Molecular Microbiology (2005) **55**(5), 1553–1565 doi:10.1111/j.1365-2958.2005.04492.x

Comparative genome hybridization reveals widespread aneuploidy in *Candida albicans* laboratory strains

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Summary

Clinical strains of *Candida albicans* are highly tolerant of aneuploidies and other genome rearrangements. We have used comparative genome hybridization (CGH), in an array format, to analyse the copy number of over 6000 open reading frames (ORFs) in the genomic DNA of *C. albicans* laboratory strains carrying one (CAI-4) to three (BWP17) auxotrophies. We find that during disruption of the *HIS1* locus all genes telomeric to *HIS1* were deleted and telomeric repeats were added to a 9 nt sequence within the transforming DNA. This deletion occurred in ~10% of transformants analysed and was stably maintained through two additional rounds of transformation and counterselection of the transformation marker. In one example, the deletion was repaired, apparently via break-induced replication. Furthermore, all CAI-4 strains tested were trisomic for chromosome 2 although this trisomy appears to be unstable, as it is not detected in strains subsequently derived from CAI-4. Our data indicate CGH arrays can be used to detect monosomies and trisomies, to predict the sites of chromosome breaks, and to identify chromosomal aberrations that have not been detected with other approaches in *C. albicans* strains. Furthermore, they highlight the high level of genome instability in *C. albicans* laboratory strains exposed to the stress of transformation and counterselection on 5-fluoro-orotic acid.

Introduction

Many wild-type fungal species undergo chromosome length polymorphisms and chromosome loss events during growth, often while in contact with their host (reviewed in Zolan, 1995). The human fungal pathogen *C. albicans* is a heterozygous diploid (Whelan *et al.*, 1980; 1981; Whelan and Soll, 1982) that lacks a complete sexual cycle (reviewed in Johnson, 2003). Haploids have not been detected and thus genetic manipulations are generally done using molecular approaches. Clinical isolates of *C. albicans* often have karyotypes that deviate from the standard pattern [detected on contour-clamped homogeneous electrical field (CHEF) gels of whole chromosomes; Magee and Magee, 1987; Rustchenko-Bulgac, 1991; Barton *et al.*, 1994; Magee, 1994a; Magee and Chibana, 2002].

Like a number of other pathogenic fungi, *C. albicans* has a remarkable tolerance for aneuploidy (gain and loss of whole chromosomes or chromosome fragments), which appears to provide a selective advantage under specific stress conditions. For example, one copy of chromosome 5 (Ch5) is often lost in cells forced to grow on sorbose as the sole carbon source (Janbon *et al.*, 1998; 1999). After growth on rich medium, the single Ch5 copy reduplicates so that two completely homozygous chromosomes are present. Similarly, Ch6 trisomy or Ch2 alterations were seen in cells grown on D-arabinose (Rustchenko *et al.*, 1994) and specific karyotype changes have been correlated with colony morphology switching (Suzuki *et al.*, 1989; Rustchenko-Bulgac *et al.*, 1990), growth on 5-fluoro-orotic acid (5-FOA; Chen *et al.*, 2003; Rustchenko, 2003), and growth on fluconazole (Rustchenko *et al.*, 1994; 1997; Marichal *et al.*, 1997; Perepnikhatka *et al.*, 1999; see below). Yet, the specific gene(s) that provide a selective advantage to strains with these aneuploidies have not been identified.

Candida albicans strain BWP17 is a widely used laboratory strain with three auxotrophies (*ura3/ura3 his1/his1 arg4/arg4*). It was derived from SC5314, the sequencing type-strain, by sequential deletion of both copies of *URA3* (yielding strains CAF-2 and CAI-4) (Fonzi and Irwin, 1993), both copies of *HIS1* (yielding strains RM1, RM10, RM100 and RM1000; Alonso-Monge *et al.*, 2003), and both copies of *ARG4* (yielding the final strain BWP17; Wilson *et al.*, 1999) (Table 1). Strains BWP17 and the RM1000 isolate used to construct BWP17 (RM1000#6) exhibited altered mobility of one Ch5 homologue (Ch5b) on CHEF gels, accompanied by loss of heterozygosity at a polymorphic marker distal to the *HIS1* marker on Ch5

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Table 1. Strains and primers used in this work.

Strain[a]	Genotype	Source
SC5314	*URA3/URA3*	Gilllum *et al*. (1984)
CAF2	*URA3/ura3Δ::imm434*	Fonzi and Irwin (1993)
CAI-4	*ura3Δ::imm434/ura3Δ::imm434*	Fonzi and Irwin (1993)[b]
RM1	*ura3Δ::imm434/ura3Δ::imm434 HIS1/his1::URA3*	Alonso-Monge *et al*. (2003)
RM10	*ura3Δ::imm434/ura3Δ::imm434 HIS1/his1Δ*	Alonso-Monge *et al*. (2003)
RM100#13*	*ura3Δ::imm434/ura3Δ::imm434 his1::URA3/his1Δ*	Alonso-Monge *et al*. (2003)
RM100#11, #16, #19, #41, #50, #62, #66, #85	*ura3Δ::imm434/ura3Δ::imm434 his1::URA3/his1Δ*	F. Navarro-Garcia
RM1000#6*	*ura3Δ::imm434/ura3Δ::imm434 his1Δ/his1Δ*	Alonso-Monge *et al*. (2003)
RM1000#2	*ura3Δ::imm434/ura3Δ::imm434 his1Δ/his1Δ*	F. Navarro-Garcia
RM1000#8*, #20*, #28*	*ura3Δ::imm434/ura3Δ::imm434 his1Δ/his1Δ*	F. Navarro-Garcia
BWP17	*ura3Δ::imm434/ura3Δ::imm434 his1Δ/his1Δ arg4Δ/arg4Δ*	Wilson *et al*. (1999)

Primer	Sequence	Relevant information
EST1F1	5′-CCAAGGCGTTGCAATACTAC	Forward primer for *EST1* amplification
EST1R1	5′-CGCCTCAAATATCCCATCGT	Reverse primer for *EST1* amplification
URA3F1	5′-AGACCTATAGTGAGAGAGCA	Forward primer for *URA3* amplification
URA3R1	5′-CAAACAATCCTCTACCAACA	Reverse primer for *URA3* amplification
PRE1F1 (1683-2B4)	5′-TGACGGCACCCTTATTGC	Forward primer anneals just centromere-proximal to *HIS1*[c]
TELR1 (1716)	5′-ACACCAAGAAGTTAGACATCCGTACACCAAGAAGTT	Contains 1.5 telomere repeats[c]
F2 (1682)	5′-CATGGTCTTTACTCCATCACAGGG	Sequencing primer within *hisG*
F3 (1704)	GCACGTTTTCGCCGATAATACCG	Sequencing primer within *hisG*
F4 (1707)	5′-CAGAAAATTAAGTACACATGTTG	Sequencing primer 175 nt 5′ (telomere-proximal) to *HIS1*
F5 (1709)	5′-GTCCCCAAATTGTTACTACTA	Sequencing primer 295 nt 5′ (telomere-proximal) to *hisG*
2340/2493 F	5′-GCTTGGGGGTTCTGATACTT	Forche *et al*. (2004)
2340/2493 R	5′-AGCGACCATTACGCAAGGTA	Forche *et al*. (2004)
1341/2493 F	5′-GTCAGCTTCACCACAGTT	Forche *et al*. (2004)
1341/2493 R	5′-AGTTCCAACTCCAAAGCC	Forche *et al*. (2004)
SNF1 F5	5′-TCCATAACAAAGGTGGGA	Forche *et al*. (2004)
SNF1 R7	5′-GTTACCTCTCTCTCAGGTG	Forche *et al*. (2004)
SNF1 F7	5′-CACCTGAGAGAGAGGTAAC	Forche *et al*. (2004)
SNF1 R9	5′-TAGCACTTTAGCCACAGG	Forche *et al*. (2004)

a. Asterisk indicates strains with both Ch5a and Ch5b.
b. Isolates CAI-4 (F2) and CAI-4 (F3) were provided by B.B. Magee; CAI-4 (J1) was provided by Perez-Martin and Johnson (Perez-Martin *et al*., 1999).
c. Positions of these primers are illustrated in Fig. 3A.

(Forche *et al*., 2004), suggesting that a distal portion of Ch5 was lost from Ch5b in these strains. Several other isolates of RM1000 sibling strains also appear to have two copies of Ch5 with normal length (Ch5a). The mechanism by which Ch5 was shortened, and subsequently lengthened in some strains, is not known.

Recently, trisomy of *C. albicans* Ch1 in some CAI-4 strains was detected using quantitative Southern hybridization to five genes located along Ch1 (Chen *et al*., 2003). Furthermore, when inoculated into mice, 13 out of 20 strains that were originally trisomic for Ch1 gave rise to strains with trisomy of Ch4. Of these 13 strains, nine were no longer trisomic for Ch1 (Chen *et al*., 2003). Thus, when *C. albicans* is propagated in the host, multiple changes in chromosome number can occur with high frequency.

Until now, alterations in *C. albicans* chromosome copy number have been detected using CHEF gels of whole chromosomes and quantitative Southern hybridization. Separating whole chromosomes on CHEF gels detects large genome changes, deletions or insertions, especially in the smaller chromosomes (4–7), which are more readily resolved. It is much more difficult to use CHEF gel analysis to detect karyotypic changes in the larger chromosomes. Quantitative Southern hybridization accurately detects changes in copy number for chromosomes of any size (Chen *et al*., 2003), but is limited by the number of different probes used in the analysis.

In this work we used comparative genome hybridization (CGH) to over 6000 *C. albicans* open reading frames (ORFs), in a microarray format, to analyse the genomic alterations that occurred in BWP17 and in different CAI-4 isolates. We determined that the deletion within Ch5 in BWP17 occurred upon disruption of *HIS1* in strain RM100 and that the deletion is due to loss of one copy of all ORFs distal to *HIS1* on Ch5 and the addition of telomere sequence to 9 nt of telomere-like sequence adjacent to the *HIS1* locus. In addition, our comprehensive analy-

sis of gene copy number in CAI-4 isolates confirmed the trisomy of Ch1 in previously characterized strains but also revealed an unexpected trisomy of chromosome 2 (Ch2) in all CAI-4 isolates analysed. These studies highlight the comprehensive nature of data obtained from CGH arrays, as well as the prevalence of aneuploidies in *C. albicans* laboratory strains, some that arise as a consequence of the molecular manipulations used in strain construction.

Results

Analysis of Ch5b in strain BWP17

Strain BWP17 has one copy of Ch5 (Ch5b) that is shorter than the standard-sized copy (Ch5a) in SC5314 (Forche *et al*., 2004). BWP17 was constructed by two sequential disruptions of the *ARG4* gene in the *ura3/ura3 his1/his1* strain RM1000 (Wilson *et al*., 1999). RM1000 was constructed by two sequential insertions of the Ura-blaster (Fonzi and Irwin, 1993) into the *HIS1* locus (RM1 and RM100), each followed by selection on 5-FOA for loss of uracil prototropy (by recombination between the flanking *hisG* repeats: RM10 and RM1000) (Table 1) (Alonso-Monge *et al*., 2003). To determine when Ch5 was altered during the construction of BWP17, we analysed the karyotypes of the RM series of strains by CHEF gel electrophoresis (Fig. 1A). This analysis clearly shows that Ch5b first appeared in strain RM100, in which the second copy of the *HIS1* gene was disrupted with the Ura-blaster cassette. Furthermore, Ch5b was present in strain RM1000#6, the strain used to construct BWP17. This suggests that the change in chromosome size is relatively stable and was maintained during the two subsequent transformations, and two rounds of selection on 5-FOA, that yielded strain BWP17.

The RM100 strain analysed in Fig. 1A is isolate #13, which was used to construct the subsequent RM1000 series. We asked if Ch5b was present in other RM100 sibling strains by using CHEF analysis (Fig. 1B). Of the nine RM100 isolates tested, only RM100#13 carried the shorter Ch5b. This suggests that the event that caused loss of Ch5 DNA occurred in ~10% of the independent transformants.

We also asked if strain RM100#13 retained the *URA3* gene within the disrupted *HIS1* gene, by performing Southern blot analysis on chromosomes separated by CHEF analysis. As expected, the *URA3* sequence was present on chromosome 5 in strain RM100#13. Furthermore, the *URA3* was present on Ch5b (and not on Ch5a) in RM100#13 (Fig. 1C). Thus, the disruption of *HIS1* accompanied the appearance of the shortened Ch5b.

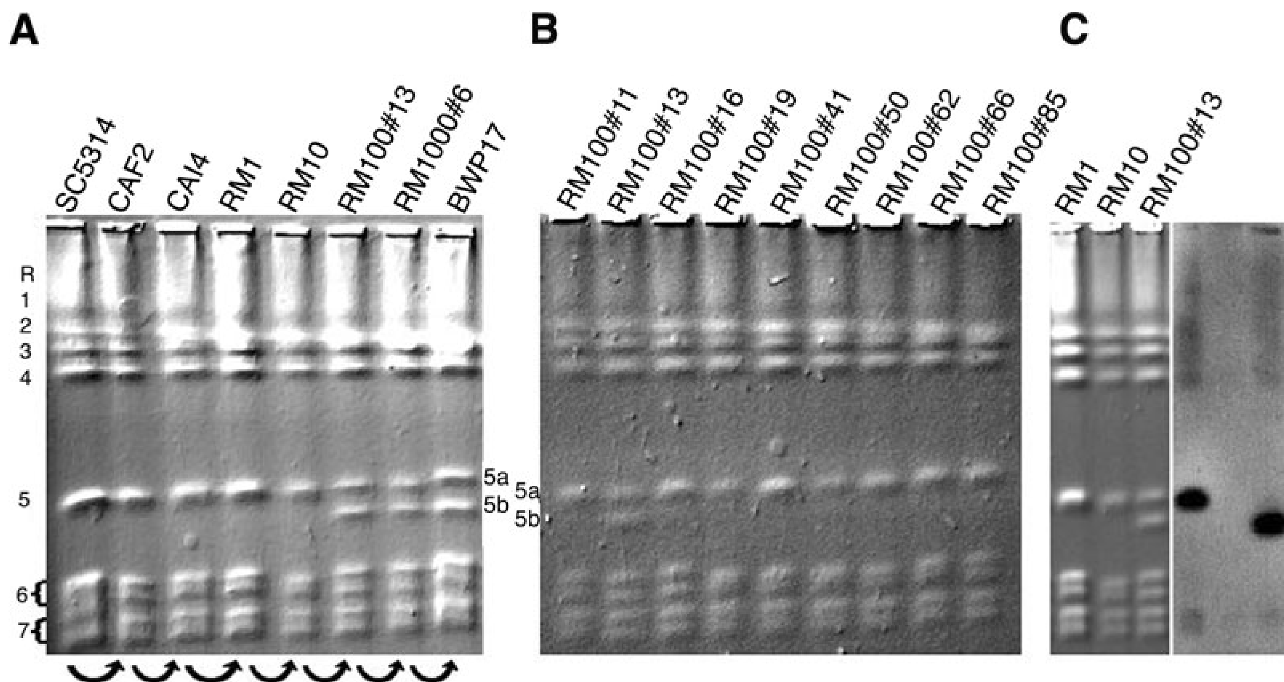


Fig. 1. Contour-clamped homogeneous electrical field (CHEF) gel electrophoresis of whole chromosomes from strains used to construct the common laboratory strain BWP17.
A. Strains derived from SC5314 (Table 1) are presented in order from left to right. First appearance of Ch5b was in strain RM100#13.
B. Sibling RM100 strains, all derived from RM10 by transformation with a Ura-blaster construct to disrupt the second copy of *HIS1.* Ch5b appeared only in strain RM100#13.
C. Southern analysis of CHEF gel using a *URA3* probe. *URA3* is present on Ch5 in strain RM1 and on Ch5b in RM100#13.

To ask about the changes that occurred in strains RM100, RM1000 and BWP17, we next performed CGH to whole genome microarrays, using labelled genomic DNA from each of these strains as the experimental sample (labelled with Cy3) and genomic DNA from SC5314 as the reference control sample (labelled with Cy5). First, we performed several control experiments, where SC5314 was used as both the experimental and the reference control sample. No genes were consistently seen to show a copy number difference (Figs 2 and 5 and *Supplementary material* Figs S1 and S6). The results are plotted on a log2 scale where the baseline (0) indicates no difference between the copy number in the experimental and reference control strains or between the intensity of the Cy3 and Cy5 labelling of the DNA. Based on the assumption that in the control (SC5314) all genes have two copies, the *y*-axis is labelled to reflect the gene copy number of the experimental strain (i.e. a log2 ratio of 0 corresponds to 2 copies, −1 to 1 copy, 0.58–3 copies and 1–4 copies). For mating type locus genes such as *MTLa1* and *MTLalpha2*, which are known to be present at one copy per genome, the copy number in these plots should be divided by two.

Comparative genome hybridization array analysis of the RM series of strains identified several salient features consistent with known aspects of strain construction. As expected, strains RM1 and RM100 had one copy of *URA3* (because of insertion of the Ura-blaster, Table 1) while strains RM10, RM1000 and BWP17 had no *URA3*-hybridizing DNA ($\log_2 < -2.5$) and BWP17 had less than one copy hybridizing to *ARG4* ($\log_2 < -1.2$). This value is not surprising given that the *ARG4* probe on the microarray spans the ~700 N-terminal nucleotides, much of which remained intact after disruption of *ARG4* in BWP17 (Wilson *et al.*, 1999). In addition, all RM series strains had approximately zero copies ($\log_2 < -2.5$) of the *IRO1* gene, which is adjacent to *URA3* and was deleted in the process of generating strains CAI-4 and CAF-2 (Fonzi and Irwin, 1993; Garcia *et al.*, 2001).

As expected, strains RM1 and RM10 had approximately one copy of *HIS1*, and strains RM100, RM1000#6 and BWP17 all exhibited approximately zero copies of *HIS1* (Fig. 2B). Importantly, these latter three strains also exhibited approximately one copy (rather than two copies) of each gene between *HIS1* and the telomere on Ch5 (Fig. 2A and B). This suggests that the insertion of the Ura-blaster into the *HIS1* locus generated a deletion of all genes distal to this site. Consistent with this conclusion, the *EST1* gene, which maps between *HIS1* and the Ch5I telomere (Fig. 2), hybridized only to the full-length Ch5a band in strains RM1000 and BWP17 (data not shown). These data indicate that CGH arrays are sensitive enough to detect the difference between two copies and one copy of

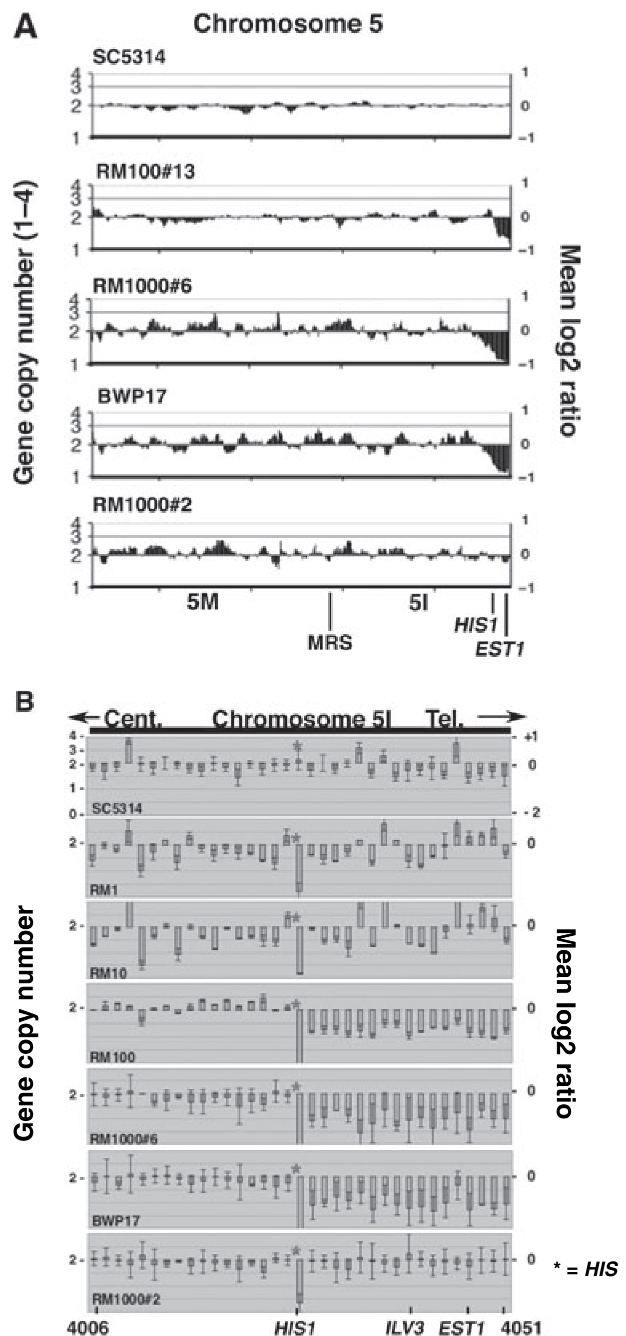


Fig. 2. Chromosome_map display of CGH data on chromosome 5 for strains used to construct BWP17. CGH data were ordered along the chromosome as described in *Experimental procedures*. Data are plotted as log2 values on the right *y*-axis, which corresponds to the chromosome copy numbers labelled on the left *y*-axis.
A. View of whole chromosome 5 using chromosome_map and displayed as a running average with a nine-ORF window. Tick marks designate every 100th predicted ORFs along the chromosome. Note that genes telomere-proximal to *HIS1* are present in approximately one copy in RM100#13, RM1000#6 and BWP17. 5I and 5M refer to the two *Sfi*I fragments that make up Ch5 (Chu *et al.*, 1993). Whole genome chromosomal_map data for these strains are available in *Supplementary material*, Figs S1–S5.
B. Close-up view of genes surrounding the *HIS1* locus (* = *HIS*).

genomic DNA, and imply that a deletion event at *HIS1* gave rise to Ch5b.

To identify the exact position of the chromosome break and to determine the mechanism by which the deletion occurred, we sequenced the terminal fragment from the right arm of Ch5b. Based on the assumption that there must be telomere sequence distal to *HIS1*, and that this telomeric sequence was within a few kb of the *HIS1* gene, we first used polymerase chain reaction (PCR) to amplify (from RM1000#6) a 2.4 kb fragment from *PRE1* (the gene just centromere-proximal to *HIS1*) through the remaining *hisG* repeat. The reverse primer was composed of one and a half copies of the *C. albicans* telomere repeat. The ability to amplify this PCR fragment from strains RM1000#6 and from BWP17 indicates that telomere sequence was added very close to the *HIS1* gene in these strains. Furthermore, absence of a PCR fragment in RM1000#2 suggests that the telomere was too far from the *HIS1* locus to yield a detectable PCR fragment.

The amplified fragment (Fig. 3A) was then sequenced and aligned with the known sequence of *HIS1* and adjacent genes in the *C. albicans* genome [http://www-sequence.stanford.edu/group/candida/index.html (Stanford *C. albicans* sequencing); Jones *et al.*, 2004; Fig. 3B]. A model of the breakpoint is illustrated in Fig. 3C. Telomere sequence was added 319 bp telomere-proximal to the *HIS1* start codon. Interestingly, the sequence at the breakpoint was CTAACTTCT, which is identical to the penultimate 9 nt (Fig. 3B, underlined) of the *C. albicans* telomere repeat sequence GGTGTACGGATGTCTAACT

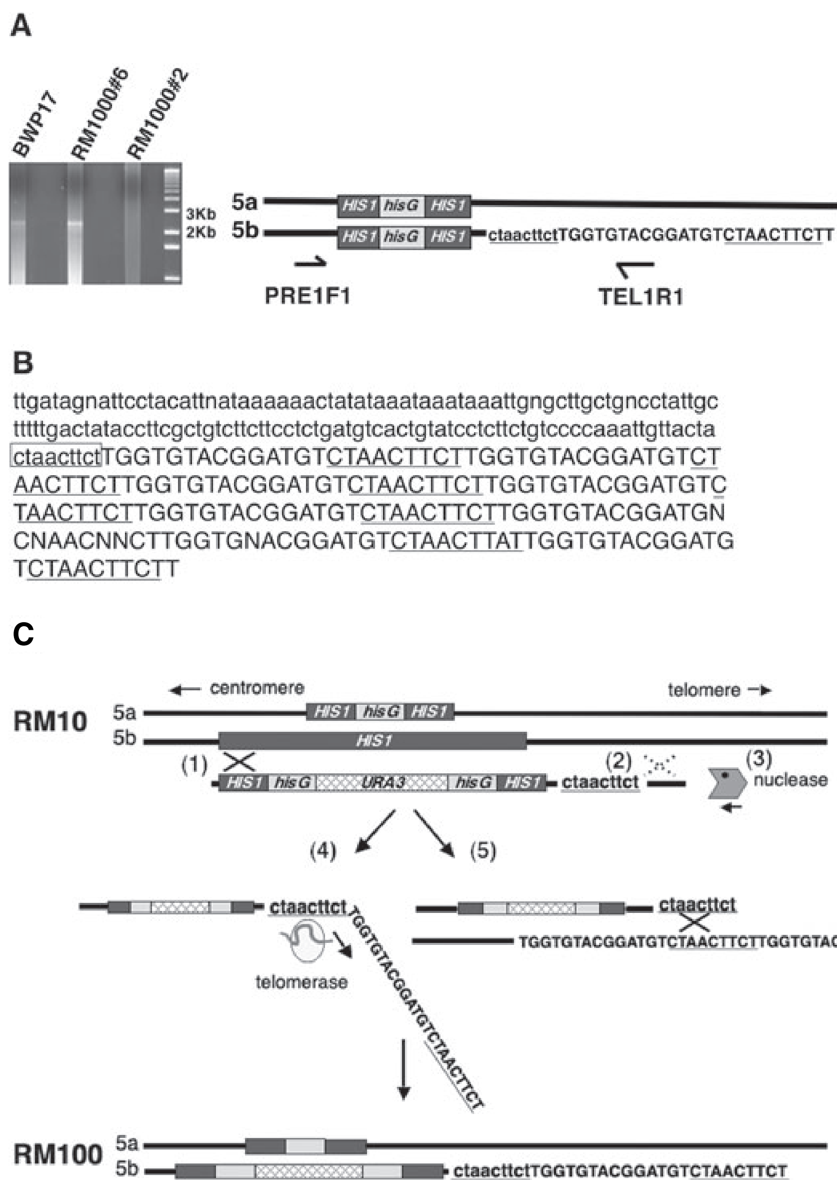


Fig. 3. Telomere sequence was added adjacent to *HIS1* in RM100#13.
A. PCR of strains indicated using forward primer PRE1F1 and reverse primer TELR1 resulted in a 2.4 kb amplified fragment from strain RM1000#6 and from BWP17. This indicates that telomeric sequence was present immediately adjacent to the *HIS1* locus in these strains.
B. Sequence of the telomere-proximal *HIS1* sequence from Ch5b in RM1000#6. Sequence telomere proximal to *HIS1* (lowercase letters) was joined to the 23 bp telomere repeat sequence (uppercase letters) just after a 9 nt sequence present 319 nt 5′ to the *HIS1* start codon (box) that is identical to a portion of the telomere repeat (underlined).
C. Model for the mechanism by which the terminal fragment of Ch5b was deleted. A single cross-over on the centromere-proximal side of the transforming DNA (1) resulted in insertion of the Ura-blaster into the second copy of *HIS1*. Removal of 34 bp at the telomere-proximal side of the transforming DNA, probably by nuclease activity (3), exposed 9 nt within the transforming DNA (ctaacttct) that is identical to the penultimate 9 nt of the *C. albicans* telomere repeat sequence (CTAACTTCT). Alternatively (2) a second cross-over event near the telomere proximal end of the transforming DNA may have failed to resolve, leaving a break near the 9 nt underlined sequence. Additional telomere repeats were added to 5b via a telomerase-mediated event (4) or via recombination with another chromosome end (5). This resulted in the loss of one copy of sequences distal to *HIS1*.

TCTT (McEachern, 1993). Furthermore, this 9 nt sequence was only 34 nt from the telomere-proximal end of the DNA fragment used for transformation (Alonso-Monge *et al.*, 2003). This suggests that the event that yielded Ch5b occurred by a single cross-over between the transforming DNA and Ch5 in the centromere-proximal (3′) region of the *HIS1* gene [Fig. 3C (1)]. This was then followed by loss of 34 bp of the DNA homologous to the 5′ end of *HIS1* either by exonuclease activity [Fig. 3C (3)] or by a failure to resolve a second cross-over in this region [Fig. 3C (2)]. Finally, telomere sequence was added to the 9 nt either by telomerase [Fig. 3C (4)] or by recombination with telomere repeats on another chromosome end [Fig. 3C (5)]. An alternative mechanism, in which two cross-over events, one in the centromere-proximal region and the second between the 9 nt telomere repeat and telomeric sequence on any chromosome end, cannot be ruled out but appears less likely given the short tract of telomere homology. The above results demonstrate that CGH analysis has the sensitivity to predict the position of a chromosomal break that resulted in a segmental aneuploidy.

Analysis of chromosome 5 instability in RM1000 sibling strains

Analysis of other RM1000 sibling strains revealed that most carried Ch5b, although one (RM1000#2) did not (Fig. 4A). One explanation for this finding is that the Ch5b state was unstable in RM100 and only became stabilized after excision of the *URA3* gene. Alternatively, some of these sibling strains may have undergone an additional change in karyotype, because of a non-reciprocal recombination event (Signon *et al.*, 2001) that likely occurred upon excision of the *URA3* gene during selection for the RM1000 series of strains.

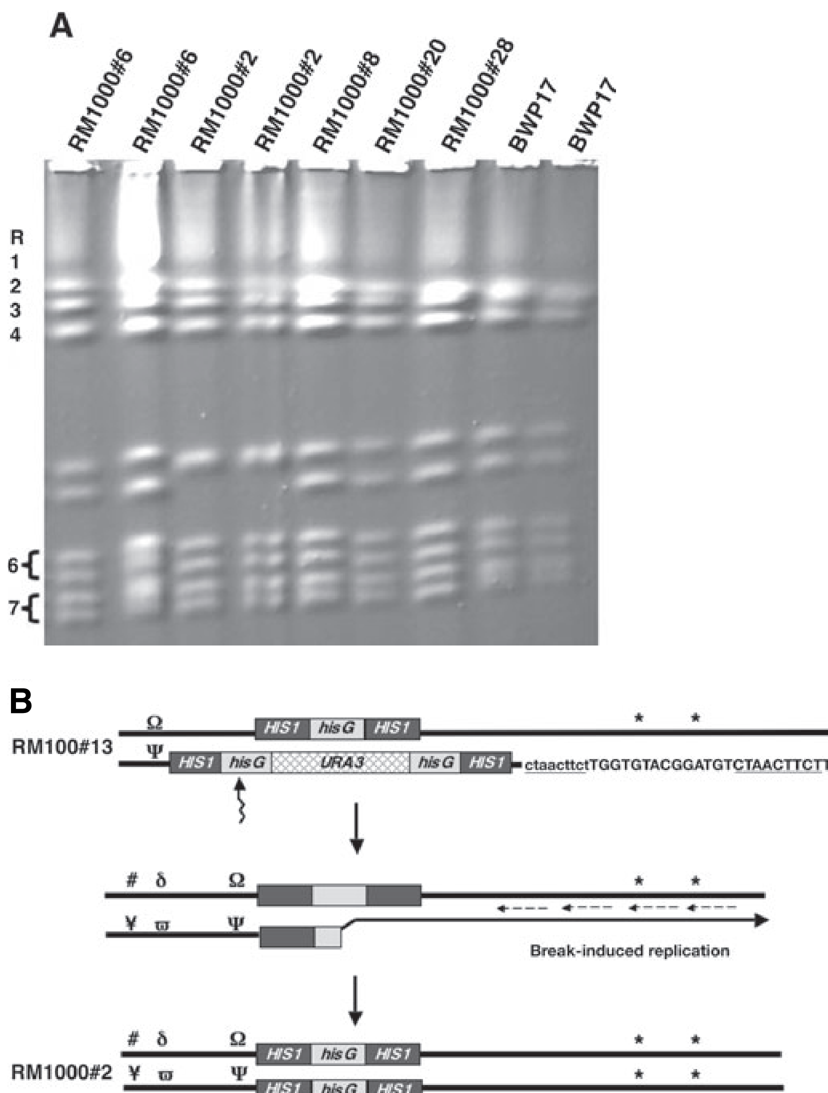


Fig. 4. Contour-clamped homogeneous electrical field electrophoresis of whole chromosomes from RM1000 sibling strains all derived from RM100#13.
A. The karyotypes of strains indicated, including two independent stocks of RM1000#6 and RM1000#2, were determined on CHEF gels. The chromosome 5 truncation (Ch5b) observed in RM100#13 was stably propagated to BWP17 and to all but one (RM1000#2) of the strains.
B. Model for mechanism by which RM1000#2 likely regained a full-size Ch5 homologue. Selection for strains that lost *URA3* was accompanied by a chromosome break centromere-proximal to the *URA3* sequence. This break appears to have been healed by strand invasion of the broken Ch5b into Ch5a followed by replication using Ch5a as the template. Lagging strand replication (dashed arrows) completed the synthesis of the new Ch5a (Kraus *et al.*, 2001). The end result was a second full-length copy of Ch5a that is homozygous for sequences telomere-proximal to the Ura-blaster insertion into *HIS1*, including at the two nucleotides (asterisks) that were heterozygous in the parental strains (Table 2; Forche *et al.*, 2004). Markers to the left of *HIS1*, on both sides of the centromere (#, δ, Ω, ¥, Ψ, ω), remained heterozygous.

Table 2. Analysis of polymorphisms distal to *HIS1* on the right arm of chromosome 5.

SC5314	A**R**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**R**AACGAGTA[a]
CAI-4[b]	A**R**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**R**AACGAGTA[a]
RM1	A**R**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**R**AACGAGTA[a]
RM10	A**R**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**R**AACGAGTA[a]
RM100	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA
RM1000#6[b]	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA
RM1000#2	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA
RM1000#8	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA
RM1000#20	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA
RM1000#28	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA
BWP17[b]	A**A**GCTCTGTAGAGGTGTACTTTCTTTTGGAT**G**AACGAGTA

a. R indicates A or G.
b. Also detected in Forche *et al.* (2004).

To distinguish between these two mechanisms, we analysed several single nucleotide polymorphisms (SNPs) along chromosome 5, on both sides of *HIS1.* Two closely spaced polymorphisms that are distal to *HIS1*, are heterozygous in SC5314 and became homozygous (since they are monosomic) in BWP17 and RM1000#6 (Forche *et al.*, 2004). If the Ch5b state was unstable in RM100#13, then RM1000 strains that maintained two normal-sized copies of Ch5 should have also maintained both alleles of the heterozygous SNPs. In contrast, if the RM1000 strains were all derived from a stable RM100#13 strain by a non-reciprocal recombination event, then both alleles of Ch5 distal to *HIS1* would have been derived from the single Ch5a present in the strain, and the two alleles distal to *HIS1* would be homozygous for the SNPs. Our data suggest that the latter case is true. First, CGH analysis of RM1000#2 confirmed that the genes distal to *HIS1* are present in two copies, indicating that the strain is disomic for all of Ch5 (Fig. 2C). Second, sequence analysis of *HIS1*-distal SNPs in the RM series of strains (Table 2) revealed that the polymorphisms in this region were heterozygous in strains RM1 and RM10 and were homozygous in RM100 (Table 2), and in all of the RM1000 sibling strains. Thus, the generation of Ch5b in RM100 was stable in RM1000#6 and in the subsequent strains that gave rise to BWP17. Finally, we also analysed three SNP markers each containing several polymorphisms, that are present to the left of the *HIS1* gene. All three of these markers remained heterozygous in all of the strains, indicating that the events that restored the right end of chromosome 5 did not affect genes that are not distal to *HIS1.* These data also indicate that the presence of two full-length copies of Ch5 in RM1000#2 occurred upon excision of *URA3*, most likely via a break within the *hisG* repeats that was resolved by a non-reciprocal recombination event, such as break-induced replication (which involves long tracts of gene conversion and often extends from a break to the telomere), that duplicated all of the predicted *HIS1*-distal ORFs to the telomere on Ch5a (Fig. 4B).

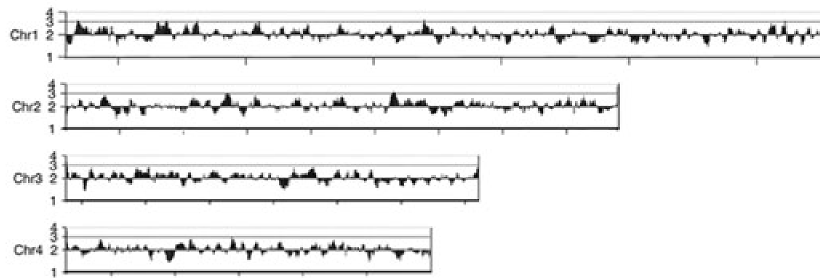
Analysis of trisomy in CAI-4 strains

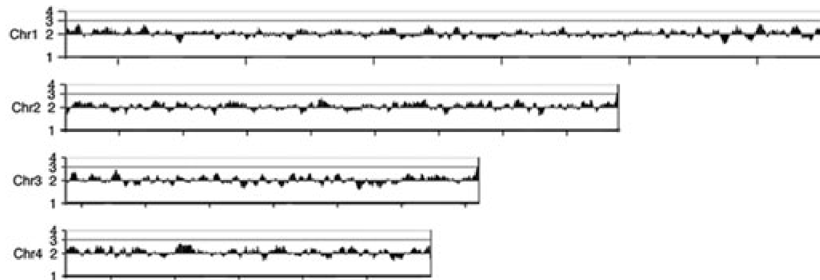
Recent work by Chen *et al.* (2003) used quantitative Southern hybridization to analyse several different isolates of CAI-4, the *ura3/ura3* derivative of SC5314 (Table 1). They found that one isolate was trisomic for Ch1, which explained why disruption of several genes that map to Ch1 appear to be present in three copies (Mio *et al.*, 1997; Wysong *et al.*, 1998; McNeil *et al.*, 2000; Riggle and Kumamoto, 2000; Chen *et al.*, 2003). In contrast, a different isolate [CAI-4 (F2)], was disomic for Ch1. To ask if CGH arrays would be sensitive enough to detect the difference between two and three chromosomal copies, we analysed strains F2 and F3, along with an isolate of CAI-4 (J1), which was provided by A. Johnson and coworkers (Perez-Martin *et al.*, 1999).

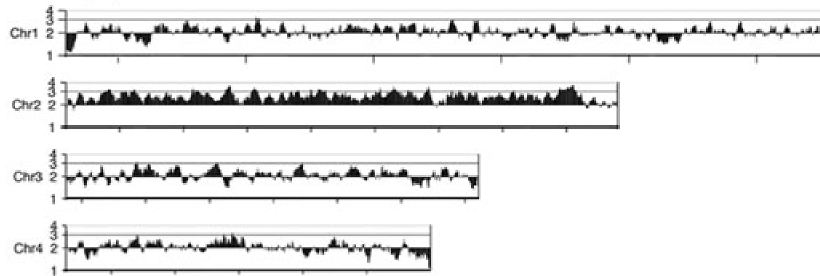
Comparative genome hybridization analysis of the CAI-4 strains is illustrated in Fig. 5. The levels of hybridization are plotted on a log2 scale, where the baseline indicates that levels of hybridization of the experimental DNA (in this case CAI-4 isolates) are similar to levels of hybridization of SC5314 DNA. Two conclusions can be reached from visual analysis of the data. First, as expected, in strain CAI-4 (F2) most of the genes on Ch1 hybridize as approximately two copies. This is consistent with the work of Chen *et al.* (Chen *et al.*, 2003), indicating that this chromosome is disomic in CAI-4 (F2). Furthermore, Ch1 appeared trisomic in strains CAI-4 (F3) and CAI-4 (J1) (Fig. 5). Second, and unexpectedly, in all three of these strains, most of the genes on Ch2 hybridized as approximately three copies (Fig. 5).

Discussion

Genome rearrangements have been described in many fungal species (reviewed in Zolan, 1995). These include large deletions between repeated sequences, chromosome breakage and healing and translocations, as well as the gain or loss of chromosomes. Such events have been best characterized in *Saccharomyces cerevisiae*, where molecular tools and the genome sequence have







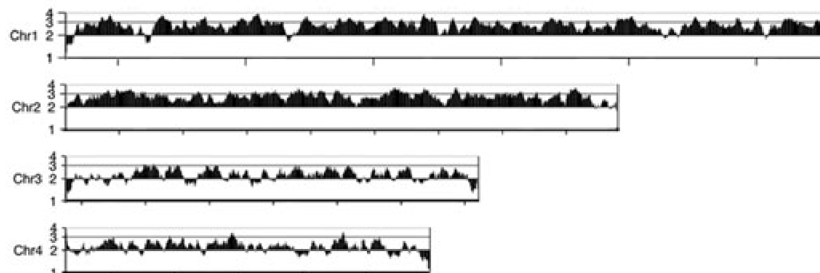


Fig. 5. Chromosome_map display of CGH data on chromosomes 1, 2, 3 and 4 for CAI-4 isolates and parental strains. Data are plotted as described in Fig. 2. CAI-4 (F2) was previously reported to be disomic for chromosome 1 (Chen *et al.*, 2003). CAI-4 (F3) displayed a pattern similar to that of CAI-4 (J1). Whole genome chromosomal_map data for these strains are available in *Supplementary material*, Figs S6–S9.

facilitated detailed studies of chromosomal changes. For example, during the production of the complete set of gene deletion strains in diploid parental strains, 8% of the strains tested underwent an aneuploidy that provided a selective advantage to the strain (Hughes *et al.*, 2000). In many cases these aneuploidies arose from recombination between repeated sequences such as transposons. Similarly, growth in limited glucose conditions selected for strains with aneuploidies that often shared common breakpoints (Dunham *et al.*, 2002). Other fungi also exhibit high levels of chromosome instability, during growth *in vitro* as well as when propagated in the host.

For example, serial transfer of *Magnaporthe grisea* strains in culture or through a rice cultivar resulted in altered karyotypes (Skinner *et al.*, 1993) and heat shock treatment of *Ustilago hordei* resulted in the deletion of 50 kb near the terminus of a 940 kb chromosome. In *Neurospora crassa*, isolates escaping from *het-c* heterokaryon incompatibility were found to have undergone large deletions of up to 31 kb, including up to 11 ORFs (Xiang and Glass, 2004).

Whole chromosome aneupoidy (2N-1) is seen for a specific subset of *S. cerevisiae* chromosomes (generally chromosomes I, V and VII) but has not been observed in *U. horedi*, presumably because none of the chromosomes are dispensable. In other fungi such as *Cochliobolis heterostrophus* and *Colletotrichum gloeosporioides*, dispensable supernumerary chromosomes have been seen as well (Tzeng *et al.*, 1992; Masel *et al.*, 1993). While we observed aneuploidies primarily for *C. albicans* chromosomes 1, 2 and 5, the number of events analysed here is too small to allow us to conclude whether such events will occur on every *C. albicans* chromosome.

Comparative genome hybridization arrays produce data that are as comprehensive as the arrays used. In our case, CGH arrays contained 14 688 total spots, representing 6175 ORFs, in which more than 93% were detected in any given CGH experiment. The approach also can detect the complete deletion of single genes (e.g. *HIS1* or *URA3*), although the noise in the system makes it difficult to discover individual deleted or duplicated genes without many replicate experiments. Nonetheless, visualization of the data across the chromosomes readily identifies large-scale aneuploidies, such as monosomies, trisomies and segmental aneuploidies, despite the presence of some noise in the data. Use of a running average for the data as it is presented across the chromosomes (Dunham *et al.*, 2002) assists in visualization of chromosomal regions that have undergone aneuploidies. Furthermore, CGH arrays reveal the specific breakpoint position(s) (within the interval between one and two ORFs) of segmental aneuploidies. In our study, all genes telomeric to *HIS1* appeared to be monosomic and this copy number was confirmed by Southern analysis with *EST1* (A.S., data not shown), as well as with the *ILV3* gene (unpubl. data in Forche *et al.*, 2004).

Comparative genome hybridization arrays can identify aneuploidies on short as well as on long chromosomes, providing a much more complete view of genome organization than has been possible with previous methods. For example, the CAI-4 strain has been analysed by pulsed field gel electrophoresis (PFGE) of whole chromosomes or *Sfi*1 digested chromosome fragments (unpubl. data in Chen *et al.*, 2003), and SNP analysis (Forche *et al.*, 2004) as well as by quantitative Southern hybridization (Chen *et al.*, 2003). PFGE readily detects whole chromosome changes in the smaller chromosomes (Ch7, 6 and 5) but has difficulties with resolution of the larger chromosomes, such as Ch1 and Ch2. Quantitative Southern blots, which require multiple replicates, can determine chromosome copy number (Chen *et al.*, 2003), but the method is limited by the number of probes used and the need to perform a large number of replicate experiments to get significant differences in the copy number measurements. Since CGH arrays measure virtually all of the ORFs in the genome, the amount of data available is sufficient to reliably detect changes in the copy number of relatively small regions across a chromosome.

Our data support the idea that *C. albicans* laboratory strains exhibit aneuploidies, including loss of chromosome segments and gain of an extra chromosomal copy. Some of these changes clearly accompany (or are a result of) molecular manipulations (e.g. disruption of *HIS1* in RM100), although they occur in a minority of the transformants. The loss of one copy of Ch5 upon growth on sorbose is postulated to provide a selective advantage, because of the presence of a repressor of sorbose utilization on Ch5 (Janbon *et al.*, 1998). However, the gene encoding this repressor has not been identified. Other changes may be due to selection on 5-FOA for excision of the Ura-blaster marker (Chen *et al.*, 2003; Rustchenko, 2003). Yet others, such as trisomy of chromosomes 1 and 2, do not have an immediately obvious cause.

The genomic changes in these strains are not always stable: RM1000#2 exhibits two apparently full-length copies of Ch5 on CHEF gels (Fig. 4A) and on CGH arrays (Fig. 2C), yet is homozygous for the polymorphic marker distal to *HIS1*. This suggests that RM1000#2 was derived from RM100#13 by gene conversion in which break-induced replication duplicated the missing information from the Ch5a information onto the Ch5b copy (Fig. 4B). In contrast, the deletion on Ch5b is relatively stable: the series of strains from RM100#13 to BWP17 retained the deletion. Similarly, other *C. albicans* strains have been shown to have stable karyotypes when they are propagated in rich medium *in vitro* (Rustchenko-Bulgac *et al.*, 1990; Chen *et al.*, 2003). Interestingly, none of the RM series of strains displayed trisomy of Ch1 (data not shown). Either they were derived from a CAI-4 strain that was not trisomic for Ch1, or they were derived from a Ch1 trisomic strain, but the Ch1 trisomy was lost during construction of RM1 and did not re-occur in any of the subsequent strains in the series. Similarly, Ch2 trisomy was not seen in the RM100, RM1000 or BWP17 strain series (*Supplementary material* Figs S1–S4).

Karyotype changes have been observed in *C. albicans* clinical isolates using CHEF analysis (Magee and Magee, 1987; Snell *et al.*, 1987; Lasker *et al.*, 1989; Rustchenko-Bulgac, 1991) as well as DNA fingerprinting (Schmid *et al.*, 1990; Lockhart *et al.*, 1995; 1996) and multilocus

sequence tag assays (Bougnoux *et al.*, 2002; 2003). Some of these changes may be due to changes in the length of the multiple repeat sequence (MRS) repeat or due to recombination events between MRS repeats on different chromosomes (Chibana *et al.*, 1998; Pujol *et al.*, 1999). Other karyotypic changes could be due to aneuploidies. It will be interesting to use CGH arrays with clinical strains to determine if genome changes that occurred in *C. albicans* clinical strains *in vivo* are similar to, or differ from, changes that occurred in *C. albicans* laboratory strains propagated *in vitro*.

Experimental procedures

Strains and growth conditions

Strains used in this study are listed in Table 1. Strains of the RM series and their exact pedigrees were kindly provided by Federico Navarro-Garcia. The RM1 and RM10 isolates we used are consecutive parents of RM100#13, which was used to generate the RM1000 series. RM1000#6 was used to generate BWP17. All strains were grown in Yeast Peptone Dextrose medium (Rose *et al.*, 1990) containing 40 mg l^{-1} adenine (YPAD) at 30°C.

Contour-clamped homogeneous electric field (CHEF) electrophoresis

Karyotypic analysis of *C. albicans* strains (Magee, 1994a,b) was carried out with a CHEF DRIII (Bio-Rad) apparatus using gels prepared from 1% Megabase agarose (Bio-Rad) in 0.5× TBE. DNA was prepared in agarose plugs (Iwaguchi and Magee, 1994). Whole chromosomes were separated using the following conditions: 60–120 min switch, 6 V cm^{-1}, 120° angle, for 36 h, followed by 120–300 min switch at 4.5 V cm^{-1}, 120° angle for 12 h.

Southern hybridization

DNA was transferred from CHEF gels to Magnacharge nylon membranes (Osmonics) (Sambrook and Russell, 2001). Membranes were probed overnight at 42°C and detected with anti-Digoxigenin-Alkaline Phosphatase and CSPD essentially as described (Smith and Summers, 1980). Probe preparation was done by PCR using DIG-labelled trinucleotides according to manufacturer's instructions (Roche). The DIG-*EST1* probe was prepared using forward primer EST1F1 and reverse primer EST1R1. The DIG-URA3 probe was prepared using forward primer URA3F1 and reverse primer URA3R1. Primer sequences are provided in Table 1.

Comparative genome hybridization arrays

DNA was prepared from *C. albicans* strains grown overnight to saturation in 5 ml YPAD medium using phenol/chloroform essentially as described (Hoffman and Winston, 1987). A total of 3 µg of DNA was digested with *Hae*III (Invitrogen) at 37°C for 1–2 h to give an average fragment size of ~1 kbp. DNA was purified using a Mini-Elute PCR purification kit (Qiagen) according to manufacturer's directions except that columns were washed twice with PE and eluted twice with 10 µl of EB. DNA was then mixed with 20 µl of 2.5× Random Primers Solution (Invitrogen), denatured for 5 min at 95°C, and allowed to reanneal for 5 min on ice. DNA was then labelled at 37°C in a reaction containing the following: 1 mM Cy3-dUTP or Cy5-dUTP (Amersham Biosciences), 1.2 mM dATP, 1.2 mM dCTP, 1.2 mM dGTP and 0.9 mM dTTP, 40 units Exnuclease-free Klenow DNA polymeraseI (Invitrogen), 10 mM Tris 8.0, and 1 mM EDTA. Then 500 µl of dH_2O was added to the labelled DNA and the reaction was filtered using a Microcon YM-30 centrifuge filter device at 12 000 r.p.m. for 10 min. This filter procedure was repeated once and the eluted DNA from both the experimental (Cy3) and control (Cy5) elutions was pooled. Eighty microlitre of DIG Easy Hyb solution (Roche) was added, DNA was filtered again using an Ultrafree-MC (Millipore Amicon), and the reaction was heated to 85°C for 5 min. DNA was hybridized to microarrays containing 14 688 total spots, representing 6175 ORFs, designed using assembly six *C. albicans* ORFs as described previously (Bensen *et al.*, 2004). Arrays were scanned (ScanArray 5000) using QuantArray v.2.01 software (GSI Lumonics, Watertown, MA). Data were analysed using GenePixPro 5.1 and GeneTraffic 3.1. The mean log2 ratio average of two duplicate spots per microarray slide and the average of 3–4 different microarrays (4–8 different spots) were analysed. A minimum of three arrays per strain were analysed. In most cases, four arrays per strain were analysed. Data were highly consistent between arrays.

Chromosomal_map software

To visualize CGH array data in its chromosomal context, the physical chromosomal map was ordered based on Assembly 19 contigs [Forche *et al.*, 2004; http://candida.bri.nrc.ca/candida/contigs/index.html (Canadian chromosome to contig hybridization site); http://www-sequence.stanford.edu:8080/haploid19.html (Assembly 19 supercontigs)] and *C. albicans* sequence information (http://www-sequence.stanford.edu:8080/haploid19.html). When contig orientation was not known, we arbitrarily used the 5′ toward 3′ direction of the contig to order the genes. A MatLab script was written to re-order the CGH array data, giving each ORF an arbitrary gene number (plotted on the *x*-axis) corresponding to its order along the chromosomes. Only genes present on contigs with known map locations were plotted. Relative hybridization levels were plotted as a running average over nine ORFs (Dunham *et al.*, 2002) and clipped to the range corresponding to 1–4 copies (*y*-axis, log2 ratios between −1 and +1). In Fig. 2, experimental strain data were normalized by subtraction of the average log2 ratio of six control (SC5314/SC5314) experiments from each experimental dataset.

Chromosome 5 sequencing

Polymerase chain reaction was used to amplify genomic sequence flanking the HIS1 gene in strains RM1000#6 and RM1000#2. Forward primer PRE1F1 hybridizes to *PRE1* which is the centromere-proximal gene adjacent to *HIS1*.

TELR1 primer contains one and a half *C. albicans* telomere repeats. The 2.4 kb genomic fragment obtained from strain RM1000#6 was sequenced, using forward primers F1, F2 or F3.

Determination of chromosome 5 sequence heterozygosity/homozygosity

Forward and reverse primers 2340/2493 (Forche *et al*., 2004) were used to amplify a 160 bp fragment within chromosomal *Sfi*I fragment 5I and distal to *HIS1*, which was shown previously to contain two single nucleotide polymorphisms (A/G) exactly 30 nt apart in SC5314 (Forche *et al*., 2004). The resulting fragments were sequenced using primer 2340. Similarly, three other SNP markers were used to assess heterozygosity along chromosome 5 in strains RM1000#2 and RM1000#6. Two primer pairs within *Sfi*I fragment 5M (SNF1 F5/R7 and SNF1 F7/R9) and one primer pair within *Sfi*I fragment 5I that is centromere-proximal to *HIS1* (1341/2493) were used for amplification and sequencing.

Acknowledgements

We thank Frederico Navarro-Garcia for providing the RM strain series and helpful discussions and B.B. Magee and A. Johnson (Rustchenko-Bulgac *et al*., 1990) for providing CAI-4 strains. We thank Maryam Gerami-Nejad for expert assistance with PCR and sequencing of Ch5b, Anja Forche for advice with the polymorphism analysis and help with determining map positions of contigs and genes, and Justin Fendos for technical assistance with CHEF gel analyses. We are grateful to the Magee lab for providing information prior to publication and to B. Magee and A. Forche for critical reading of the manuscript. We thank members of the Berman laboratory, in particular Eric Bensen, Michelle Desruisseaux and Mark McClellan, for help with microarray production. *C. albicans* microarray production was supported by a supplemental award from NIDCR to Mira Edgerton. This work was also supported by funds from a Burroughs Wellcome Senior Scholar Award and from the Minnesota Medical Foundation to J.B.

Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4492/mmi4492sm.htm

Figs S1.–S9. Whole genome chromosome_map display of CGH data for *C. albicans* laboratory strains ultimately used to construct strain BWP17. CGH data were ordered along the chromosome as described in *Experimental procedures*. Data are plotted as log2 values on the right *y*-axis, which corresponds to the chromosome copy numbers labelled on the left *y*-axis.

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