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Microsatellite Polymorphism in the Promoter Sequence of the Elongation Factor 3 Gene of *Candida albicans* as the Basis for a Typing System

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The polymorphism of a TTC/TTTC microsatellite in the promoter sequence of the elongation factor 3 gene of *Candida albicans* was investigated by PCR. One primer was fluorescein labeled, and PCR signals were read with an automatic sequencer. Twenty-nine reference strains and 31 independent clinical isolates were studied. Eleven different alleles were identified, giving 16 different profiles among the 60 strains tested, with a discriminatory power of 0.88. This marker is stable upon subculture, and reproducibility was achieved by automated procedures. When several microsatellite markers are available, many isolates can be rapidly and reproducibly tested for epidemiological questions, such as the prevalence of a given strain in a hospital setting and transmission between patients.

Candida albicans is a commensal yeast which acts as an opportunistic agent in immunocompromised patients and which has emerged as a major fungal pathogen in recent years (2). The need for a better understanding of the epidemiology of *C. albicans*, for example, route of acquisition, transmission between patients, the prevalence of a strain causing nosocomial infections, or the emergence of antifungal agent-resistant strains, has led to the development of different techniques to characterize strains and isolates. None of the phenotypic or genotypic typing procedures alone provides a definitive answer to epidemiological questions. Optimal approaches should include a combination of as many markers as possible.

The DNA-based methods already used include restriction endonuclease analysis by either conventional or pulsed-field gel electrophoresis (12, 15, 19), Southern blot hybridization with different probes from *C. albicans* (16, 20, 22), and electrophoretic karyotyping (13). However, these techniques either provide poorly polymorphic markers or are time-consuming due to the need for DNA preparation, digestion, electrophoresis, transfer, and hybridization, procedures not always compatible with the imperatives of the clinical laboratory. Due to their rapidity, PCR-based methods seem more appropriate. Recently, the random amplification of polymorphic DNA (RAPD) technique has been used. Primers either were arbitrarily chosen (10) or consisted of repetitive elements (23) or genomic regions known to be variable in lower eukaryotes (26). This latter typing procedure seems at least as able as other genotypic assays to distinguish between isolates. Unfortunately, the RAPD technique is poorly reproducible due to the low annealing temperatures used in the PCR; also, the pattern obtained is often complex and is hardly comparable between laboratories. Moreover, RAPD data possess a number of undesirable features for parsimony analysis and phylogenetic studies (1). PCR-based methods also include single-strand conformation polymorphism analysis, but this technique requires DNA sequencing of PCR products to identify the cause of the polymorphism observed (7).

We therefore decided to look for microsatellites, defined as short tandem repeats of two to five nucleotides, in the *C. albicans* genome. Indeed, microsatellites are known to be highly polymorphic as well as numerous and equally spread in the human genome (3) as well as the genomes of all other eukaryotes (6). From the technical point of view, the polymorphism of microsatellites can be evaluated by PCR.

MATERIALS AND METHODS

Known *C. albicans* sequences were first subjected to an endogenous microsatellite search by using a personal program. This program permitted the detection of repeated sequences containing at least five contiguous, identical motifs of one to five nucleotides each. A microsatellite DNA consisting of TTC and TTTC repeats was found in the upstream sequence of the elongation factor 3 (EF3) gene, between a putative TATA box and the transcription start site. Interestingly, the EF3 gene has been sequenced with two different strains by two different teams (4, 14), and differences in the numbers of TTC and TTTC repeats were observed. Primers were designed to amplify this sequence, and the polymorphism between reference strains was evaluated. The upstream primer 5′-TTT CCT CTT CCT TTC ATA TAG AA-3′ was located at positions 193 to 215 (sense) (GenBank accession no. Z11484) and included a putative TATA box (14). The downstream primer 5′-GGA TTC ACT AGC AGC AGA CA-3′ was located at positions 308 to 327 (antisense), including the transcription initiation start signal. The downstream primer was 5′ labeled with fluorescein to allow for determination of the sizes of PCR products with an automatic sequencer (see below).

DNA was extracted from 29 independent reference strains (Table 1) as described previously (9). The reaction mixture consisted of 100 ng of *C. albicans* DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.25 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates, 10 pmol of primers (DNAgency, Malvern, Pa.), and 1.25 U of *Thermophilus aquaticus* 1 polymerase (Perkin-Elmer Cetus, Paris, France) in a 20-μl reaction volume. Hot start was achieved by using a monoclonal antibody to *T. aquaticus* DNA polymerase (TaqStart antibody; ClonTech, Palo Alto, Calif.). All the amplifications were carried out with a Perkin-Elmer DNA thermal cycler 480 and included 30 cycles of denaturation at 94°C and annealing at 50°C for 30 s each and extension at 72°C for 1 min. The final cycle was followed by an additional 7 min at 72°C to complete partial polymerization. PCR products were diluted 1/20 in water, and 1 μl of each was run on a 12-cm-long acrylamide-urea gel (8% acrylamide, 8.3 M urea, 1× TBE [Tris-borate-EDTA]) for 2 h and 30 min at 1,500 V. An internal standard labelled with 6-carboxy-X-rhodamine dye (GenScan-500 Rox; Applied Biosystems) was

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TABLE 1. PCR results for the 29 strains from reference collections and 31 independent clinical isolates of *C. albicans*

Clinical isolate code (laboratory or provider)	Corresponding ATCC[a] strain or anatomical site of isolation	EF3 allele association[b]
SC 5314 (Calderone)		126–135
3153 A (Fonzi)	ATCC 36801	126–135
441.B (Fonzi)		126–135
A81.Pu (Fonzi)		126–135
IP 884/65 (Dromer)		126–135
IP 993/69 (Dromer)		126–135
IP 1332/82 (Dromer)	ATCC 26278	126–135
IP 996/69 (Dromer)	ATCC 36803	126–135
IP 2146/93 (Dromer)	ATCC 66027	126–135
B 792 (Kwon-Chung)		131–139
IP 886/65 (Dromer)		131–139
ATCC 28367 (Fonzi)	ATCC 28367	131–139
Ca 4918 (Calderone)	ATCC 44808	130–136
IP 887/65 (Dromer)		130–136
IP 1548/84 (Dromer)	ATCC 44858	130–136
IP 1407/82 (Dromer)		130–136
H 12 (Calderone)	ATCC 56879	130–144
B 311 (Fonzi)	ATCC 32354	130–144
IP 1214/80 (Dromer)	ATCC 38248	130–144
ATCC 38696 (Fonzi)	ATCC 38696	133–144
ATCC 10231 (Calderone)	ATCC 10231	139–139
IP 1118/75 (Dromer)		130–130
IP 1213/80 (Dromer)	ATCC 38245	130–130
IP 1880/89 (Dromer)		131–131
IP 1180/79 (Dromer)	ATCC 2091	131–131
IP 1878/89 (Dromer)		131–131
IP 1663/86 (Dromer)		131–135
IP 1876/89 (Dromer)		135–135
IP 1877/89 (Dromer)		135–135
CI 1	Upper respiratory tract	130–136
CI 2	Upper respiratory tract	131–142
CI 3	Upper respiratory tract	126–135
CI 4	Upper respiratory tract	130–136
CI 5	Upper respiratory tract	131–131
CI 6	Upper respiratory tract	131–131
CI 7	Skin	126–135
CI 8	Skin	126–135
CI 9	Skin	126–135
CI 10	Urine	133–144
CI 11	Urine	136–136
CI 12	Urine	136–146
CI 13	Urine	130–145
CI 14	Urine	136–146
CI 15	Urine	136–136
CI 16	Urine	130–136
CI 17	Urine	130–136
CI 18	Urethra	130–130
CI 19	Vagina	131–131
CI 20	Abdominal drainage	126–135
CI 21	Abdominal drainage	136–145
CI 22	Stool	131–131
CI 23	Stool	126–126
CI 24	Stool	126–135
CI 25	Stool	130–136
CI 26	Blood	136–145
CI 27	Blood	130–144
CI 28	Blood	131–131
CI 29	Blood	133–145
CI 30	Blood	136–145
CI 31	Bone	131–131

[a] ATCC, American Type Culture Collection (Rockville, Md.).
[b] The number assigned to the alleles corresponds to the length of the PCR products (in base pairs).

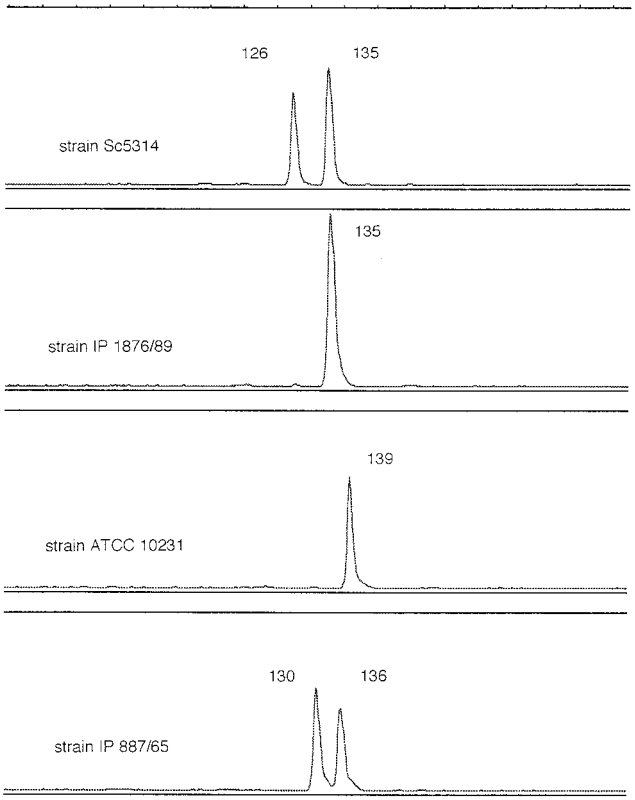

FIG. 1. Representative profiles obtained upon amplification with a fluorescein-labeled primer and analysis with an automatic sequencer by using GenScan software (Applied Biosystems). PCR products were run on an acrylamide-urea gel with an internal standard labeled with 6-carboxy-X-rhodamine dye loaded in each well. The numbers refer to the size of the PCR products (in base pairs). Strains SC 5314 and IP 887/65 were found to be heterozygous, whereas strains IP 1876/89 and ATCC 10231 were found to be homozygous.

loaded into each well along with the PCR products. Signals were read with an automatic sequencer (Applied Biosystems), and the data were stored and analyzed with GeneScan software (Applied Biosystems). Secondarily, 31 independent clinical isolates were tested as described above. These isolates were collected from different patients in different wards and did not come from the same anatomical site.

RESULTS

Most of the PCR products of the 29 reference strains consisted of two bands between of 126 and 144 bp (Fig. 1). Because *C. albicans* is thought to be diploid and the EF3 gene is known to be a single-copy gene (14), each band was assigned to an allele. Eight alleles were detected and their combination led to 10 different profiles: 21 strains were heterozygous and 8 were homozygous (Table 1). Identical results were obtained upon two different amplifications of the same DNA and upon amplification of two different DNA extractions for each strain. Four reference strains (B 792, Ca 4918, H 12, and ATCC 38696) were subcultured 25 times in yeast potato dextrose, corresponding roughly to more than 300 generations, and the alleles were unchanged. Moreover, strains ATCC 10231 and ATCC 32354 strains, obtained from two different laboratories, had the same pattern. In addition, a *ura3*-deficient mutant of the SC 5314 strain had the same pattern as its original strain, despite numerous subcultures (data not shown). The amplifications were specific to *C. albicans* because no bands were observed upon amplification of *Candida tropicalis* and *Candida glabrata* DNAs.

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TABLE 2. Number of TTC and TTTC repeats found for the eight alleles of the 29 reference strains and the 31 independent isolates tested[a]

EF3 allele (bp)	Microsatellite sequence	Frequency (% [total no.])
126	(TTC)3 (TTTC)4	14 (17)
130	(TTC)3 (TTTC)5	17 (20)
131	(TTC)5 (TTTC)4	19 (23)
133	(TTC)3 (TTTC)6	3 (3)
135	(TTC)5 (TTTC)5	17 (20)
136	(TTC)4 (TTTC)6	15 (18)
139	(TTC)5 (TTTC)6	4 (5)
142	Not done	1 (1)
144	(TTC)4 (TTTC)8	6 (7)
145	Not done	3 (4)
146	Not done	2 (2)

[a] A total of 120 alleles were tested. The PCR products of reference strains were cloned into PT7Blue plasmids, and two clones were sequenced for each allele. The sequences of the alleles were identical except for the number of TTC and TTTC repeats, which account for the differences in the lengths of the PCR products.

PCR products were subsequently subcloned into the pT7Blue vector which contains a single overhanging T nucleotide. After ligation, DH5α competent cells were transformed and two white colonies were randomly selected on the plates for each PCR product. The plasmids were subjected to alkali denaturation and were sequenced using the Sequenase kit (U.S. Biochemicals, Cleveland, Ohio). Sequencing shows that the differences observed were due to the different numbers of microsatellites (Table 2).

In a second step, the 31 clinical isolates were tested. Three new alleles were found at 142, 145, and 146 bp. Along with these new alleles, new associations were observed leading to the observation of six new profiles (Table 1). The most common profile (profile 126–135) among the reference strains was also the most common profile among the clinical isolates (Table 3). No clear link between a given profile and an anatomical site was obvious with these samples.

When the results for the reference strains and the clinical isolates were pooled, 16 different profiles were found among 60 independent *C. albicans* isolates (Table 2). This led to a numerical index of discriminatory power (D) of 0.88 from the following formula (8):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j (x_j - 1)$$

where s is the number of profiles ($s = 16$), x_j is the number of the population falling into the jth type, and N is the size of the population ($N = 60$).

Some alleles were more frequently observed than others, varying from 1.7 to 19.2% (Table 2). For instance, allele 126 was almost always associated with allele 135, but once it was found to be homozygous. It is also noteworthy that some associations were more frequent than others, such as association 126–135, which accounted for 25% of the 60 strains tested (Table 3).

TABLE 3. Frequency of the 16 allele associations for the 29 reference strains and the 31 independent isolates tested

EF3 allele association	No. of reference strains	No. of clinical isolates	Total no. (%)[a]
126–135	9	6	15 (25)
131–139	3	0	3 (5)
130–136	4	5	9 (15)
130–144	3	1	4 (7)
133–144	1	2	3 (5)
139–139	1	0	1 (2)
130–130	2	1	3 (5)
131–131	3	6	9 (15)
131–135	1	0	1 (1)
135–135	2	0	2 (3)
131–142	0	1	1 (2)
136–136	0	2	2 (3)
136–146	0	2	2 (3)
130–145	0	1	1 (2)
136–145	0	3	3 (5)
126–126	0	1	1 (2)

[a] Percentage among the 60 strains tested.

DISCUSSION

Among PCR techniques, amplification of a short DNA sequence at a high annealing temperature, as used for the analysis of microsatellites, increases the level of reproducibility upon sequential tests and between laboratories. Moreover, the pattern observed is easier to read than the patterns observed after RAPD analysis; standardization can be achieved by using automated procedures with fluorescent primers, and the profiles are stable upon subculture, even if the reference strains came from different laboratories. Thus, the reproducibility of the technique is high.

The polymorphism observed with the microsatellite in the promoter region of the EF3 gene is restricted to chromosome 5 because the EF3 gene is a single-copy gene located on this chromosome. Although the spacing between yeast initiation sites and TATA box elements is much more flexible and somewhat larger than that in the genomes of higher eukaryotes (24), the presence of such a polymorphic sequence in a region critical to the initiation of transcription is surprising. The level of transcription does not seem to be influenced by the number of repeats. Indeed, we carried out Northern blots with total RNA of previously studies strains SC 5314 and B792 (4, 14) and three homozygous reference strains (strains ATCC 10231, IP 1118/75, and IP 1876/89). Probing with the EF3 gene and the actin gene as a control did not show any correlation between the level of transcription of the five strains tested and the allelic polymorphism (data not shown). Other studies should be designed to determine the role of this polymorphism in a promoter region because EF3 mRNA levels change depending on the culture conditions (25).

The aim of this work was to develop a marker for epidemiological studies. From this point of view, the discriminatory power of this marker is close to the discriminatory power of other markers obtained by previously published techniques (8), although it remains below the discriminatory power of fingerprinting with a moderately repetitive sequence (22). RAPD analysis can also provide more discriminatory markers, but its reproducibility is questionable. Seven microsatellite markers have recently been described, and 10 of the 11 *C. albicans* isolates tested were found to have unique genotypes with these microsatellites (5). Therefore, multiple microsatellites on other chromosomes will probably be described soon, which should increase the discriminatory power of this approach.

The delineation of new microsatellites will allow investigators to address the question of the clonal nature of *C. albicans* in patients supported by phenotypic (17) and genotypic (7, 11) studies. The presence of a linkage disequilibrium supports the

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hypothesis that populations of *C. albicans* are mostly clonal (17). The finding in our work that one allele association (126–135) accounted for 25% of the 60 strains tested might support the hypothesis that a main *C. albicans* population develops more efficiently than others. Microsatellite markers on other loci should be studied to confirm this hypothesis or to show that the microsatellite marker described is simply not polymorphic enough to distinguish between clinical isolates. Moreover, comparison with markers obtained by different techniques should be done.

In contrast to RFLP analysis, microsatellite markers cannot currently address the question of genetic distance between *C. albicans* isolates. Indeed, RFLP analysis is mainly based on the presence or absence of restriction enzyme sites. Thus, the number of mutations from a given nucleotide to another can be used to calculate a genetic distance between isolates. In contrast, the polymorphism of microsatellites is based on a completely different mechanism. The predominant means by which new alleles are generated is thought to be intra-allelic polymerase slippage during replication (21). It is the accumulation of mutations resulting in alleles with different lengths which renders microsatellites among the most variable classes of repetitive DNAs and widely used in human genetics (3). The mutation rate at microsatellite loci has been estimated to vary between 1×10^{-4} and 5×10^{-6}. Microsatellite DNA sequences usually mutate by gaining or losing one or two repeats units. This evolution is probably not random, and allele frequency distributions at a few loci are under some constraints (18). It therefore seems possible to use microsatellites to study genetic relatedness between *C. albicans* isolates and to compare the results with those obtained by RFLP analyses, as has been done for humans. For that, it will be necessary to obtain microsatellite mutation rates specific for *C. albicans* because microsatellite evolution seems to be species specific (18). Moreover, the mutation rate depends on the type of repeats: the mutation rate is higher for tri- or tetranucleotide repeats than for dinucleotide repeats (27). However, without knowing the precise mutation rate specific for *C. albicans*, the knowledge of polymorphic microsatellite markers on different chromosomes will soon enable investigators to track the evolution of chromosome associations and to estimate relatedness between *C. albicans* isolates.

Microsatellite polymorphism is easy to detect by PCR, and highly reproducible results can be obtained by using automated procedures, as has already been done for human genetics. When several microsatellite markers are available, many isolates could be rapidly and reproducibly tested to answer epidemiological questions, such as the prevalence of a given strain in a hospital setting and transmission between patients. Moreover, the microsatellites present an opportunity to construct a genome map of *C. albicans.*

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