A highly polymorphic microsatellite for the identification of *Candida albicans* strains

Paula Sampaio[1], Leonor Gusmão[2], Cíntia Alves[2], Cidália Pina-Vaz[4], António Amorim[2,3] and Célia Pais[1*]

[1]*Centro de Ciências do Ambiente, Departamento de Biologia, Universidade do Minho, 4710-057 Braga, Portugal*

[2]*Instituto de Patologia e Imunologia Molecular da Universidade do Porto, R. Roberto Frias, s/n 4200 Porto, Portugal*

[3]*Faculdade de Ciências, Universidade do Porto, Portugal*

[4]*Departamento de Microbiologia, Faculdade de Medicina, Universidade do Porto, Portugal*

Running Title

NEW POLYMORPHIC MICROSATELLITE IN *CANDIDA ALBICANS*

Author for correspondence:

Célia Pais
Tel: +351 253 604312
Fax: +351 253 678980
Email: cpais@bio.uminho.pt

ABSTRACT

The polymorphism of a new microsatellite locus (CAI) was investigated in a total of 114 *C. albicans* strains, including 73 independent clinical isolates, multiple isolates from the same patient, isolates from several episodes of recurrent vulvovaginal infections and two reference strains.

PCR genotyping was performed automatically, using a fluorescent labelled primer and, in the 73 independent isolates, 26 alleles and 44 different genotypes were identified, resulting in a discriminatory power of 0.97.

CAI revealed to be species specific and with a low mutation rate, since no amplification product was obtained when testing other pathogenic *Candida* species and no genotype differences were observed when testing over 300 generations.

When applying this microsatellite to the identification of strains isolated from recurrent vulvovaginal infections in eight patients, it was found that 13 out of 15 episodes were due to the same strain. When multiple isolates obtained from the same patient and plated simultaneously, were typed for CAI, the same genotype was found in each case, confirming that the infecting population was clonal. Moreover, the same genotype appeared in isolates from the rectus and the vagina, revealing that the former could be a reservoir of potentially virulent strains.

This new microsatellite proves to be a valuable tool to differentiate *C. albicans* strains. Furthermore, when compared to other molecular genotyping techniques, CAI proved to be very simple, highly efficient and reproducible, being suitable for low quantity and very degraded samples and for application in large scale epidemiological studies.

INTRODUCTION

It is known that opportunistic yeast pathogens are common residents of the mucosal surfaces of the gastrointestinal tract, genitourinary system and oral cavity in warm-blooded animals. Although several yeast species can be associated to infection, the predominant causal agent of candidiasis is *Candida albicans.* This yeast causes several infections in humans including a wide variety of life threatening conditions triggered by bloodstream infections, especially in immunocompromised patients. Since pathogenecity and antifungal susceptibility often vary among strains, a rapid and accurate identification of the disease causing strains of *C. albicans* is crucial for clinical treatment and epidemiological studies.

Advances in molecular biology in the last two decades have allowed the development of rapid molecular genotyping techniques for clinical and epidemiological analysis. Several molecular typing methods have been developed to differentiate *C. albicans* strains, including electrophoretic kariotyping (2), the use of species-specific probes such as Ca3 or 27A in restriction enzyme analysis (20, 23, 27, 29, 32, 33) and PCR-based methods (1, 10, 21, 24, 28, 37). More recently, short tandem repeats (STRs) or microsatellites have assumed increasing importance as molecular markers in fields so diverse as oncogenetics, population genetics and strain identification and characterisation. They occur in several thousands of copies dispersed throughout the genome and display high polymorphism, Mendelian co-dominant inheritance and PCR typing simplicity. Only a few polymorphic microsatellite loci have been identified so far in *C. albicans* genome and most of them located near or inside coding regions and exhibiting a discriminatory power between 0.77 and 0.91 (3, 4, 7, 19, 25). However, it is known that the degree of polymorphism is much higher in microsatellite loci from non-coding regions and, to date, few studies have been developed towards the analysis of loci from those regions in *C. albicans* (18, 19).

The aim of this work was to identify and describe a new highly informative microsatellite locus (CAI), outside a known coding region, in the genome of the pathogenic yeast *C. albicans* and evaluate its applicability to accurately differentiate strains. Another goal of this study was to use this microsatellite marker to assess genetic relatedness of *C. albicans* isolates obtained from sequencial episodes of recurrent vaginal candidoses and from multiple simultaneous isolations of the same patients.

METHODS

Microsatellite selection and design of PCR primers. A search in *Candida albicans* genome sequences, available in databases from Stanford´s DNA Sequencing and Technology Centre, (http://www-sequence.stanford.edu/group/candida) was conducted for sequences containing microsatellite repeats. The aim of this search was to select repetitive sequences that were expected to have a very high degree of polymorphism, based on two criteria, the number of simple repeat units (more than 20) and the location, outside a coding region. Ten microsatellites were selected and primers were designed, in the non variable flanking regions, for locus-specific amplification. Based on the results of preliminary studies on amplification efficiency, species specificity and observed polymorphism, a sequence containing 32 CAA repeats, 396062C04.s1.seq, was selected for further characterisation and for application in strain identification purposes.

Yeast strains. A total of 112 clinical isolates of *Candida albicans*, obtained from two Hospitals and a Health Center located in Braga and Porto (North Portugal), the reference strain WO-1 as well as the type strain PYCC 3436 (ATCC 18804), were selected for this study. All isolates were previously identified by their assimilation patterns on ID 32C strips (Biomerieux, SA, Marcy – L'Étoile, France) and by PCR fingerprinting with primer T3B using the methodology described by Thanos *et al.*, (37). The type strains of *C. parapsilosis* PYCC 2545 (ATCC 22019), *C. krusei* PYCC 3343 (ATCC 6258), *C. tropicalis* PYCC 3097 (ATCC 750), *C. glabrata* PYCC 2418 (ATCC 2001), *C. guilliermondii* PYCC 2730 (ATCC 6260), *C. lusitaniae* PYCC 2705 (ATCC 34449) and *C. dubliniensis* CBS 7987 (ATCC MYA-646) were also tested. All reference strains were obtained from the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Portugal except the isolates of *C. dubliniensis* that were from Centraalbureau voor Schimmelcultures (CBS),The Netherlands. Stock cultures were maintained on Sabouraud glucose agar medium at 4°C.

DNA isolation and PCR amplification. Prior to DNA isolation, cells were grown overnight on Sabouraud medium at 30°C. DNA extraction followed procedures previously described (15). PCR reactions were performed in 25 µl reaction volume containing 1x PCR buffer (20 mM Tris HCl, pH 8.4, 50 mM KCl), 0.2 mM of each four dNTPs (Promega), 0.25 µM of each primer (Forward: 5'- ATG CCA TTG AGT GGA ATT GG -3', Reverse: 5'- AGT GGC TTG TGT TGG GTT TT -3'), 25 ng of genomic DNA and 1 U of *Taq* DNA polymerase (Gibco). For automatic allele size determination, the forward primer was 5´ fluorescently labelled with FAM.

Amplification was carried out in a DNA thermocycler 2400 (AB Applied Biosystems) with a program consisting of an initial denaturing step at 95°C for 5 min; followed by 30 cycles of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C; and a final extension step of 7 min at 72°C.

Fragment size determination. For allele size determination, the PCR products were run in an ABI 310 Genetic Analyser (AB Applied Biosystems). Fragment sizes were determined automatically using the GeneScan 3.1 Analysis software. Alleles have been designated by the number of trinucleotide repeats (Table 1).

DNA sequence analysis. After PCR amplification, DNA fragments were separated by electrophoresis in 6% polyacrylamide gels in denaturing conditions (6.5M Urea) using the buffer systems described by Gusmão *et al*. (12), and visualised by the silver staining method (5). Allele bands were cut individually from the gel, eluted in 250 μl of TE buffer, frozen and thawed three times, reamplified and purified with Microspin S-300 HR columns (Pharmacia). The purified products were submitted to dideoxy cycle sequencing reaction using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (AB Applied Biosystems). Sequence analysis was performed on an ABI 377 Genetic Analyser using the Data Collection Software 377-18.

Stability. To test the stability of the marker, three different clinical isolates and the type strain were grown over 300 generations in 1-liter Erlenmeyer flasks containing 500ml of Sabouraud medium and incubated at 30ºC in an orbital shaker (169 rpm). At the end of the exponential phase, a 1/10 dilution with new medium was made in order to allow continuing of cell duplication. This procedure went on for 4 weeks until completion of around 300 generations. Cells were harvest at the end of approximately 100, 200 and 300 generations, and DNA was extracted for amplification.

Reproducibility. Reproducibility of the method was assessed by testing three strains ten times in three separate experiments.

Statistical analysis. Genotype frequencies were estimated by genotype counting. Statistical analysis for Hardy-Weinberg equilibrium was performed using an exact test (11), running the statistical software package GENEPOP. The discriminatory power of the marker was calculated according to Hunter and Gaston (14).

RESULTS

With the aim of identifying highly informative microsatellite polymorphisms for molecular discrimination of *Candida albicans*, a search for short repetitive sequences was conducted as described in Methods. It is well established that a higher degree of polymorphism is expected for microsatellites outside coding regions (26) as well as for long tracts of simple repeats (34). For these reasons, our search was based upon the number of uninterrupted repeats outside known coding regions. Dinucleotide repeats were not considered, since they are described as being more prone to stutter bands due to DNA polimerase slippage during amplification (9).

For the ten sequences selected with more than 20 uninterrupted tri to penta repeat units, specific primers were designed, for annealing in the non variable flanking regions, and used for preliminary studies on amplification efficiency and specificity and for evaluation of the informative content of polymorphism. Only a sequence containing 32 CAA units showed the required characteristics and was selected for further studies and to address the question of applicability in the differentiation of related strains. This new microsatellite locus was designated CAI.

Sequence analysis. Sequencing analysis of 37 amplified fragments revealed that the consensus structure was in accordance with the originally published (396062C04.s1.seq), confirming locus specific amplification and structure of the alleles. The variation in length of the CAI alleles was always due to differences in the number of trinucleotide repeat units and, therefore, the alleles were designated by the total number of trinucleotide repeats. For instance allele 21 when it possessed a size consistent with that number of repetitions independently of the structure variation. The sequence analysis revealed three different levels of polymorphism in CAI, (i) the number of repeats, (ii) the structure of the repeated region and (iii) point mutations outside the repeated region (data not shown). In the context of this paper, the genotyping was made based only in the first level of polymorphism, but the second and third levels of variation may contribute to further differentiate *C. albicans* strains.

CAI locus analysis. One hundred and twelve clinical isolates of *C. albicans* and two reference strains were genotyped for CAI (examples shown in Figure 1-A). For an easier and more accurate size determination, genotype analysis was performed automatically using a fluorescent labelled primer (Figure 1-B). In order to check for method reproducibility, for each of four selected samples, the PCR reaction was performed at least 10 different times (including different DNA extractions), displaying always the same result.

The PCR products obtained consisted of fragments with different lengths, varying between 189 bp (11 repeats) and 303 bp (49 repeats). Since *C. albicans* is thought to be diploid, each fragment was assigned to an allele and, the strains showing two PCR products were typed as heterozygous (72.6%), while when a strain presented a single amplification product it was considered as homozygous.

Using only the results obtained for the sample of 73 non related strains, isolated from non related patients, a significant departure from Hardy-Weinberg equilibrium expectations was found ($P< 0.001$). This finding supports the previous conclusions (10, 17) that the inheritance in *C. albicans* is mainly clonal. For this reason, the CAI diversity content and discriminatory power can only be evaluated using genotype frequencies rather than allele frequencies. In the 73 non related strains, a total of 26 different alleles and 44 distinct genotypes were observed. The genotype frequencies vary between 0.014 and 0.082. The most frequent genotype (21-25) was present in only six out of the 73 non related strains (Table 1). The number of CAI genotypes is much higher than the ones described so far for other loci (3, 4, 6, 7, 13, 18, 19, 25, 34), resulting in a discriminatory power of 0.97.

Stability and specificity. *In vitro* stability of CAI marker was tested by growing four independent strains over approximately 300 generations. For all the strains tested the genotypes were the same after the 300 generations, suggesting that CAI has an expected mutation rate less than $3.33x10^{-3}$.

The CAI microsatellite revealed also to be species specific, since no amplification products were obtained when using the described primers in the amplification of other pathogenic *Candida* species, namely *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. lusitaniae* and *C. dubliniensis*. It is noteworthy to notice the specificity regarding *C. dubliniensis* which is very closely related to *C. albicans* and only very recently was recognised as a different species (36).

Similar results were found in previously described STRs, by testing other *Candida* species, *C. krusei*, (7), *C. tropicalis* and *C. glabrata* (4) and *C. dubliniensis* (25).

The use of CAI for strain distinction. CAI genotyping results obtained in eight cases of multiple isolates from the same patient and plated at the same time are shown in Table 2. As it can be observed, in each case, all the strains isolated showed exactly the same genotype suggesting that only one strain is present in the infecting population.

To verify if the infecting population is the same at different body locations, isolations were made from the same patients displaying multiple local infections. The results showed that two strains from patient I, isolated from the upper respiratory tract, were identical, but different from the urine isolate. The same occurred for patient J, where distinct genotypes were observed for the two strains isolated, one from the vagina and the other from the urine. These results show clearly that in different body sites, patients can have different clones but the infecting population at each body site is monoclonal.

The analysis of 15 cases of recurrent vulvovaginal infections in eight patients revealed that the infecting *C. albicans* strains isolated sequentially in different relapses of the illness displayed the same CAI genotype, except in two cases (Table 3). The second and third strain isolations from patients L and N presented a different genotype from the first episode indicating possible cases of strain replacement. However, further analysis with three additional STRs, including the one described by Bretagne, EF3 (4) confirmed that all three L isolates had the same genotype (data not shown). So, most probably, they do not represent different strains, and just differing by a mutation at CAI locus. The microvariation observed inside one of the alleles, from 30 to 32 repetitions, shows a different scenario of recurrent vaginitis with maintenance of a strain which is undergoing microevolution. For patient N a case of strain replacement had really occurred and was confirmed with further analysis using the same additional STRs (results not shown). These observations are in accordance with the literature (16) where three basic scenarios are described for the genetic relatedness of strains isolated from patients with recurrent vaginitis, (i) maintenance of the same strain, (ii) maintenance of a strain which is undergoing microevolution and (iii) strain replacement.

In five patients with recurrent vulvovaginal candidoses, anorectal and vulvovaginal isolates were simultaneously obtained and typed with CAI (results on table 3). In all cases, the strains shared the same genotype, confirming that the anorectal region can be a reservoir of *C. albicans* infecting strains, in accordance with previous observations (16, 31, 33).

DISCUSSION

Many investigations were undertaken in order to search for molecular variation in the genome of *C. albicans* for a large set of applications, such as identification, phylogenetic analysis, resistance development and gene association studies (4, 17, 25). As referred in the introduction, nowadays there are different DNA-based methodologies available for these purposes. It has been demonstrated that STR-PCR based methods have several advantages over the other methodologies used in strain identification, since microsatellites are known to be highly polymorphic, the PCR is a less time-consuming technique and results can be easily reproduced and compared between laboratories (4, 6).

Numerous microsatellites have been reported in various organisms (8, 13, 34, 38), but, until now, only a few polymorphic microsatellite loci were described in *C. albicans*, most of them located near, EF3 (4), CDC3 and HIS3 (3), or inside coding regions, ERK1, 2NF1, CCN2, CPH2 and EFG1 (25). The discriminatory power calculated for these STRs, was between 0.77 (for CDC3) and 0.91 (for HIS3), and, the most discriminant microsatellite approach was obtained when combining three STRs in a single multiplex amplification reaction, yielding a discriminatory power of 0.97 (3), the same obtained for CAI in the present work. Thus, CAI revealed to be more polymorphic than other STRs described so far for *C. albicans*, this result confirming that the criteria we defined to choose this microsatellite proved to be adequate. A probable explanation lies in the fact that CAI is, as far as it is now known, probably located in a non coding region, thus being less prone to selective forces (26) and presents a long non interrupted repetitive tract, as an evidence of previously accumulated diversity.

This high degree of polymorphism exhibited by CAI could be correlated to a high mutation rate which would result in a limitation for its use in strain identification. However, our results demonstrate that CAI is stable not only in laboratory culture but also in vivo, since in most cases of recurrent infections we found the same CAI genotype, including in one patient where strains from five recurrent episodes were genotyped.

Development of multiplex systems, co-amplifying several STRs, in order to test rapidly and reproducibly a great number of isolates, is of great importance in biomedical mycology. CAI and EF3 STRs clearly stand out as candidates to be included in such a multiplex system since they are very well characterised and the same typing methodology is used. A special care must be paid to typing standardisation since the use of different primers or separation techniques have shown to prevent the comparison of results from the same locus. Standardisation of allele nomenclature, based on the repeat number rather than fragment size,

is also essential for the construction of public databases in light of what is already in current use in human genetics (22, 30).

It is clear that the analysis of multiple STR loci may enable high-speed typing in the near future, and the high number of available markers allows a previous selection of the best markers to be included in multiplexes, based on typing performance, mutation rates and discriminative power.

Furthermore, they can be used to complement studies such as RAPDs and DNA fingerprinting, by which evolutionary related strains can be distinguished, particularly when searching for micro-evolutionary events (4, 25).

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Table 1 – Frequency of genotypes identified for CAI microsatellite.

Observed Genotype	Number of strains	Frequency	Observed Genotype	Number of strains	Frequency
11-18	2	0.027	22-34	1	0.014
11-28	1	0.014	23-24	1	0.014
13-32	1	0.014	23-27	2	0.027
16-27	2	0.027	24-24	1	0.014
17-17	1	0.014	24-26	1	0.014
17-21	3	0.041	24-27	1	0.014
17-23	3	0.041	25-25	2	0.027
18-18	3	0.041	25-26	1	0.014
18-25	2	0.027	25-27	2	0.027
18-27	1	0.014	26-26	3	0.041
18-34	1	0.014	26-33	1	0.014
18-47	1	0.014	27-27	2	0.027
20-20	1	0.014	27-42	1	0.014
20-27	1	0.014	27-45	1	0.014
20-28	3	0.041	27-47	1	0.014
20-37	1	0.014	27-49	1	0.014
21-21	2	0.027	28-28	1	0.014
21-22	3	0.041	28-47	1	0.014
21-25	6	0.082	30-30	1	0.014
21-26	4	0.055	36-36	1	0.014
21-27	1	0.014	39-46	1	0.014
22-22	2	0.027			
22-23	1	0.014			

Table 2- Multiple strains isolated from the same patient and cultured simultaneously.

Patient	Isolate	Body location	CAI genotype
A	1M	Urine	21-25
	2M	Urine	21-25
	31M	Urine	21-25
B	4M	Peritoneal exsudate	26-26
	15M	Peritoneal exsudate	26-26
	17M	Peritoneal exsudate	26-26
	19M	Peritoneal exsudate	26-26
C	10M	Upper respiratory tract	17-17
	12M	Upper respiratory tract	17-17
D	52M	Urine	21-21
	55M	Urine	21-21
E	41M	Urine	21-22
	43M	Urine	21-22
	45M	Urine	21-22
	47M	Urine	21-22
	48M	Urine	21-22
F	82M	Urine	18-47
	84M	Urine	18-47
G	49M	Urine	36-36
	51M	Urine	36-36
H	69M	Upper respiratory tract	21-25
	75M	Upper respiratory tract	21-25
	86M	Upper respiratory tract	21-25
I	64M	Upper respiratory tract	22-22
	67M	Upper respiratory tract	22-22
	88M	Urine	20-28
J	33M	Vagina	23-24
	37M	Urine	17-23

Each patient is referred as a letter (A to J) and the isolates distinguished by a number followed by an M (laboratory designation).

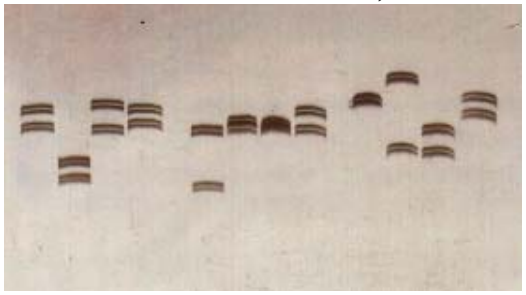
Table 3-CAI genotypes of sequential isolates from vulvovaginal recurrent infections and anorectal/vulvovaginal body locations.

Recurrent strains		**CAI genotype**
Patients	*Isolates*	
K	3J	17-23
	4J	17-23
	5J	17-23
	6J	17-23
L	7J	30-30
	8J	30-32
	9J	30-32
M	12J	18-25
	13J	18-25
	14J	18-25
	15J	18-25
	16J	18-25
N	17J	21-21
	18J	20-29
	19J	20-29
O	20J	26-26
	21J	26-26
P	22J	23-27
	23J	23-27
Q	27J	22-23
	28J	22-23
S	29J	20-20
	30J	20-20
Vagina/Rectus		**CAI genotype**
T	31J-V	21-26
	32J-R	21-26
U	35J-V	25-25
	36J-R	25-25
V	37J-V	18-27
	38J-R	18-27
X	39J-V	21-22
	40J-R	21-22
Z	41J-Ra	23-27
	42J-Rb	23-27
	43J-V	23-27

Each patient was referred as a letter (K to Z) and the isolates distinguished by a number followed by a J (laboratory designation). Strains from vagina/rectus were added an additional letter, V or R for vagina and rectus, respectively.

Figure 1- **A**) Denaturing gel electrophoresis of the fragments obtained by PCR of 12 *C. albicans* clinical isolates for CAI marker **B)** CAI GeneScan profiles observed for four *C. albicans* strains also represented in the gel.

16C 14C 13C 11C 10C 9C 7C 6C 5C, 10M 2C 1C



+

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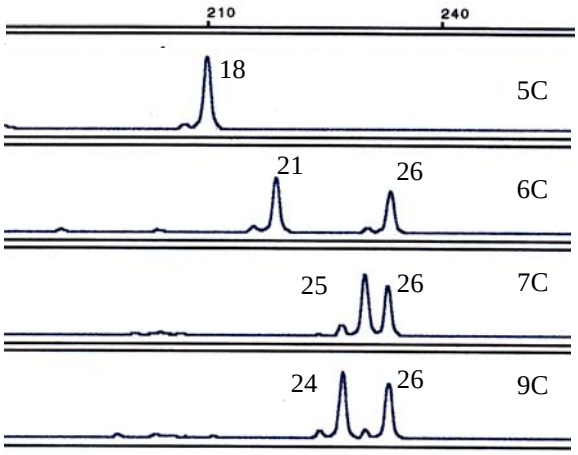
A



B