

We obtained one or more fusants from 15 of 25 possible combinations of parents, although in some cases only after several attempts (Figure 3). One strain, FJ11α, did not mate with any of the five *MTL***a** derivatives, because of a mutation in the *MTL*α1 gene essential for mating (Tsong *et al.* (2003); File S2). All remaining strains could mate with at least one of the five strains of the opposing mating type.

The DNA content of fusants was often less than that of both parents combined (Figure 3), indicating chromosome loss during the selection procedure. Thus loss of the markers that we used to select and identify fusants (drug resistance and *MTL* alleles) may have prevented us from recovering fusants from additional matings. However, there was no

	FJ11 α	HUN97 α	OD8916 α	W17 α	YSU63 α
Au35**a**		2.00	1.50 ±0.25	1.60 ±0.06	1.43 ±0.04
Au90**a**		2.00 ±0.11	1.40	1.00 ±0.29	1.86 ±0.27
RIHO11**a**			1.81 ±0.25	1.77 ±0.11	1.32
W43 **a**			2.03 ±0.11	2.23 ±0.50	1.71 ±0.30
Au7**a**					1.34

Figure 3 Matrix of outcomes of matings between *MTL*-homozygous derivatives. Black boxes indicate combinations yielding seven or more fusants per experiment and in which ≥10% of all colonies tested were fusants. Grey boxes indicate combinations in which fewer fusants were obtained and open boxes indicate combinations yielding no fusants in three or more independent experiments (some matings involving FJ11α were attempted only once after it was discovered that a genetic defect prevented it from mating). Numbers in boxes indicate the DNA content of fusants (mean ±SD where multiple fusants were analyzed) relative to the mean DNA content of the parents. Fusants were selected and identified, and their DNA content assessed by flow cytometry as described in *Materials and Methods*. All fusants were mononucleate apart from a small percentage of W17α cells and a small percentage of W17α offspring, which were multinucleate (see Figure S3).

correlation between the reduction of DNA content of fusants and the frequency with which fusants were obtained (Figure 3). Furthermore, there was no indication of selection for loss-of-resistance cassettes even in the absence of the drugs and we never observed loss of *MTL*-heterozygosity when serially propagating fusants (Figure S4 and File S2). It thus seems more likely that, apart from one case of mutational decay of a key gene (in FJ11), it was incompatibility of genetic backgrounds that prevented, or severely curtailed, the generation of fusants in 10 of the 25 pairwise combinations.

Fusants often grew better than their parents in laboratory culture

To test if mating can generate genotypes that are fitter than parental genotypes, we assessed the growth rates of 34 fusants (3 per parent combination unless fewer fusants were obtained; listed in Table 3) and their 9 MTL-homozygous parents.

C. albicans' largely clonal population structure (Gräser *et al.* 1996; Tibayrenc 1997; Tavanti *et al.* 2004; Bougnoux *et al.* 2008) suggests that mating may only rarely increase fitness in the host. We therefore tested fitness under conditions that should favor fusants. We assessed growth rates during serial propagation, for 100–120 generations, in YPD medium at 37°, and under harsh conditions under which not all *MTL*-homozygotes could grow, namely at 42° in a low-glucose minimal medium containing 0.2 M NaCl. Under the latter conditions we assessed only 11 fusants that were close

Table 5 ***MTL*** **status of colonies recovered from sorbose plates**

Strain	% *MTL***a**/*MTL*α[a] (no. of colonies)	% *MTL***a**[a] (no. of colonies)	% *MTL*α[a] (no. of colonies)
AU35	71 (17)	29 (7)	0 (0)
AU7	94 (45)	6 (3)	0 (0)
Au90	25 (6)	75 (18)	0 (0)
RIHO11	96 (23)	4 (1)	0 (0)
W43	4 (1)	25 (6)	71 (17)
FJ11	4 (1)	17 (4)	79 (19)
HUN97	50 (6)	0 (0)	50 (6)
OD8916	79 (19)	0 (0)	21 (5)
W17	42 (10)	0 (0)	58 (14)
YsU63	25 (6)	0 (0)	75 (18)

[a] determined by colony PCR as described in File S1.

to tetraploid upon their isolation. In other fusants, parental alleles beneficial to the harsh environment may have been lost, because the conditions under which fusants were isolated differed substantially from the extreme conditions (see Table 3 for a list of fusants tested).

Both conditions represent novel environments in which (i) the benefits of mating in the form of accelerated adaptation should be high, (especially under harsh conditions under which the strains could barely survive) and (ii) the cost of breaking up compatible parental allele combinations should be lower than in the environment in which they were selected (Goddard *et al.* 2005; de Visser and Elena 2007; Morran *et al.* 2011). Reproductive success in culture will depend on maximizing growth rates in the novel environment and reducing the cost of features that enhanced fitness in the host but are now superfluous (Agrawal *et al.* 2010; Hill *et al.* 2015). Unlike their parents, fusants initially contain two entire parental genomes. Chromosome loss subsequently generates a variety of aneuploid, and eventually diploid, lineages from which the best-adapted combination of parental alleles can be selected. If some fusant lineages were to become *MTL*-homozygous as they reduce their genome size, their mating could further speed up adaptation to the new environment. However, we never observed *MTL* allele loss in serially propagated fusants (Figure S4).

Mating clearly enhanced reproductive success, over that of continued clonal reproduction, for the mating-competent *MTL*-homozygous isolates (Table 6). Even prior to serial propagation about one-third of fusants grew faster than their parents and some faster than any of the *MTL*-homozygotes. After serial propagation fusants' growth rates had increased more than those of their parents and most fusants grew better than parents. Under harsh conditions most fusants grew better than any of the *MTL*-homozygotes.

We also assessed the benefits of mating under conditions more closely resembling *C. albicans*' natural environment, in a model of oral commensal colonization. The outcome indicated that mating can occur during commensal colonization, but also that it confers less benefit in *C. albicans*' normal environment than in culture. In 13 of 51 rats, orally inoculated with equal numbers of cells of mating-compatible

parents, fusants were initially detectable (Table S2). However, even though colonization levels in the oral cavity increased over time, fusant numbers declined (Figure 4), as did the frequency of one parent (Table S2), and the percentage of mating-competent opaque cells (data not shown). This suggests that fusants formed initially when equal numbers of mating-competent *MTL***a** and *MTL*α cells were present, but could not compete well with their parents. Thus while in laboratory culture between 47% (YPD) and 82% (harsh conditions) of fusants eventually grew faster than both parents (Table 6), fusants could compete with their parents in <8% (<1/13) of animals.

Many fusants also grow faster than their MTL-heterozygous ancestors

One likely reason why fusants grew faster than their parents in laboratory culture was that mating reversed LOH at the *MTL* locus and the remainder of chr 5. That such LOH events reduce fitness is indicated by the low prevalence (~10%) of *MTL*-homozygous *C. albicans* clinical isolates (Legrand *et al.* 2004) and their reduced virulence (Ibrahim *et al.* 2005; Lockhart *et al.* 2005; Wu *et al.* 2007). We also observed that *MTL*/chr 5 LOH resulted in a significantly reduced fitness under our growth conditions. When first isolated after sorbose selection, the median growth rate of *MTL*-homozygotes was 45% lower than that of their heterozygous ancestors in YPD (Figure 5A) and 15% lower under harsh conditions (Figure 5B; one *MTL*-homozygote, W17α, could not grow under harsh conditions at all - not included when calculating the median). Growth rates recovered within 30 generations of serial propagation but remained ~3% below those of the heterozygous ancestors. The rates measured after 30 generations were close to those of a set of 23 naturally occurring *MTL*-homozygous clinical isolates (Figure 5). Two *MTL*-homozygotes that spontaneously arose from isolates OD8916 and W43 on YPD medium grew 20% slower than their *MTL*-heterozygous ancestors (Figure 5). Their growth rates did not improve over time.

A comparison of fusants with their *MTL*-heterozygous ancestors (Table 7) indicated that mating could lead to fitness increases beyond those explicable by restoration of chr 5 heterozygosity. After ≥100 generations of serial propagation in YPD, 15% of fusants grew faster than both *MTL*-heterologous ancestors, and 27% after serial propagation under harsh conditions—although no fusant grew faster than the fastest growing of all *MTL*-heterologous ancestor strains. Approximately 10% of fusants already grew faster than either ancestor prior to serial propagation.

These results may underestimate the true fitness gain achievable by mating in culture, because the fusants' parents, but not the *MTL*-heterozygous ancestors, had been transformed with drug-resistance cassettes. The cassettes themselves apparently had little effect on fusants' fitness since they were retained during serial propagation in the absence of drug selection (File S2 and Figure S4). However, transformation did, in many cases, reduce the fitness of

Table 6 Comparison of the growth rates of fusants and the *MTL*-homozygotes that parented fusants

	Initial				After serial propagation for 100-120 generations			
	n	Average growth rate (doublings/hr)	% of fusants with rates higher than rates of both parents/higher than parental average	No. of fusants growing faster than all *MTL*-homozygotes[a]	*n*	Average growth rate (doublings/hr)	% of fusants with rates higher than rates of both parents/higher than parental average	No. of fusants growing faster than all *MTL*-homozygotes[a]
YPD at 37°								
MTL-homozygotes	9	0.960	n/a	n/a	9	0.972	n/a	n/a
Fusants	34	0.964	32/44	5	34	1.037	47/79	10
Harsh conditions								
MTL-homozygotes	8	0.502	n/a	n/a	8	0.481	n/a	n/a
Fusants	11	0.566	27/36	0	11	0.655	82/91	8

[a] All *MTL*-homozygotes with resistance cassettes, which parented one or several of the fusants tested.

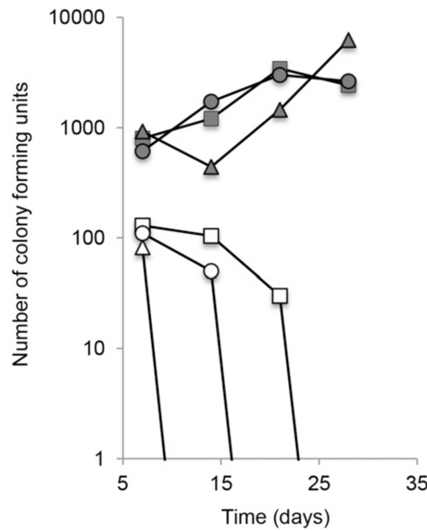

Figure 4 Number of fusants detected over 28 days in a rat model of oral colonization. The figure shows the total number of colony forming units (solid symbols) and the number of fusants (open symbols) recovered by swabbing the oral cavities of a set of three rats (each represented by a differently shaped symbol) co-inoculated orally with opaque cells of strains W43a–pNZ4 and OD8916α–pNZ11. This is one of several experiments with similar outcomes (see Table S2 for outcomes of all experiments and more details).

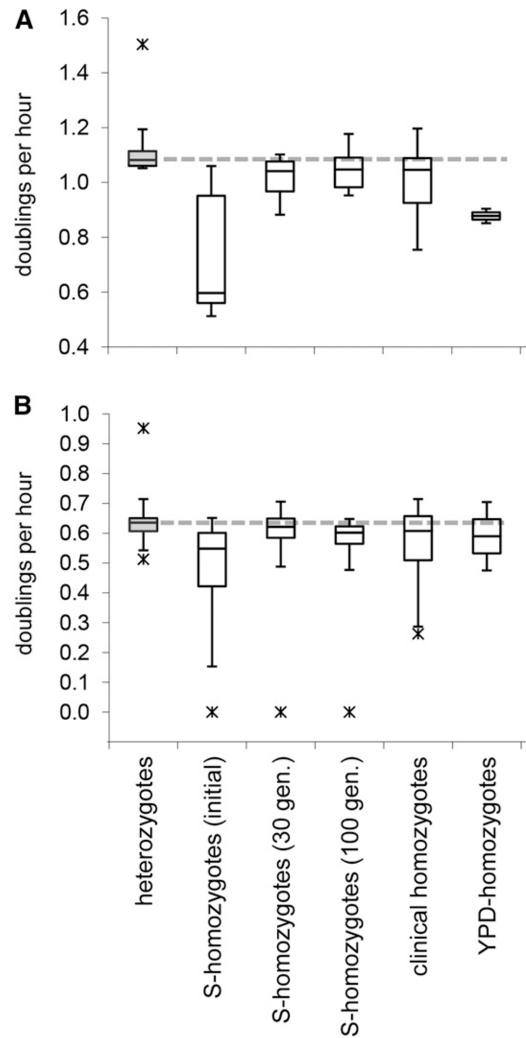

Figure 5 Impact of *MTL*-homozygosity on growth rates. Box and whisker plots of growth rates of the 10 clinical *MTL*-heterozygous clinical isolates used in this study (shaded boxes), their *MTL*-homozygote derivatives, selected on sorbose (S-homozygotes) initially and after 30 and 100 generations of serial propagation, *MTL*-homozygous clinical isolates (clinical homozygotes) and spontaneous *MTL*-homozygotes isolated on YPD medium (YPD-homozygotes); serial propagation for 30 generations of the latter did not increase the growth rates over the initial rates depicted in the figure. Asterisks, outliers. A dashed line indicates the median growth rate of the *MTL*-heterozygous clinical isolates. (A) Strains grown on YPD, (B) strains grown under harsh conditions (W17α could not grow under harsh conditions).

MTL-homozygotes (Table S3). This suggests that spurious genetic defects acquired by parents during their transformation (rather than the cassettes) might be inherited by fusants and reduce their fitness.

To compensate for the potential reduction in fusant fitness caused by transformation-related parental genetic defects, we computationally increased the growth rates of fusants according to the fitness differential between transformed and original *MTL*-homozygotes (see Table 7 footnote *a* for details on the computational adjustment). This provides an upper estimate of the benefits of mating, since it assumes that mating cannot ameliorate these genetic defects by complementation or their elimination through chromosome loss in fusants. Indeed there was, at least in YPD, a reasonably good correlation between the extent by which transformation had reduced parents' growth rates and the extent by which the rate of their fusant progeny exceeded it, as expected if mating eliminated these defects (Figure S5). The most striking example was strain W17α. Transformation reduced its growth rate by almost 40%, compared to 0–5% in other *MTL*-homozygotes (Table S3), making its transformed derivative the slowest growing of all transformed parent strains. Nevertheless this strain parented the fastest-growing fusants (Table 7).

Applying the computational adjustment, 47% of fusants grew better than both of their ancestors after serial propagation in YPD and 73% after serial propagation under harsh conditions. Four grew faster than the fastest growing of the *MTL*-heterologous ancestors (Table 7).

We limited propagation to 100–120 generations, and it is possible that fusants can eventually reach higher growth rates. One likely cause would be further reduction in ploidy. Indeed a decrease of DNA content of fusants during serial propagation was associated with an increase in growth rate (tested in YPD; Figure S6A). Nevertheless after 100 generations only ~20% of fusants tested had a DNA content indicative of diploidy. Also, the DNA content after 100 generations did not predict how well fusants grew, relative to their parents or *MTL*-heterologous ancestors (Figure S6, B and C). This suggests that euploidy may be less important in

Table 7 Comparison of the growth rates of fusants and their *MTL*- heterozygous ancestors

	Initial					After serial propagation for 100-120 generations				
	n	Average rate adjustment factor ± SD[a]	Average growth rate (doublings/hr)	% of fusants with rates higher than both ancestors/ higher than ancestral average	No. of fusants growing faster than all *MTL*-hetero-zygotes[b]	*n*	Average rate adjustment factor ± SD[a]	Average growth rate (doublings/hr)	% of fusants with rates higher than both ancestors/higher than ancestral average	No. of fusants growing faster than all *MTL*-hetero-zygotes[b]
YPD at 37°										
MTL-heterozygotes	9	n/a	1.133	n/a	n/a	9	n/a	1.152	n/a	n/a
fusants	34	n/a	0.936	12/15	0	34	n/a	1.037	15/29	0
fusants adjusted[a]	34	0.92 ± 0.11	1.063	38/38	0	34	0.91 ± 0.10	1.158	47/50	1
adjusted w/o W17α[a,c]	24	0.99 ± 0.04	0.961	17/21	0	24	0.96 ± 0.03	1.063	8/25	0
Harsh conditions										
MTL-heterozygotes	8	n/a	0.626	n/a	n/a	8	n/a	0.642	n/a	n/a
fusants	11	n/a	0.566	9/27	0	11	n/a	0.655	27/64	0
fusants adjusted[a,d]	11	0.89 ± 0.05	0.638	45/55	1	11	0.93 ± 0.06	0.704	73/91	4
adjusted w/o W17α[a,e]	8	0.90 ± 0.06	0.606	38/38	0	8	0.94 ± 0.05	0.679	63/75	1

[a] In addition to data based on actual growth rates, the table includes data based on fusant growth rates that were adjusted to compensate for the impact of transformation on their parents. To generate the adjusted rates, the actual growth rate of each fusant was divided by an adjustment factor. The adjustment factor was calculated as follows: The average of the rates of a fusant's actual parents was determined. This was then divided by the average of the rates of the same strains prior to their transformation with the resistance cassettes.

[b] All *MTL*-heterozygotes, whose *MTL*-homozygous derivatives parented one or several of the fusants tested.

[c] Values excluding W17α-derived fusants. Strain W17α was the fastest growing of all initially recovered *MTL*-homozygotes. After each of two transformation attempts, W17α's growth rate was reduced by almost 40%, compared to 0–5% in other *MTL*-homozygotes strains (Table S3). This made the cassette-bearing W17α derivative the slowest growing of all transformed parent strains. However, the strain parented the fastest-growing fusants, indicating that the growth defect was not passed on to fusants and that a 40% adjustment of growth rate was excessive.

[d] The normal procedure for growth rate adjustment (described in footnote *a*) could not be applied to all fusants. It is based on growth rates of parents, but neither W17α nor its resistance cassette-bearing-transformed derivative could grow under harsh conditions. Growth rates of fusants parented by W17α were therefore adjusted assuming that W17α's growth rate reduction after transformation equaled the average reduction in other *MTL*-homozygotes.

[e] Values excluding rates for W17α-derived fusants, since these had to be adjusted using a different procedure, as explained in footnote *d*.

determining fitness than other factors, such as two copies of a chromosome homolog containing fitness-enhancing alleles. Maximal fitness and diploidy may be attainable only after a lengthy series of mitotic recombination events.

Discussion

To date, mating in *C. albicans* has been observed only in the laboratory. Judging by our analysis of mutations in mating genes, the ability to mate is under selection, indicating that *C. albicans* mates in its natural environment and that mating is a biologically relevant process. To be under selection, mating must generate genotypes of increased fitness. Even if it does so rarely these genotypes will increase the reproductive success of the species because their progeny will, over time, replace or reduce in frequency other less-fit genotypes. Furthermore, even if the fitness increase is small, compounded over many generations it can have a substantial impact on the reproductive success of the species. However, each mating also involves costs, diminishing the species' reproductive success. To be under selection, *C. albicans* mating must thus generate fitter genotypes often enough to outweigh the cost of mating.

Our laboratory growth rate data show that mating can generate fusants that are fitter than their parents and their *MTL*-heterologous ancestors. One possible reason is that mating can assist in adaptation to novel environments. Goddard *et al.* (2005) showed that mating led to faster adaptation of a long-cultured (Tauro and Halvorson 1966) *S. cerevisiae* laboratory strain to novel unusual growth conditions than clonal reproduction. In normal growth medium, mating did not increase fitness (Goddard *et al.* 2005). Similarly, selective advantages, in terms of growth rate, of *C. albicans* were observable in many of our culture experiments but not, in terms of survival, in a small number of oral model colonization experiments, conditions more similar to *C. albicans*' normal environment. Mating probably also increased fitness in our experiments by removal, or complementation, of deleterious mutations present in parental genomes. The latter is suggested by the correlation between mating-associated increase in fitness and the reduction of fitness associated with genetic modification of parental strains (Figure S5) and also by the observation that some fusants already grew faster than ancestors prior to serial propagation in the novel environment.

A likely category of frequent natural genetic defects that mating, and only mating, can repair are LOH events that occur frequently throughout the *C. albicans* genome and that can "expose" heterologous deleterious mutations (Diogo *et al.* 2009; Forche *et al.* 2009, 2011; Hickman *et al.* 2013). The immediate fitness increase of some fusants, over that of their *MTL*-homozygous parents, may appear as striking examples of benefits of mating in the form LOH correction, but a significant part of the fitness reduction associated with chr 5 LOH may be caused by the expression of genes required for mating in mating-competent *MTL*-homozygous cells. In *S. cerevisiae* mating competency is associated with a 2% growth rate reduction attributed to expression of such genes (Lang *et al.* 2009), equivalent to more than half of the ~3% growth rate difference between chr 5 homozygotes and chr 5 heterozygotes that we measured.

The fitness reduction associated with mating competency is one of many factors that contribute to the cost of mating. Slow-growing mating-competent cells produce fewer progeny, decreasing their reproductive success relative to that of continued clonal reproduction of faster-growing *MTL*-heterozygotes—and the longer it takes for them to mate after reaching mating competency, the greater this cost becomes. In the host, *MTL*-homozygotes may even become extinct when competing for limited resources with faster-growing *MTL*-heterozygotes, eliminating any chance to eventually recoup this cost in the form of fast-reproducing fusants. The act of mating itself reduces reproductive success, yielding fewer new cells than could be generated in the same time period by continued clonal propagation (Lockhart *et al.* 2003). Other costs of mating include matings that "fail" in that they do not yield fusants fitter than parents or *MTL*-heterologous ancestors. The parental alleles combined in some fusants may never lead to genotypes fitter than those of ancestors or parents, even after prolonged periods of fusant growth. In the natural host, competition with parents and ancestors for limited niches and resources in the presence of host clearance mechanisms can also eliminate, before they have a chance to do so, fusants that could, over time, become fitter than parents and ancestors. Even fusants that are fitter than the parents or ancestors may thus often become extinct and contribute to the number of "failed" matings. If mating is rare in the host, a small number of resulting fusants will be at risk of extinction regardless of their fitness, among the more abundant parents or *MTL*-heterozygous ancestor cells (Kimura and Ohta 1969). This may be part of the reason, with a reduced probability of mating to generate genotypes better than existing genotypes in an organism's normal environment (Goddard *et al.* 2005; De Visser and Elena 2007; Morran *et al.* 2011), why fusants apparently struggled to survive in competition with their parents in the animal model. Similarly, a low probability of survival of fusants in the human host would explain why mating, although under selection, has so little impact on the population structure of the species, which is largely clonal (Gräser *et al.* 1996; Tibayrenc 1997; Tavanti *et al.* 2004; Bougnoux *et al.* 2008).

While fusants that survive but only eventually grow faster than parents or ancestors do not contribute to the failed matings, the time it takes them to reach a higher growth rate is also a cost. It reduces the differential between the number of their progeny generated over time compared to that generated by continued clonal reproduction.

A difficulty in deducing from our findings how the benefits of mating outweigh its costs in *C. albicans*' natural environment is that mating has never been observed in the

human host and may occur via different scenarios. We have focused on one of these, the mating of genetically different strains after chr 5 loss. While chr 5 loss is the most common mechanism of spontaneous loss of *MTL*-heterozygosity (Wu *et al.* 2005), in most *MTL*-homozygous clinical isolates *MTL*-homozygosity has arisen by localized recombination events (Wu *et al.* 2007). It seems unlikely though that the net benefit of mating is significantly affected by the mechanism of *MTL*-heterozygosity loss. The growth rates of clinical *MTL*-homozygous isolates were indistinguishable from those we generated by chr 5 loss (Figure 5); *i.e.*, the fitness of the two is comparable. Also, while parents heterozygous for large parts of chr 5 bring more alleles to the initial tetraploids, because of selection for *MTL*-heterozygosity (our data; Ibrahim *et al.* 2005; Lockhart *et al.* 2005; Wu *et al.* 2007), the final diploid products of mating will contain one copy of chr 5 from each parent—except possibly when rare circumstances allow mating of strains of the same mating type (Alby and Bennett 2011). The costs and benefits of these two scenarios are thus probably fundamentally similar (although the prevalence of isolates with partial chr 5 LOH indicates they may have better chances of long-term survival (Wu *et al.* 2007) while chr 5 LOH, stress induced (Forche *et al.* 2011) in pairs of cocolonizing strains, could more often allow mating shortly after mating competency is reached).

Mating of diploid or aneuploid *MTL*-homozygotes derived from the same strain is possible (Magee and Magee 2000) at least in some strains (Table 5). Such matings offer benefits in terms of restoring *MTL*- and/or chr5-heterozygosity but cannot correct other LOHs or generate better-adapted allele combinations, beyond those also achievable by genome rearrangements during clonal reproduction (Chibana *et al.* 2000; Forche *et al.* 2005; Rustchenko-Bulgac *et al.* 1990).

Mating is also possible between *C. albicans* haploids spontaneously arising by chromosome loss (Hickman *et al.* 2013), immediately yielding diploid fusants and restoring chr 5 heterozygosity. However, the mating of haploids should have a lower probability of generating genotypes of improved fitness than mating between diploids. Fusants derived from haploid parents inherit one of two chromosome homologs from each parent, and the future compatibility of parental alleles in fusants plays no role in selecting which homolog a parent passes on. In contrast, when diploids mate, the merger of two diploid genomes followed by chromosome loss allows selection for the best pairs of parental homologs, including the option of retaining only homologs from one parent. Fusants generated by mating of haploid derivatives of the same strain grow poorly (Hickman *et al.* 2013).

Thus, among these possible scenarios, the mating of strains of different ancestry following chr 5 LOH or LOH at the *MTL* locus appears to offer the greatest net benefit. However, with the exception of haploid mating, all are likely to offer some net benefits that could contribute to the selection of mating ability.

After subtracting the estimated costs, the remaining net benefit of *C. albicans* mating appears considerably lower than that of a fully functional sexual cycle as exemplified by the yeast *S. cerevisiae*. One important reason is the absence of meiosis in *C. albicans*. In the absence of meiosis all of the initial fusants arising from the mating of two strains are genetically more or less identical tetraploids. Subsequent competition among clonally reproducing fusant lineages can only slowly improve fitness by removing allelic incompatibilities and reducing ploidy through chromosome loss and mitotic recombination. In *S. cerevisiae*, in contrast, each offspring is immediately diploid and each has a different combination of parental alleles, having inherited only one of two homologous parental chromosomes, with parental alleles on these partially unlinked by chiasma formation (Carlile *et al.* 2001). *S. cerevisiae*'s sexual cycle further increases efficacy of selection of the best allele combinations by allowing frequent return to the mating-competent state, not by spontaneous LOH but in response to environmental cues (Merlini *et al.* 2013), increasing the likelihood that mating-competent cells will quickly find a compatible partner and reducing the cost of the slower growth of mating competent cells.

A fully functional sexual cycle offers powerful protection against deleterious mutations (Felsenstein 1974). It is thus difficult to conceive why mutations reducing sexual ability swept through the species in the past if such loss was deleterious. Possibly loss of sex was advantageous at the time, as a means of protecting rare allele combinations (Vrijenhoek and Parker 2009) that allowed *C. albicans* to become one of the very few *Candida* species that colonize the human intestinal tract (Skinner and Fletcher 1960; Odds 1988). Bottlenecks and genetic drift among small numbers of initial colonizers of humans could also have contributed to the fixation of mutations that reduced the ability to mate, even if mating was advantageous (Lynch *et al.* 1995). Since then, long periods of predominantly clonal reproduction are likely to have generated clonal lines whose allele combinations are often poorly compatible (Carlile *et al.* 2001); this would also explain why only 15/25 pairwise combination of mating-capable strains yielded fusants in our experiments.

The mutational decay of the sexual cycle in *C. albicans*, while increasing the cost and reducing the benefits of mating for strains that engage in mating, has also effectively eliminated the cost of mating as far as the species as a whole is concerned and this may be pivotal to why mating is under selection. Fitness-decreasing LOH events are frequent in *C. albicans* (Diogo *et al.* 2009; Forche *et al.* 2009, 2011; Hickman *et al.* 2013) and the strains affected are doomed to become extinct unless these defects are corrected, which is achievable quickly only by mating. Thus mating is restricted to those members of the species that otherwise are destined for extinction, offering them a small chance of survival by mating if the LOHs include *MTL*-LOH. Any instance in which mating averts their extinction, or leads to fitness increases

beyond restoration of their initial fitness, constitutes a net benefit of mating to the species.

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Selective Advantages of a Parasexual Cycle for the Yeast *Candida albicans*

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Table S1 Primers

Name	Oligonucleotide Sequence (5' to 3')
M13pr	AATTAACCCTCACTAAAGGGAACAAAAGCTGG
Sacpf1	ACTGGAGCTCATTTTATGATGGAATGAATGGG
*MTL***a**1-F [a]	TTGAAGCGTGAGAGGCTAGGAG
*MTL***a**1-R [a]	GTTTGGGTTCCTTCTTTCTCATTC
MTLα1-F [a]	TTCGAGTACATTCTGGTCGCG
*MTL*α1-R [a]	TGTAAACATCCTCAATTGTACCCGA
ENOpf	GGGATCAAGATTTGTTTACAG
tetRWH11pr	CCATGGTGAGACGCGACAGA
TRpf	CTCGAGCCCGGGTGGACTTCTTCG
TRpr	CTGCAGGTCGACTTTTCTGA G
IMH3pfatg	ATGGTGTTTGAAACTTCAAAAG
IMH3pr	CTCGAGTCTAGAACTCAGTATATCTT CA
TRpIMHp	ATCTCAGAAAAGTCGACCTGCAGATGGTGT TTGAAACTTC AAAAG
TS1pf	GCGGCCGCCCCGGGTCCTTTCTTACTAAAATATAGA
TS1pr	GCGGCCGCGATCTTACACACACAATCAG
TS2pf	AAGCTTAGAAGGAAAGAAGGAAAGAA
TS2pr	AAGCTTCCCGGGCCTTATCATCATGATCACCA
pNZ11pf	CGAATTGGAGCTCATTTTATG
CaNATpr	GGACATGGCATAGACATATAC
pACTFpf	TGCCTCTTACCAACTATTTCA
TS1pr-p	GCGGCCGCGATCTTACACA
TS1Fpf	CACTACTACTACCTACTACTAC
TRsepr	GGTGTGGTCAATAAGAGCGA
TS2Fpr	TACCTATGCACTACTACTACTC
CaACTpr	CTAAAACATACCACCGTCCA
pENOpr	ATCGTTAGTCAACTTTTGCAAC
SSR1-2 [b]	CTAGTTCAGCCAAGGCTTCTTC

Continued on next page

Table S1, continued

Name	Oligonucleotide Sequence (5' to 3')
SSR1-3 [b]	AGAAGAAGCCTTGGCTGAAC
MCEA1outpf2 [c]	TGGGTCATGTGGTATTGGAG
MCEA1outpr2 [c]	ATGGCCGGATGTTTCCAGAA
MC0repf [c]	AACCATCATGACGATCACCA
MC0repr [c]	GATAAATCTCATCTGCAGGC
tetRpf	ATGTCTAGAT TAGATAAAAG TAAA
tetRpr2	AGTCGTCATCAGTACCGGC
TS1se	CTCTCTCCTT TTGTTACGAG
CaNATpf	CTACTACTTTGGATGATACTG

[a] designed by M. Legrand et al. (Legrand *et al.* 2004)

[b] designed by Z. Zhou (Zhou 2010).

[c] designed by N. Zhang et al. (Zhang *et al.* 2009)

Table S2 Parental strains and fusants detected in rat co-colonization model

Mating	Inoculum		Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Fusants Detected [a,b]			
OD8916α x W43**a**			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	2.7E+03	3.0E+02	8.4E+03	0.0E+00	1.4E+04	0.0E+00	4.3E+03	0.0E+00	70	10	0	0
		Rat2	2.2E+03	2.5E+02	1.1E+04	0.0E+00	1.1E+04	5.8E+02	1.7E+03	0.0E+00	80	20	0	0
		Rat3	2.9E+01	1.5E+00	1.5E+04	7.7E+02	1.8E+04	0.0E+00	4.5E+03	0.0E+00	0	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	3.7E+02	3.0E+02	6.6E+02	4.4E+02	3.2E+03	3.2E+03	2.1E+02	4.1E+03	10	10	0	0
		Rat2	3.8E+02	4.6E+02	2.4E+02	2.0E+02	1.8E+03	2.8E+03	5.6E+02	5.1E+03	0	0	0	0
		Rat3	3.0E+02	2.0E+02	6.7E+02	1.0E+03	3.3E+03	4.9E+03	3.4E+02	6.5E+03	30	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque	Rat1	3.7E+02	3.0E+02	6.6E+02	4.4E+02	1.2E+03	2.2E+03	1.2E+02	2.3E+03	129	104	30	0
		Rat2	3.8E+02	4.6E+02	2.4E+02	2.0E+02	5.8E+02	8.7E+02	6.3E+02	5.6E+03	82	0	0	0
		Rat3	3.0E+02	2.0E+02	6.7E+02	1.0E+03	1.2E+03	1.8E+03	1.3E+02	2.5E+03	110	50	0	0

Continued on next page

Table S2, continued

Mating	Inoculum		Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Fusants Detected [a,b]			
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	1.3E+03	8.7E+02	3.7E+03	0.0E+00	1.1E+03	0.0E+00	5.7E+02	0.0E+00	30	30	0	0
		Rat2	2.4E+02	2.0E+02	2.8E+03	3.1E+02	1.0E+03	0.0E+00	3.6E+02	0.0E+00	10	50	10	0
		Rat3	1.1E+03	2.8E+02	4.0E+03	0.0E+00	9.9E+02	0.0E+00	5.0E+02	0.0E+00	30	0	0	0
W17α x W43**a**			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	3.4E+02	2.8E+02	1.5E+03	0.0E+00	5.0E+03	2.7E+02	4.1E+03	2.2E+02	0	0	0	0
		Rat2	0.0E+00	0.0E+00	1.0E+01	0.0E+00	1.3E+03	6.8E+01	4.5E+03	2.4E+02	0	0	0	0
		Rat3	5.8E+02	3.8E+02	5.1E+02	2.7E+01	2.9E+03	0.0E+00	7.8E+03	0.0E+00	40	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque	Rat1	3.4E+02	2.8E+02	1.5E+03	0.0E+00	1.1E+03	5.6E+01	9.5E+02	5.0E+01	227	0	0	0
		Rat2	0.0E+00	0.0E+00	2.0E+02	0.0E+00	8.4E+02	4.4E+01	7.8E+02	4.1E+01	0	0	0	0
		Rat3	6.5E+02	4.3E+02	5.1E+02	2.7E+01	4.0E+03	0.0E+00	1.0E+03	0.0E+00	70	0	0	0

Continued on next page

Table S2, continued

Mating	Inoculum		Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Fusants Detected [a,b]			
YSU63α x W43**a**			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	5.0E+03	1.3E+03	2.9E+03	3.2E+02	1.3E+03	6.9E+01	3.8E+03	0.0E+00	0	0	0	0
		Rat2	8.1E+03	9.0E+02	1.8E+03	0.0E+00	4.0E+03	0.0E+00	3.4E+03	0.0E+00	0	0	0	0
		Rat3	1.2E+03	6.5E+01	1.4E+03	1.6E+02	2.7E+03	0.0E+00	3.9E+03	0.0E+00	0	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	9.1E+02	1.6E+02	6.5E+01	0.0E+00	2.0E+03	1.0E+02	2.3E+03	0.0E+00	0	0	0	0
		Rat2	4.3E+02	1.1E+02	3.2E+01	7.0E+00	7.6E+03	4.0E+02	2.6E+02	0.0E+00	0	0	0	0
		Rat3	1.4E+03	3.5E+02	3.9E+02	0.0E+00	2.7E+03	0.0E+00	2.8E+02	0.0E+00	0	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque	Rat1	3.9E+03	3.2E+03	3.6E+03	2.4E+03	1.3E+03	2.4E+03	2.4E+03	4.5E+04	0	0	0	0
		Rat2	3.2E+03	4.0E+03	4.0E+03	3.3E+03	3.6E+03	5.4E+03	3.2E+04	2.9E+05	0	0	0	0
		Rat3	4.7E+03	3.1E+03	4.3E+02	6.4E+02	3.0E+03	4.4E+03	1.1E+04	2.1E+05	0	0	0	0

Continued on next page

Table S2, continued

Mating	Inoculum		Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Fusants Detected [a,b]			
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque [c]	Rat1	6.1E+02	1.1E+03	7.3E+02	1.1E+03	1.1E+03	4.5E+02	1.2E+03	1.3E+02	0	0	0	0
		Rat2	7.5E+02	1.4E+03	8.8E+02	8.8E+02	8.2E+02	2.1E+02	1.4E+03	7.6E+01	0	0	0	0
		Rat3	4.8E+02	1.1E+03	7.0E+02	8.5E+02	8.4E+02	2.1E+02	9.7E+02	1.1E+02	0	0	0	0
HUN97α x Au90**a**			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	0.0E+00	7.3E+03	0.0E+00	2.1E+04	1.8E+02	3.5E+03	3.6E+02	6.9E+03	0	0	0	0
		Rat2	1.9E+03	1.1E+04	0.0E+00	4.8E+02	3.2E+02	6.1E+03	3.7E+01	7.0E+02	0	0	0	0
		Rat3	1.1E+03	9.8E+03	4.4E+02	8.4E+03	0.0E+00	5.8E+02	0.0E+00	5.8E+02	0	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque	Rat1	3.7E+02	4.5E+02	0.0E+00	2.2E+03	8.4E+01	1.6E+03	4.6E+01	8.8E+02	0	0	0	0
		Rat2	5.9E+02	5.9E+02	0.0E+00	3.2E+03	9.8E+01	1.9E+03	1.9E+01	3.5E+02	0	0	0	0
		Rat3	5.0E+02	7.5E+02	1.6E+02	3.1E+03	0.0E+00	2.2E+03	0.0E+00	4.1E+03	0	0	0	0

Continued on next page

Table S2, continued

Mating	Inoculum		Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Fusants Detected [a,b]			
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque [d]	Rat1	6.4E+02	1.9E+03	5.5E+02	1.3E+03	7.0E+02	8.5E+02	9.0E+02	3.9E+02	0	0	0	0
		Rat2	2.2E+02	6.0E+02	7.3E+02	1.1E+03	3.9E+02	5.8E+02	1.1E+03	3.7E+02	0	0	0	0
		Rat3	3.7E+02	1.0E+03	4.6E+02	1.0E+03	4.2E+02	6.0E+02	8.7E+02	9.6E+01	0	0	0	0
FJ11α x Au90**a**			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	1.3E+03	1.5E+02	1.7E+03	0.0E+00	2.6E+03	0.0E+00	2.6E+03	0.0E+00	0	0	0	0
		Rat2	3.1E+03	3.4E+02	3.3E+04	0.0E+00	2.3E+03	1.2E+02	2.4E+03	0.0E+00	0	0	0	0
		Rat3	2.8E+02	1.5E+01	1.9E+03	1.0E+02	3.1E+03	0.0E+00	2.8E+03	0.0E+00	0	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque	Rat1	2.3E+03	4.0E+02	4.9E+03	0.0E+00	3.3E+03	1.7E+02	9.9E+02	0.0E+00	0	0	0	0
		Rat2	2.5E+03	6.3E+02	2.0E+03	2.2E+02	2.1E+03	1.1E+02	5.1E+03	0.0E+00	0	0	0	0
		Rat3	3.0E+03	7.4E+02	2.7E+03	0.0E+00	1.1E+03	0.0E+00	7.6E+02	0.0E+00	0	0	0	0

Continued on next page

Table S2, continued

Mating	Inoculum		Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Parent 1	Parent 2	Fusants Detected [a,b]			
FJ11α x W43**a**			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	white	Rat1	4.0E+02	4.4E+01	4.4E+02	0.0E+00	9.9E+03	0.0E+00	1.0E+04	0.0E+00	0	0	0	0
		Rat2	2.1E+02	2.3E+01	2.3E+02	0.0E+00	8.8E+03	4.6E+02	1.1E+03	0.0E+00	0	0	0	0
		Rat3	8.3E+01	4.4E+00	8.3E+01	4.4E+00	4.8E+03	0.0E+00	9.5E+03	0.0E+00	0	0	0	0
			t = 7 d		t = 14 d		t = 21 d		t = 28 d		t = 7 d	t = 14 d	t = 21 d	t = 28 d
	opaque	Rat1	1.3E+03	3.3E+02	4.7E+03	0.0E+00	2.2E+03	2.5E+02	1.7E+03	0.0E+00	0	0	0	0
		Rat2	1.5E+03	3.6E+02	3.2E+03	3.5E+02	1.5E+03	8.0E+01	3.1E+03	0.0E+00	0	0	0	0
		Rat3	3.3E+03	8.3E+02	5.4E+03	0.0E+00	3.2E+03	0.0E+00	2.7E+03	0.0E+00	0	0	0	0

[a] No fusants were detected when any of the inocula were plated out at t = 0 d

[b] Identification of fusants was not significantly affected by marker loss. When groups of three rats were inoculated with either an OD8916α x W43**a** or a W17α x W43**a** fusant, and cells were recovered from the rats over a period of four weeks and checked for the presence of *MTL***a**, *MTL* , NAT^{r} or MPA^{r} markers by PCR, only one out of 469 cells tested had lost one marker (MPA^{r})

[c] ratio of YSU63α:W43**a** in initial inoculum was 1:2

[d] ratio of HUN97α:Au90a in initial inoculum was 2:1

Table S3 Effect on growth rate of marking parental *MTL*-homozygotes with resistance cassettes

	Growth rate of strain with resistance cassette relative to that of the unmarked ancestral strain[a], (actual rate in doublings /h)	
strain	YPD	Harsh conditions [b]
Au35**a**	0.988 (1.019)	1.006 (0.551)
Au7**a**	1.001 (1.065)	n/a
Au90**a**	0.969 (0.932)	0.939 (0.601)
RIHO11**a**	0.819 (0.963)	n/a
W43**a**	0.949 (0.904)	1.001 (0.606)
HUN97α	0.999 (1.098)	0.971 (0.587)
OD8916α	0.997 (1.014)	0.994 (0.562)
W17α	0.612 (0.685)	1[c] (0.000)
Ysu63α	1.000 (1.063)	0.964 (0.583)

[a] Calculated by dividing the average growth rates after ≥ 100 generations of serial propagation of strains with resistance cassettes by those of their unmarked *MTL*- homozygous ancestors.

[b] Rates were only determined for those strains that produced progeny that were tested under harsh conditions

[c] NeitherW17α, nor its marked version W17α –pNZ11 grew under the harsh conditions.

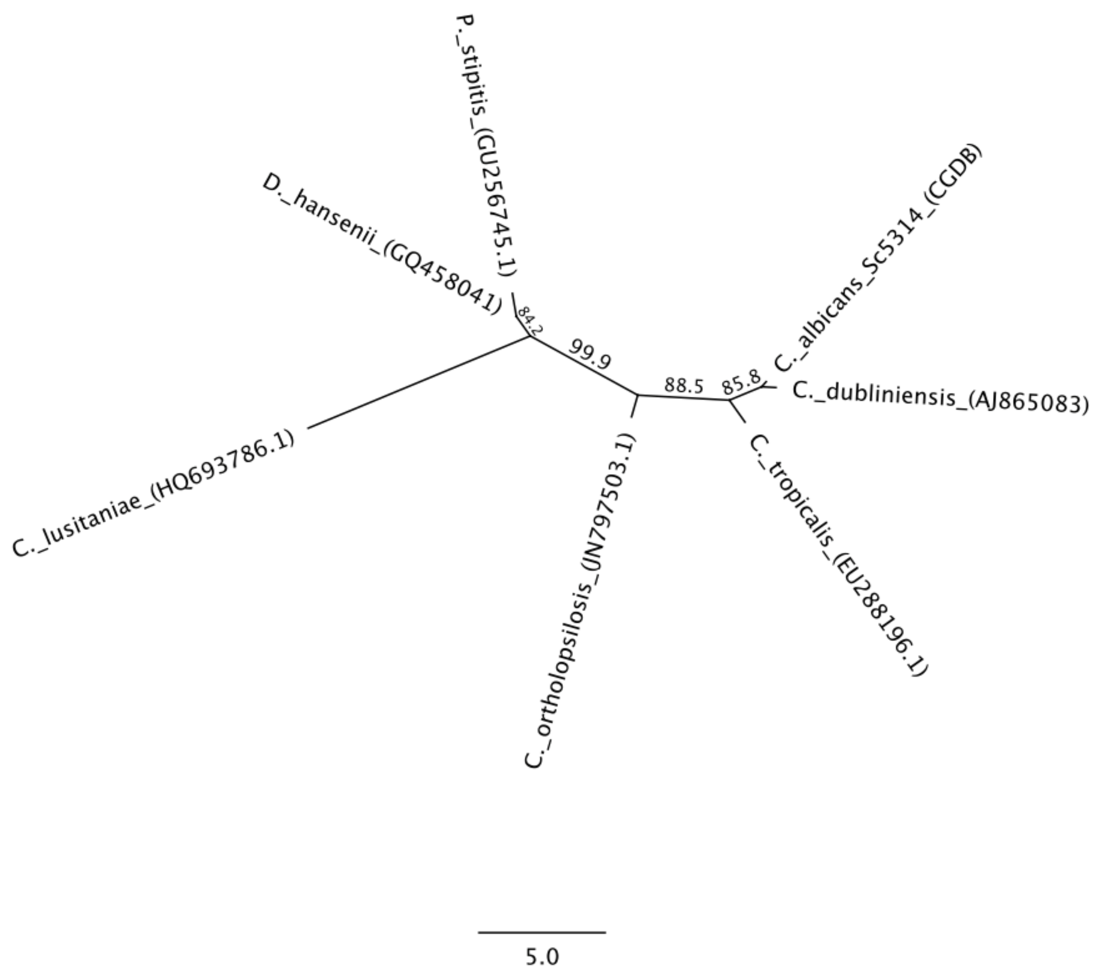

Figure S1 Neighbor-joining consensus tree showing ITS1-based phylogenetic relationships between the species used in d_n/d_s analysis. Percentages of consensus support values of nodes below 100% are shown. The bar indicates 5 substitutions.

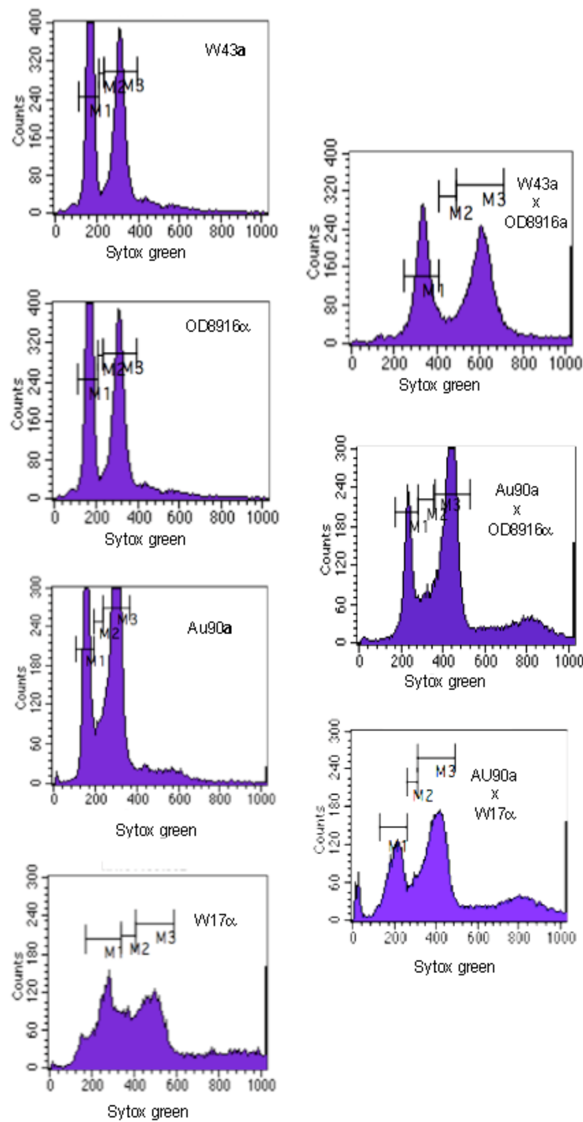

Figure S2 Examples of flow cytometry analyses by SYTOX staining of fusants and parents. Four parents, W43a, OD8916α, AU90a, W17α, and their fusants W43a x OD8916α, AU90a x OD8916α and AU90a x W17α are shown. M1 and M3 are peaks in the DNA content distribution corresponding to exponentially growing cells in their G1 and G2 phases, respectively. M2 corresponds to S phase cells. The peak regions were selected visually based on the shape of the histogram.

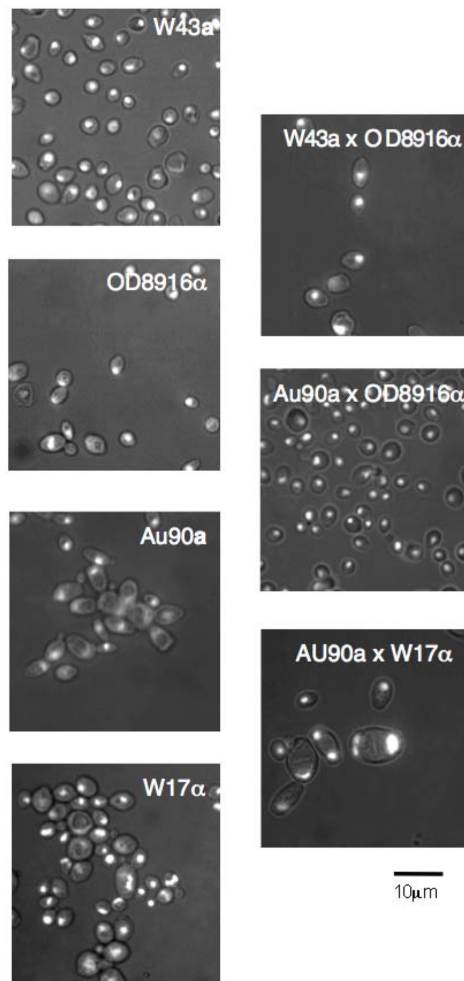

Figure S3 Examples of nuclear staining (DAPI) of parents and fusants. Four parents, W43a, OD8916α, AU90a, W17α, and their fusants W43a x OD8916α, AU90a x OD8916α and AU90a x W17α are shown. The photographic images were generated by combining fluorescent images and bright field images.

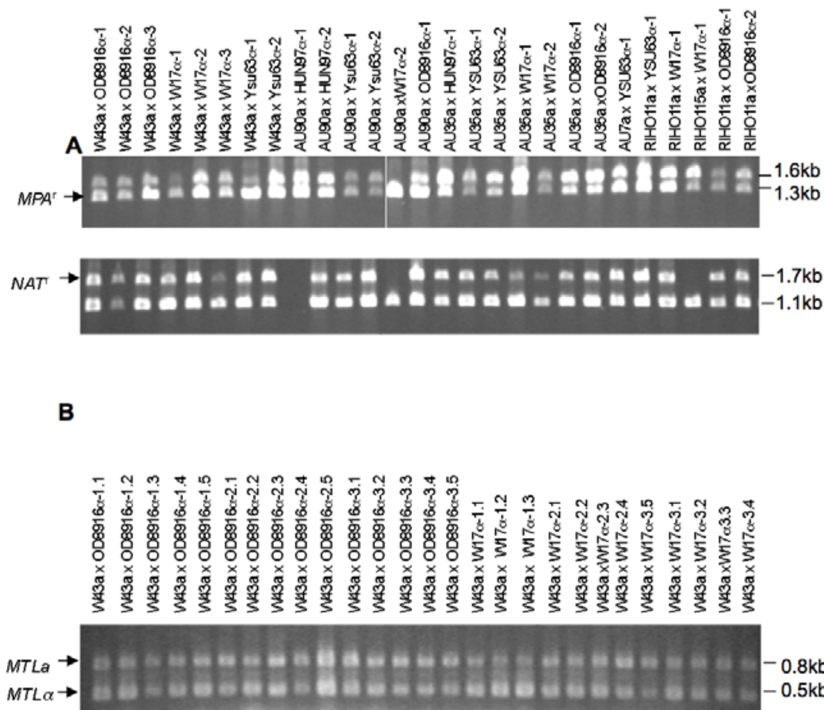

Figure S4 Retention of resistance cassettes and *MTL* alleles in serially propagated fusants. (A) Representative \fusants of each successful mating were serially transferred in YPD medium for 100 generations and the presence of the MPA and NAT resistance markers was investigated in a single colony of each fusant. Three-primer PCRs were used (primers TS2Fpr/pENOpr/TS1se for assays of the *MPA*^{r} cassette and primers pACTFpf/CaACTpr/CaNATpr for assays of the *NAT*^{r} cassettes), to distinguish between the insertion site with a cassette (1.3 kb and 1.7 kb products for the *MPA*^{r} and *NAT*^{r} cassette respectively; marked with arrows), and the insertion site without cassettes (1.6 kb and 1.1 kb, respectively). In 27/27 fusants tested the *MPA*^{r} cassettes were retained and in 24/26 fusants tested the *NAT*^{r} cassettes were still present. (B) In 27/27 fusants tested after serial propagation for 100 generations, both mating type loci (0.8 and 0.5 kb as indicated on the right and marked with arrows on the left), were also still present. The offspring from six fusants of two matings were streaked and 3 to 5 single colonies were tested from each fusant. The names above the figure show the parents, followed by the number of fusants, followed by the number of the colony tested.

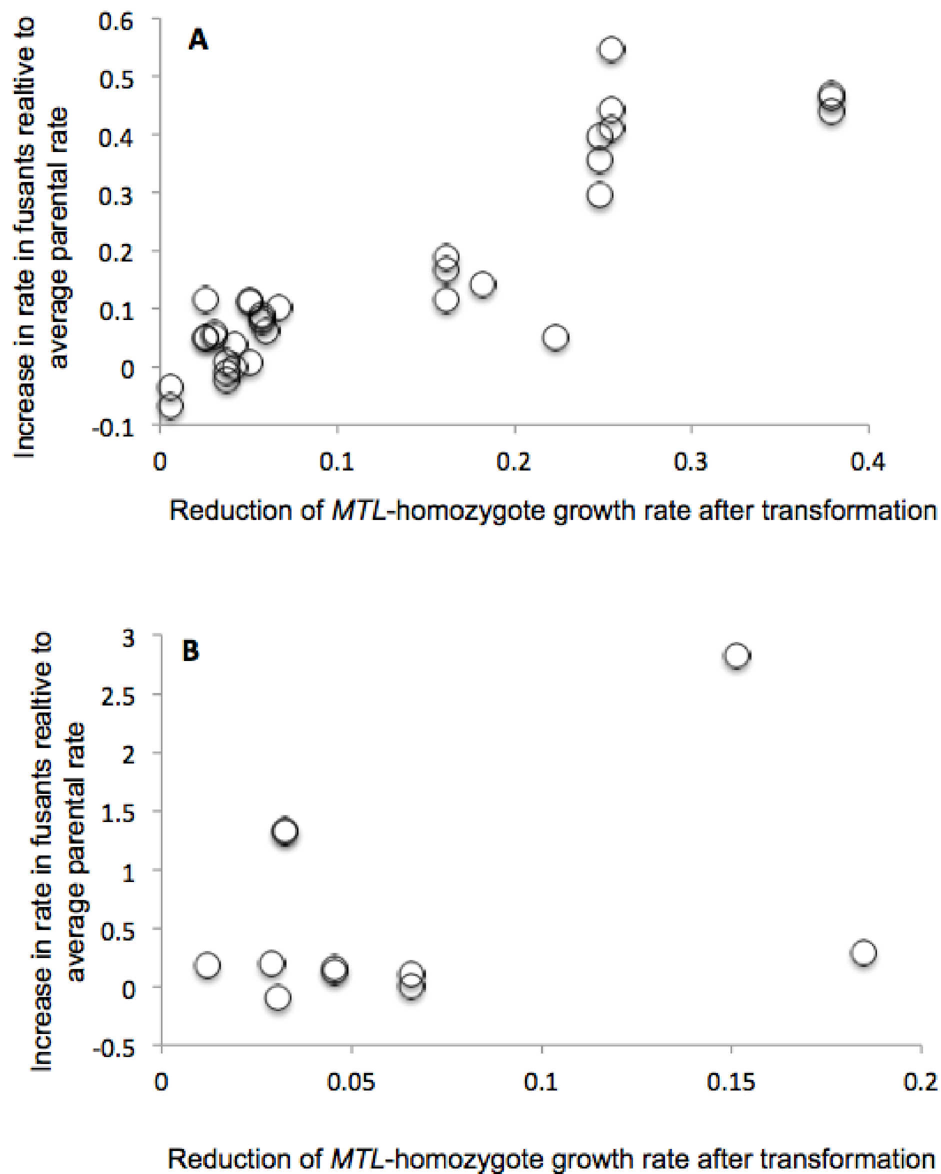

Figure S5 Increase in growth rates of fusants relative to the average rate of their parents (calculated as fusant rate / average parent rate -1) plotted against the reduction of growth rates of *MTL*-homozygotes after transformation with resistance cassettes (the average of the reductions for both parents each calculated as 1-rate of transformed homozygote / rate of original homozygote). Calculations are based on rates determined after ≥ 100 generations of serial propagation. (A) YPD medium at 37ºC; (B) harsh conditions (excluding transformed and untransformed W17α and the fusants it parented, since only the fusants could grow in harsh conditions).

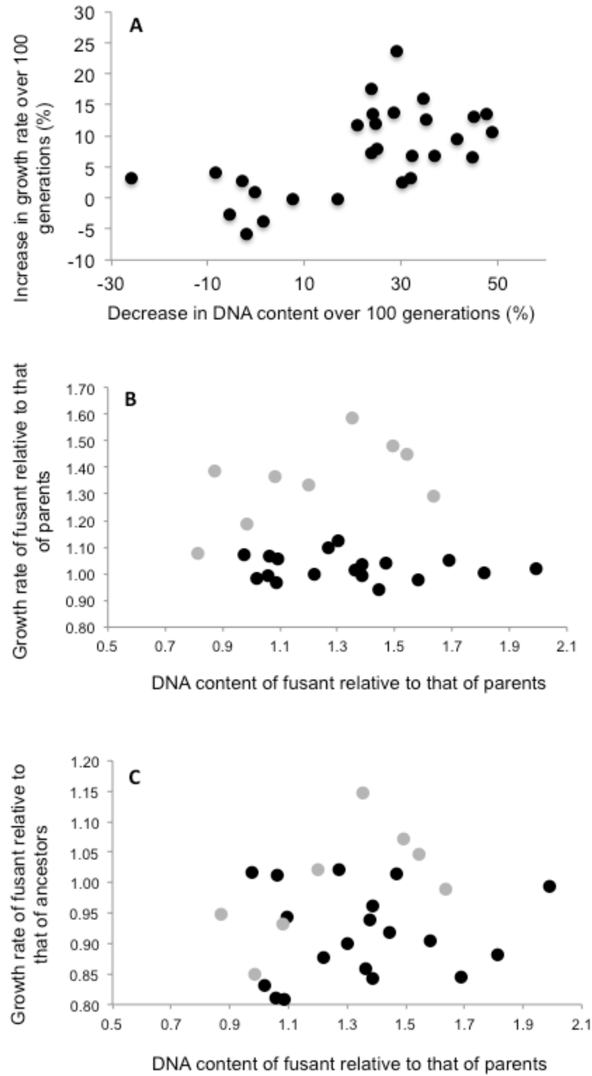

Figure S6 Relationship between reduction in DNA content and increase in growth rate. (A) Fusant growth rate increase over 100 generations of serial propagation versus fusant DNA content decrease over 100 generations of serial propagation, in YPD for 28 representative fusants from all 15 successful matings (names of fusants used are shown in Figure S4). DNA content changes were estimated from flow cytometry. Apparent increases (negative values on x-axis) are most likely attributable to inaccuracy of this method and mainly associated with W17-derived fusants where flow cytometry peaks are ill-defined (Figure S2). (B) Relationship between growth rate and DNA content of fusants, both relative to those of parental averages, after 100 generations of propagation in YPD. Grey symbols represent values for W17-derived fusants. Growth rates of parents prior to propagation in YPD were used in these calculations. (C) Relationship between growth rate, relative to those of *MTL*-heterozygous ancestors, and DNA content of fusants, relative to those of their parents, after 100 generations of propagation in YPD. Growth rates of *MTL*-heterozygous ancestors after propagation in YPD were used in these calculations. Grey symbols in (B) and (C) represent values for W17-derived fusants.

File S1

SUPPORTING METHODS

Estimating the frequency of mutations which abolish mating

The frequency at which mutations can arise which prevent mating (*m*) is the product of several parameters. The first is the mutation frequency per bp (3.3×10^{-10} for point mutations, determined in *S. cerevisiae* (Lynch *et al.* 2008)). The second is the number of genes required for parasexual recombination. The likely number of genes required for mating is between ~25 (genes whose disruption is listed by the *Candida* Genome Database (CGD) as affecting mating) and ~80 (the former plus genes described in CGD as having a role or predicted role in mating) (Arnaud *et al.* 2015). The third and fourth are the average ORF and estimated promoter sizes of these genes which are 1439bp (Braun *et al.* 2005) and ~500 bp, respectively (Kristiansson *et al.* 2009). Lastly the frequency of mutation needs to be multiplied with the probability that they will affect function, which is 0.107 (based on a probabilities of 0.12 for coding regions and 0.07 in promoters, respectively (Doniger *et al.* 2008)).

Based on this, we arrive at an estimate of the frequency with which mutations arise which interfere with *C. albicans*' ability to mate which lies between 1.7×10^{-6} and 5.5×10^{-6} per division.

The accuracy of this estimate is influenced by two factors affecting the rate at which mutations arise that abolish mating ability, which we have not taken into consideration, because suitable estimates are lacking. Fortunately these have opposing effects of comparable size and are likely to largely cancel each other out: Firstly we used in the above calculations probabilities of mutations being deleterious (Doniger *et al.* 2008). To be deleterious, a mutation needs to only reduce functionality, and not necessarily completely destroy it. Thus the probability of a mutation destroying function is less, by an unknown degree, than the figure we used. Secondly ~ 1% of protein-coding *C. albicans* DNA is repetitive (Schmid *et al.* 2012)with a mutation rate of ~10^{-6} (Lynch *et al.* 2008), which would increase the overall probability of a mutation in mating-related genes ~30 fold. However repeat regions mutate by insertion and deletion of repeat units and the extent of impact of insertion or deletion of repeat units is uncertain.

PCR amplifications

Most polymerase chain reactions (PCRs), were performed in a final volume of 20 µl containing 1 U *Taq* DNA polymerase (Qiagen Pty Ltd, Clifton Hill Vic, Australia), 4 µl of Q-buffer and 1x PCR buffer supplied by the manufacturer (Qiagen), 10 pmol of each primer, 200 µM of each dNTP (Roche Diagnostics, Auckland, New

Zealand), and 10–100 ng DNA. For amplification of products used in construction of resistance cassettes KOD polymerase (Novagen) was used instead of Taq polymerase. The cycling conditions varied according to primer sets and the size of the products (Ausubel *et al.* 2014) and included an initial incubation for 2 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 50–60°C, and 30 s to 3 min at 72°C. All PCR protocols included a final 5 min extension step at 72°C. For colony PCR, a portion of a *C. albicans* colony was picked with a 10 µl pipette tip and mixed with 20 µl PCR reaction mixture; the initial step in the cycling program was altered to 5 min at 96°C. Reactions were carried out in an Eppendorf Mastercycler thermocycler (Eppendorf, Hamburg, Germany).

Selection of *MTL*-homozygous derivatives

MTL-homozygous derivatives of clinical isolates were obtained through chr 5 loss by sorbose selection (Rustchenko *et al.* 1994). Approximately 10^6 *C. albicans* cells of each strain were spread on sorbose medium agar plates (2% w/v sorbose, 0.67% w/v yeast nitrogen base w/o amino acids [sterilized by filtration], 1.5% agar) (Rustchenko *et al.* 1994). The plates were incubated at 37°C for one to two weeks. Colonies were checked by multiplex colony PCR for the presence of *MTL***a** and *MTL*α alleles. Primer combinations used were *MTL***a**1-F/*MTL***a**1-R and *MTL*α-1F/*MTL*α1-R (Table S1) (Legrand *et al.* 2004). Colonies lacking one type of *MTL* allele were sub-cultured twice on sorbose plates, tested by PCR once again and then stored in YPD containing 30% glycerol v/v at -80°C.

To obtain spontaneously arising *MTL*-homozygotes, approximately 4-6 x 10^3 *C. albicans* cells were plated at low density (50 to 150 colonies per 85-mm plate) on YPD + phloxine B (5 µg/ml) agar and the plates were incubated at 25°C for 2 weeks, as previously described (Lockhart *et al.* 2002). Red sectors were checked by multiplex colony PCR for the presence of *MTL***a** and *MTL*α alleles. Potential *MTL*-homozygotes were streaked onto YPD plates to obtain single colonies and the colonies were checked again for presence of *MTL* alleles.

Transformation of *MTL*-homozygotes

The most frequently occurring *MTL*-homozygous derivative of a clinical isolate was transformed with a resistance cassette, except for strain W43, where the slightly less frequent derivative was used to balance the *MTL* allele distribution among the derivatives used for mating. The *IMH3*^{r} cassette was removed from plasmid pNZ4 by *Nae*I digestion, gel purified, and used to transform *MTL***a** derivatives, using either the lithium acetate heat shock method (Beckerman *et al.* 2001) or electroporation (De Backer *et al.* 1999). Putative transformants were selected on minimal plates (0.67% w/v yeast nitrogen base without amino acids, 2% w/v glucose, 1.5% w/v agar)

containing MPA (5 μg/ml) and re-streaked on minimal medium with MPA (10 μg/ml) to verify resistance. The presence and correct insertion of the *IMH3*^{r} cassette were tested by colony PCR (Zhang *et al.* 2010) with primer combinations TS1Fpf/TRsepr and TS2Fpr/pENOpr, and with primers TS1Fpf and TS1pr-p to detect tandem integration. Southern hybridization (Ausubel *et al.* 2014) of *EcoR*I digests of transformants' genomic DNA using a 1134 bp fragment amplified from pNZ4 plasmid with primers tetRpf/tetRpr2 as a probe confirmed that insertion had occurred only at the intended locus.

*MTL*α derivatives were transformed with uncut plasmid pNZ11 as described above, except that 100 μl portions of transformation mixture were spread on YPD plates and incubated at 37°C for 7 h, at which time a thin lawn of cells had formed. Putative transformants were selected by replica plating onto YPD plates containing NAT at 200 μg/ml. The presence of a correctly inserted *NAT*^{r} cassette was verified by PCR with primers M13pr/CaACTpr and pACTFpf/CaNATpr. A PCR reaction using primers M13pr and pNZ11pf was used to detect tandem integration. Southern hybridization (Ausubel *et al.* 2014) of *Sal*I digests of transformants' genomic DNA using a 564 bp DNA fragment amplified from pNZ11 using primers CaNATpf/CaNATpr as a probe confirmed that insertion had occurred only at the intended target area.

Serial transfers and growth rate determinations

For serial transfer experiments, cultures were grown in 2ml medium to saturation (for 24 h). Then 10μl were transferred to 2 ml for further propagation.

For growth rate determinations, a pre-culture was made by inoculating 2ml of YPD medium in a 19 mm x 20 cm test tube with one inoculating loop of cells and then placing it in an incubator and shaking it at 150 rpm. After 16 h, 80 μl of culture was used to inoculate 40 ml of medium in a 200 ml Erlenmeyer flask, which was incubated under the same conditions. Growth rates reported are averages of at least two, and usually three, independent experiments (with 8 absorbance measurements each) conducted at different times with different batches of medium. As far as possible, rates that were to be compared with each other were determined in the same experiments. Experiments under harsh conditions were carried out in a similar manner, except that growth rates were determined in 200μl cultures in 96-well plates using a plate reader.

In vivo mating in a rat co-colonization model

All animal husbandry and manipulation was carried out in government-approved facilities and procedures were approved by the University of Otago Animal Ethics Committee (approval 60/2007). Groups of three male Sprague-Dawley rats (~ 200 g in weight) were housed together and fed standard rat chow and water *ad libitium*. Immunosuppressants (doxycycline and dexamethasone) were added to the drinking water of rats one week after admission to the animal facility. Two weeks after admission to the facility, rats were orally inoculated with equal numbers of white- or opaque-phase *C. albicans* cells of the two parental strains that had been grown on YPD agar containing phloxine B (5 µg/ml) at 35°C for 24 h. Cells of each strain were washed and suspended to a concentration of 3.0×10^8 cells/ml in sterile water (confirmed by cell counting with a hemocytometer) and an equal volume of each strain suspension was mixed together to form the inoculum. Rats were inoculated by placing 100 µl of the cell suspension on their tongues. The rats were inoculated three times at 48 h intervals. The rats had their mouths swabbed weekly after initial inoculation to sample yeast present. The tongue of each rat was rubbed with a separate sterile swab moistened in sterile saline. Each swab was then vortex mixed in 1 ml sterile saline and portions (5, 50, or 100 µl) of appropriate dilutions of the oral swab samples were plated on YPD agar containing chloramphenicol and on double selection medium (DSM) consisting of 0.67 % yeast nitrogen base with amino acids, 2 % glucose, 1.5 % bacteriological agar, mycophenolic acid (MPA) 5 µg/ml, nourseothricin (NAT) 100 µg/ml. Large colonies on DSM agar were picked and streaked to single colonies on YPD and individual colonies were tested by PCR to detect *MPA*^{r}, *NAT*^{r}, *MTL***a** and *MTL*a markers. Colonies were considered as fusants if both *MTL***a** and *MTL*a markers could be amplified. The number of colonies on YPD + chloramphenicol agar plates was used to calculate the total number of *C. albicans* cells/swab. Individual colonies from these plates were picked at random and checked by PCR amplification of *MTL***a** or *MTL*a markers to determine the numbers of each parent and present in the swab sample. To determine the rate of marker loss in fusants, groups of three rats were inoculated with either an OD8916α x W43**a** or a W17α x W43**a** fusant. The mouths of rats were swabbed weekly and swabs plated on YPD + chloramphenicol agar plates. At each sampling time up to 20 colonies from each rat were selected at random and were tested by PCR to detect *MPA*^{r}, *NAT*^{r}, *MTL***a** and *MTL*a markers.

File S2

SUPPORTING DATA

The inability of FJ11α to mate is most likely caused by a truncated Mtlα1 protein

We were unable to obtain fusants from any matings that involved strain FJ11α, be it with *MTL*-homozygous derivatives of other clinical isolates or with the universal tester strain 3710 (Magee *et al.* 2002), an MPA-resistant auxotrophic *MTL***a** derivative of laboratory strain SC5314 with which fusants can be selected on minimal plates containing MPA*.* All other nine strains could mate with 3710 or its *MTL*α equivalent 3685 (Magee *et al.* 2002) (data not shown). When we found that the *MTL***a** derivative of FJ11 could mate with 3685, we suspected that a defective Mtlα protein, essential for mating (Tsong *et al.* 2003), might prevent FJ11α from mating. We sequenced the FJ11α *MTL*α locus (Genbank accession number JN099704) and found that it contained a nonsense mutation that led to the truncation of the Mtlα1 protein.

Retention of resistance cassettes in serially propagated fusants

To assess if resistance cassettes carry a fitness cost we assessed their retention in fusants serially propagated for 100 generations in YPD medium containing neither MPA nor NAT. A fitness cost should be apparent as a loss of the cassettes more frequent than expected on the basis of ploidy reduction, as monitored by flow cytometry analysis. For example if a fusant's ploidy had been reduced from 4n to 2n, we would expect on average only one cassette to be lost if they incurred no fitness cost.

We tested how often both resistance markers were still retained in a single randomly-chosen colony from each of 27 fusant cultures (at least one per successful mating) analyzed by flow cytometry after 100 generations. The *MPA*^{r} cassette was present in all of these and the *NAT*^{r} cassette was present in 92%. Figure S4A). The average DNA content reduction of these fusants was 24% (median 21%).

The presence of both resistance markers was also checked in 3-5 individual colonies from each of six fusants (three W43a x OD8916α and three W43a x W17α), that had been transferred for 100 generations on YPD medium, by PCR (primers TS2Fpr/pENOpr/TS1se for assays of the *MPA*^{r} cassette and primers pACTFpf/CaACTpr/CaNATpr for assays of the *NAT*^{r} cassettes). Of 27 colonies analyzed, only one, a W43a x OD8916α fusant, had lost one marker - a NAT resistance cassette (i.e. the frequency of loss was 0% for the MPA resistance cassette and 3.7 % for the NAT resistance cassette; Figure S4B). In contrast, the average DNA content reduction of the six fusants was 36% (median 31%).

Both experiments imply that the resistance cassettes had no negative impact on the fitness of fusants.

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