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 x 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 x10⁻⁶ and 5.5 x 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).
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 10⁶ 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 MTLα and MTLα alleles. Primer combinations used were MTLα-F/MTLα-R and MTLα-1F/MTLα1-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 10⁴ 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 MTLα and MTLα 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 M13′ cassette was removed from plasmid pNZ4 by Nae1 digestion, gel purified, and used to transform MTLα 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 \textit{IMH3}\(^\prime\) cassette were tested by colony PCR (Zhang \textit{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 \textit{et al.} 2014) of \textit{EcoRI} 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.

\textit{MTL\(\alpha\)} 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 \textit{NAT}\(^\prime\) 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 \textit{et al.} 2014) of \textit{SalI} 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 libitum*. 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 x 10^4 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*, *NAT*, *MTLa* and *MTLa* markers. Colonies were considered as fusants if both *MTLa* and *MTLa* 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 *MTLa* or *MTLa* 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 W43a or a W17α x W43a 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*, *NAT*, *MTLa* and *MTLa* markers.