Towards understanding the evolution of the human commensal yeast *Candida albicans*

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Allelic frequencies and relationships for one dimorphic locus and three unlinked polymorphic loci have been determined for 114 unrelated isolates of *Candida albicans*, including 14 laboratory reference strains and 50 strains from each of two geographic regions. Although there was no indication of geographical partitioning, there were significant correlations for specific allelic pairs among loci and little evidence that any alleles were in Hardy-Weinberg equilibrium. This gives additional support for the concept that the primary mode of genetic inheritance in this species is clonal, with other intracellular genetic events playing a lesser role in the creation of genomic diversity. Through inference of this and other known attributes of closely related *Candida* species, such as sequence analysis of IS1 and the ITS2 (internal transcribed spacer 2) region of the rDNA cistron, the deduced phylogeny suggests an evolutionarily recent origin for many frequently isolated strains. This finding will be of interest in the context of understanding pathogenicity and drug resistance in this human commensal yeast.

Keywords: *Candida albicans*, genetics, phylogeny, microsatellite, VNTR

INTRODUCTION

Candida albicans is a ubiquitous component of the normal human microflora and a frequent cause of superficial epithelial yeast infections. In immunocompromised individuals, however, this species is capable of invading host tissues, resulting in severe disseminated disease. *C. albicans* is a leading primary agent in nosocomial mycotic infections, the rate of which has been increasing during the past decade (Beck-Sague *et al.*, 1993). Further, there is evidence for emerging resistance to widely used antifungal (azole) compounds (Johnson *et al.*, 1995), which has led to a renewed interest in this organism's epidemiology and to the understanding of genetic diversity within the species.

C. albicans lacks a known sexual cycle and is diploid, containing eight chromosomes (Chu *et al.*, 1993). Population-structure studies have suggested a primarily clonal mode of reproduction (Pujol *et al.*, 1993), although some evidence for sexual recombination has been presented (Graser *et al.*, 1996) and the degree of

allelic disequilibrium needed to rule out meiosis has been debated (Tibayrenc, 1997; Vilgalys *et al.*, 1997). Moreover, relatively high rates of intracellular (mitotic) recombination, including chromosomal translocation, are known to occur (Thrash-Bingham & Gorman, 1992). This is reflected in the high degree of interstrain variation observed for all eight chromosomes, as revealed by electrophoretic karyotyping (Rustchenko-Bulgac, 1991; Iwaguchi *et al.*, 1990) and Southern blotting with cloned repetitive elements (Scherer & Stevens, 1988; Lockhart *et al.*, 1995). These have been used as typing schemes for a number of epidemiological investigations (Merz *et al.*, 1988; Diaz-Guerra *et al.*, 1997; Pfaller *et al.*, 1994).

To address the question of strain relatedness, approaches have focused on the analysis of allelic variation at multiple loci. This was first observed as phenotypic variation for growth with various carbon and nitrogen sources, tolerance to low pH, and other phenotypic characteristics (Odds *et al.*, 1983). More recent techniques include multilocus enzyme electrophoresis (MLEE; Caugant & Sandven, 1993), random amplified polymorphic DNA (RAPD; Lehmann *et al.*, 1992), ribosomal and mitochondrial polymorphisms

Abbreviation: VNTR, variable number of tandem repeats.

(REA; Magee *et al.*, 1987) and single-stranded conformational polymorphisms (SSCP; Graser *et al.*, 1996). Although relatively few studies have directly compared these approaches, there appears to be fairly good agreement between them, and two to three subgroups within the species *C. albicans* have been identified (Clemons *et al.*, 1997; Pujol *et al.*, 1997).

With the advent of additional DNA sequence information, other loci have been identified that contain multiple alleles within the population. These include, but are not limited to, the *ALS1* gene (a member of the agglutination-like substance gene family) (Hoyer *et al.*, 1995), the *ERK1* (protein kinase) locus (Metzgar *et al.*, 1998) and *CEF3* (translation elongation factor 3) (Bretagne *et al.*, 1997). For the minisatellite *ALS1*, there is a 108 bp repeat unit encoding a 36 amino acid motif within the structural gene. An allele is therefore defined as the number of observed repeat units. For *ERK1* and *CEF3* the repeats are trinucleotide microsatellites; *ERK1* encodes a polyglutamine tract within the structural gene, while *CEF3* is a variable region within the putative promoter. Similar to *ALS1*, alleles are defined on the basis of repeat number (termed variable number of tandem repeats, or VNTRs). All three loci appear to be stable within a strain over many generations (Hoyer *et al.*, 1995; Metzgar *et al.*, 1998; Bretagne *et al.*, 1997). In addition, recent evidence suggests that in the yeast *Saccharomyces cerevisiae*, at least certain classes of trinucleotide repeats, when present at or below approximately 25 repeat units, have mutation rates that can approach the overall nuclear mutation rate (Miret *et al.*, 1998). Both *ERK1* and *CEF3* have a mean number of repeat units below 25. Thus, due to the high number of alleles at each locus, these three loci may be informative not only for strain identification, but also in determining strain relatedness. In addition, Mercure *et al.* (1993) have determined that for a certain subpopulation of *C. albicans*, strains harbour an *IS1*-like element within the 28S rRNA gene, contained on the largest chromosome (designated R). There is no indication that this element is mobile in *C. albicans* (Mercure *et al.*, 1993). By definition this is not a VNTR, in that only a single copy of the element is known to be inserted. Although this insertion event could have occurred by multiple (and presumably rare) integrations, we believe that it is much more likely that these strains are related through a common ancestor, and that this may afford the possibility of finding true phylogenetic relationships between certain groups of strains. This has led us to determine allelic frequencies and relationships within a large population and to compare this with a smaller, previously characterized population (Pujol *et al.*, 1997), with particular reference to those strains harbouring the *IS1* element.

METHODS

Yeast strains and maintenance. Strains were grown and maintained on YPD (1%, w/v, yeast extract, 2%, w/v, peptone, 2%, w/v, glucose) medium at 35 °C as described

previously (Lott *et al.*, 1993). Routine manipulations followed the methods of Kaiser *et al.* (1994). One hundred *C. albicans* isolates were chosen at random from a surveillance study encompassing two metropolitan areas (San Francisco, CA, and Atlanta, GA). Each isolate was obtained from a blood sample representing an individual patient, and there was no epidemiological evidence of relatedness (Rees *et al.*, 1998). A more complete description of all survey isolates will be presented elsewhere. Only three isolates in the present study were from HIV-infected individuals. Strains were initially speciated by Chromagar (Hardy Diagnostics) and API20C (BioMérieux Vitek) profiles. In addition, 14 isolates from a previous study (Pujol *et al.*, 1997) were included, representing unrelated isolates from described subgroups.

DNA isolation and PCR conditions. Genomic DNA was extracted from 10 ml overnight cultures by using a Purgene Gram-positive and yeast DNA isolation kit (Gentra Systems) following the procedure of the manufacturer. Approximately 10 ng DNA was used per 50 µl PCR reaction. *Taq* polymerase, nucleotides, 10× buffer + MgCl₂ and PCR conditions followed the recommendation of the manufacturer (Boehringer Mannheim). Following an initial denaturation step at 95 °C, PCR conditions were 35 cycles of 1 min steps of 95, 55 and 72 °C, with a final elongation for 5 min at 72 °C. For *ALS1* and *CEF3*, the annealing temperature was lowered to 50 °C.

Primers and electrophoresis conditions. *IS1*: 5'-GGG AAT CTG ACT GTC TAA TTA A-3' (forward); 5'-CTT GGC TGT GGT TTC GCT AGA T-3' (reverse). *ALS1* 5'-GGT GGT ACA AGT TCC ACT GCC-3' (forward); 5'-GTT GAC ATA ATG AGG ACG GG-3' (reverse). *ERK1* 5'-CGA CCA CGT CAT CAA TAC AAA TCG-3' (forward); 5'-CGT TGA ATG AAA CTT GAC GAG GGG-3' (reverse). *CEF3* 5'-TTT CCT CTT CCT TTC ATA TAG AA-3' (forward); 5'-GGA TTC ACT AGC AGC AGA CA-3' (reverse).

For *CEF3*, the forward primer was end-labelled with TET (5'-tetrachlorofluorescein phosphoramidite; Glenn Research) and for *ERK1* the reverse primer was labelled with FAM (5'-fluorescein; Glenn Research). For *IS1* and *ALS1*, following PCR amplification, the DNA was precipitated with 2.5 vols ethanol, dried and resuspended in 8 µl TE (10 mM Tris/HCl, 1 mM EDTA). The entire amount was then loaded into a single well. For *IS1*, gels consisted of 1.5% (w/v) agarose (Sigma), 1.0% (w/v) Nusieve GTG agarose (FMC); for *ALS1*, gels were 1.5% (w/v) agarose (Sigma). Gels were stained with ethidium bromide, destained and photographed. For *ERK1* and *CEF3*, the PCR reactions were diluted fivefold in H₂O and analysed by an ABI system 310 (Applied Biosystems), using GENESCAN software (Applied Biosystems).

Genetic analysis. Parsimony analysis was conducted using PAUP version 4.0 (Swofford & Berlocher, 1987) running under GCG (Genetics Computer Group) with a UNIX systems alpha workstation (Digital Systems). Digenic disequilibria were calculated using Genpop (1.2) (Rousset & Raymond, 1995), and exact tests followed that of Weir (1990). An electronic copy of the data set can be obtained by contacting tj11@cdc.gov.

RESULTS

To detect repeated elements and the presence/absence of *IS1*, oligonucleotide primers for PCR were designed to flank the variable regions and the resulting fragments sized and scored as an allelic type. In the case of *ALS1*, the allele number refers to the number of 108 bp repeats;

Table 1. Allelic frequencies and distributions

Locus	Most common allele*	Next most common	Other alleles†	Total samples‡	More extreme§	Prob. of extreme
<i>ALS1</i>	16/83/0.43	13/13/0.07	98/0.50	5151	3999	0.00049
<i>CEF3</i>	135/61/0.31	126/57/0.29	78/0.40	17310	755	1.0 ⁻¹⁴
<i>ERK1</i>	239/95/0.48	231/65/0.33	38/0.19	10290	5300	1.2 ⁻⁸
<i>IS1</i>	—	—	—	15	10	0.02

* Shown as allele/allelic count/allelic frequency.

† Shown as allelic count/allelic frequency.

‡ No. of theoretically possible genotypic distributions.

§ No. of genotypic distributions less likely to occur than the observed data.

|| Summed probability of all more extreme distributions.

these could easily be scored from the 'ladder' generated in each lane of the gel by using a lower-stringency PCR. In the case of *CEF3* and *ERK1*, the alleles are indicated by the total size of the DNA fragments in bp. Finally, in the case of *IS1* the presence or absence of the element was scored as '1' or '0', respectively. When two fragments were generated from a single strain, it was assumed to be heterozygous; likewise, a single fragment was scored as homozygous. Initially, 50 isolates each from two metropolitan areas were analysed separately. Surprisingly, for all three loci with variable size repeats (*ALS1*, *CEF3* and *ERK1*), there was little evidence for a normal distribution around a mean size in the two populations. For example, in the case of *CEF3*, the highest frequency allele was 135 bp, the next most common were 126 and 130 bp, respectively. The remainder displayed a wide distribution in size range and were represented by one or a few isolates each. These frequencies were found to be similar in both populations and they did not differ significantly (Student's *t* test, $P > 0.01$). Data from the two populations were pooled. The frequency for the presence of *IS1* was also not significantly different between the two populations. The results for the combined analysis are given.

Agglutination-like substance 1 gene (*ALS1*, chromosome 6)

For the 108 bp repeat motif (Hoyer *et al.*, 1995), 24 alleles were observed ($n = 111$). Copy numbers ranged from 4 to 37 and a common type (16/16) was observed at a frequency of 0.27. Sixty-two isolates had a unique type and the remaining types (19) were represented by 2–5 isolates each. The mean heterozygosity was 0.60.

Elongation factor 3 gene (*CEF3*, chromosome 5)

A total of 16 alleles ($n = 112$) were observed for the TTC/TTTC microsatellite located 308 bp upstream of the transcriptional start site of the *CEF3* gene (Bretagne *et al.*, 1997). The most frequent type (126/135) was found at a frequency of 0.419 and there were 19 types in total. Seven strains had unique types, and the frequencies

of the other types were approximately the same. The mean heterozygosity was 0.77.

Protein kinase 1 gene (*ERK1*, chromosome 4)

For the clustered microsatellite encoding a Q/A poly-amino acid tract (Field *et al.*, 1996), 12 alleles were found ($n = 113$) giving rise to 16 allelic types. The most predominant allelic type (231/239) was present at a frequency of 0.40. The next most frequent type (239/239) was observed at a frequency of 0.16. Interestingly, type 231/231 was not observed. Eight isolates had unique types, and the heterozygosity was 0.57.

Insertion-like element 1 (*IS1*, chromosome R)

Flanking primers within the conserved 28S rDNA were used to amplify the 379 bp *IS1* insertion element (Boucher *et al.*, 1996), and three classes were observed: 0.196 (22 of 112 isolates) displayed a single fragment showing the presence of the element, 0.04 (5 of 112) were heterozygous, and the remaining majority did not contain the insertion. The mean heterozygosity was 0.04.

Composite analysis of loci

For all four loci collectively, 79 of 111 (0.71) strains where information was available displayed a unique type (i.e. each individual strain was unique) out of 85 total different types (6 types were represented by more than one strain). In addition, 22 of 111 (0.198) were monomorphic for all four loci and shared the allelic type: 16/16, 126/135, 231/239, 0/0 (*ALS/ALS*, *CEF3/CEF3*, *ERK1/ERK1*, *IS1/IS1*). For each locus separately, when the two most predominant alleles were considered, there were significant departures from the Hardy-Weinberg equilibrium under the assumption of random mating (χ^2 statistics, $P < 0.05$ for randomness). It is interesting to note, however, that when all alleles were considered for each individual locus, three loci (*CEF3*, *ERK1* and *IS1*) exhibited total frequencies of heterozygosity not significantly different from those expected

Fig. 1. Parsimony analysis, where each represented strain is of a unique type. Clusters I, II and III correspond to those described by Pujol *et al.* (1997) and the isolate numbers and their corresponding positions are shown. Asterisks indicate isolates containing at least one copy of *IS1*. The arrow indicates the position of the 22 monomorphic isolates. The heuristically searched, majority consensus tree is unrooted.

from Hardy–Weinberg expectations. For the six possible pairwise combinations, digenic disequilibrium statistics were calculated, and all but one were observed to be highly significant (*CEF3* to *ALS1* was observed at $P = 0.039 \pm 0.023$.) We then performed an exact test of Hardy–Weinberg expectations (Weir, 1990) for *ALS1*, *CEF3* and *ERK1*, and a similar two-way exact test for *IS1*. For a given locus, a sample is defined by the allelic and genotypic counts. The same number of alleles (allelic counts) may be distributed in different ways such that it is theoretically possible to observe many different genotypic distributions for the observed number of

alleles. A computer program was written to calculate the probability of observing each theoretically possible collection of genotypic counts, given the same allelic counts; it then sums the probabilities of all samples with the same allelic count that are less likely to be observed (more extreme) than the observed genotypic counts. Hardy–Weinberg expectations are based on the observed allelic counts and are calculated according to probability laws (Weir, 1990). Genotypic distributions in agreement with Hardy–Weinberg are expected. A probability near 1 indicates that the observed genotypic counts are consistent with random segregation, while a probability near 0 indicates a deviation from Hardy–Weinberg expectations. For the *ALS1*, *CEF3* and *ERK1* loci separately, alleles were grouped into three categories: most common, next most common, and all others (Table 1). None of the loci met Hardy–Weinberg expectations.

To determine allelic associations, parsimony analysis was performed for the 85 unique genotypes generated by eight alleles (per strain) and a majority consensus tree was compiled. All alleles were given equal weight, and the consensus tree is shown in Fig. 1. A bootstrap analysis to determine the statistical significance of the three identified clades failed to identify any nodes with confidence values above 70%. We therefore determined the genotypes of 14 unrelated strains from a previous study (Pujol *et al.*, 1997), including four or five strains from each of the three clusters identified by using a different phylogenetic approach. These data were then combined with the original 85 unique strain types. We observed that the three clusters identified in the present study corresponded to the three clades previously identified, and that all isolates corresponded to their previous groupings (Pujol *et al.*, 1997). Significantly, members of cluster III are associated (but not exclusively) with the presence of the *IS1* element. These isolates also exhibit higher degrees of allelic variability at the other three loci. Conversely, cluster I includes the 22 isolates monomorphic for all loci (Fig. 1).

DISCUSSION

The use of allelic variation in defining population structure within a species or between closely related species has been well established, and the constraints to this type of approach have been recognized (McIntyre & Weir, 1997). It is possible to estimate, for example, the effective population size (n) needed when x number of loci, each with y number of alleles, are analysed (Selander & Levin, 1980). In the present work we have attempted to determine the number of alleles for three highly variable loci. We did not observe that any of the isolates were unstable (i.e. a particular strain-specific pattern would vary) supporting the observations of others (Bretagne *et al.*, 1997; Field *et al.*, 1996; Hoyer *et al.*, 1995). In addition, we did not find more than two alleles per locus for any strain. This is particularly relevant in the case of *ALS1*, where it is known that this repeat is present in additional copies of a multigene

family (Hoyer *et al.*, 1995). We did find, however, that for each locus there were two to three predominant alleles and a large number at low frequency. For our defined population ($n = 114$), we observed a large number of unique alleles, and we infer from this that we have not uncovered the absolute number of alleles for the population at large. To do this (particularly in the case of *ALS1*) may require n to be in the thousands. However, our interpretation is that frequency versus size does not represent a normal distribution, and that although increasing n may determine the limits of size, the actual distribution pattern will not vary significantly.

Why then, are certain alleles predominant? One possibility is that these are selected for. In the case of *ALS1*, it has been suggested that the 36 amino acid repeat motif may have the function of determining relative 'adhesiveness' (Hoyer *et al.*, 1995), and that certain domains of repeating units may have a role in optimal protein function. This may also be true for the translated microsatellite found in *ERK1*, although it is less obvious for the untranslated repeat found in the promoter of *CEF3* (Bretagne *et al.*, 1997). Our interpretation, however, based on what is known concerning microsatellites in other eukaryotic systems (Field & Wills, 1996), is that within certain limits repeat size is neutral, and that size variation is generated at a certain rate through specific genetic mechanisms (Strand *et al.*, 1994). This has led us to consider whether certain allelic combinations are random (suggesting something like meiosis) or more tightly associated, as would be the case in a primarily clonal mode of inheritance.

In the present study we have observed little evidence for allelic equilibrium, either within a given locus or between loci. We conclude, therefore, like others (Graser *et al.*, 1996; Pujol *et al.*, 1993) that the predominant form of inheritance in *C. albicans* is clonal, and we hypothesize that departures from strict clonality may be due to other known mechanisms that create genetic diversity in this species (Thrash-Bingham & Gorman, 1992).

At present there is no evidence that VNTRs are conserved to any degree at the interspecies level within the genus *Candida*. However, due to the previously demonstrated stability of these alleles over many generations and the observations from this study, relatedness owing to allelic size may be useful in developing a phylogeny for strains within the species *C. albicans*. For multiallelic loci, phylogenetic analysis using distance matrix algorithms is complex and involves many assumptions concerning the evolution of a particular allelic state. We therefore chose a parsimony approach and made no assumptions (such as 'weighted states'). The problem, however, is that when more than a few alleles are present, the number of possible topological trees becomes very large and the statistical evidence for a particular grouping (the 'bootstrap') becomes increasingly small. Nevertheless, when a subset of strains from a previous analysis (Pujol *et al.*, 1997) were included, all of the isolates clustered in the same manner as previously reported. It should be noted that in the

Fig. 2. Cladogram for the evolution of *C. albicans* and closely related species. The initial tree was generated by an analysis of ITS2 DNA sequences (Genbank accession numbers L07796, L11352, U96719 and L47114) and was rooted at position A, which indicates the initial divergence of *C. parapsilosis* (and other *Candida* spp.) from a common ancestor containing a nonuniversal CUG^{ser} codon. B: Gain of full-length IS1 element and the divergence of *C. dublinensis*. C: modification of IS1 and divergence of *C. stellatoidea*. D: loss of IS1 and divergence of groups I and II. Branch lengths and node distances are not significant. Lineages containing simple lines do not have the IS1 element, whereas large hatched lineages contain a full-length element and smaller hatched lineages contain a truncated element.

former study an entirely different set of polymorphisms were analysed by using a different phylogenetic algorithm (Pujol *et al.*, 1997). We believe, therefore, that the fundamental topology of the three clusters is correct. Although the majority of consensus trees showed the order of groups as III, I, II, as shown in Fig. 1, there were a significant number of trees generated with a III, II, I order, and the precise assignment must therefore be considered as tentative.

There are two other aspects of Fig. 1 that we believe are important for deciphering the true phylogeny of *C. albicans*. The first is that without exception strains harbouring the IS1 element belong to group III (although the converse, that all group III strains contain the IS1 element, is not necessarily true). Second is the observation that groups I and II show, in general, less variability in allelic type than group III isolates. For example, in the present study 22 isolates in group I were monomorphic for all loci studied. If it is assumed that allelic diversity is a function of mutational rate (Rousset & Raymond, 1995), our interpretation, shown in the cladogram depicted in Fig. 2, is that group III isolates represent an evolutionarily older and more diverse class, while groups I and II are of more recent origin and show less variability. In addition, the presence of IS1 in group III isolates is also consistent with what is known about

this mobile element in other closely related *Candida* species. For example, in *C. albicans*, the element has been truncated from a more typical full-length sequence by the deletion of two stem-loop regions (Mercure *et al.*, 1993). For *Candida stellatoidea*, the element is the same length as in *C. albicans* and over 99% identical in sequence (Boucher *et al.*, 1996). In *Candida dublinensis*, the element is full-length and in the conserved regions shares a high degree of homology with *C. albicans*, although lower than the *albicans-stellatoidea* homology (Boucher *et al.*, 1996). Finally, for the related species *Candida parapsilosis*, an analysis of 42 independent isolates has not demonstrated the presence of the element (T. J. Lott, unpublished). Our interpretation of these results is also consistent with 18S and ITS2 sequence homology data (Barns *et al.*, 1991; Santos *et al.*, 1997), as well as other genetic aspects of these related species. For example, it has been suggested that from a common ancestor sharing a CUG^{ser} codon change (Pesole *et al.*, 1995), species such as *C. parapsilosis* and *Candida tropicalis* diverged (Fig. 2, position 'A'). Some later time there was the gain of IS1 in *C. albicans*, followed by the divergence of *C. dublinensis* (Fig. 2, position 'B'). At some later point following the truncation of IS1, *C. stellatoidea* diverged (Fig. 2, position 'C'). Most recently, in *C. albicans* there has been the divergence of groups I and II and the loss of IS1 (Fig. 2, position 'D'). An interesting correlate to this hypothesis is that *C. dublinensis* and *C. albicans* have undergone concerted evolution, in that phenotypically they cannot be distinguished (Boucher *et al.*, 1996).

Although we cannot exclude the possibility of population sample bias, the *C. albicans* strains used in this study have no known epidemiological relationship, and there is no evidence for geographical partitioning by strain type. Thus, it will be of interest to determine why a large number of strains represented in this population are of a common lineage (i.e. monomorphic for all loci studied), and whether these common alleles have a selective advantage in the host. However, other well-established factors may also be present in defining population structure (McIntyre & Weir, 1997). The establishment of clonal lineages will also aid in our understanding of emerging phenotypes, such as drug resistance, where there is often evidence for pleiotropic mutations of unknown genetic origin (Sanglard *et al.*, 1995). With the advent of additional genomic sequences, it is hoped that other markers will be uncovered that evenly cover all eight chromosomes. This will clearly facilitate a genetic understanding of this medically important yeast and perhaps help address other questions, such as the time framework for the evolution of distinct subgroups.

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