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New Polymorphic Microsatellite Markers Able To Distinguish among *Candida parapsilosis* Sensu Stricto Isolates[▽]

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Among the *Candida* species causing bloodstream infections, *Candida parapsilosis* is one of the most frequently isolated. The objective of the present work was the identification of new microsatellite loci able to distinguish among *C. parapsilosis* isolates. DNA sequences with trinucleotide repeats were selected from the *C. parapsilosis* genome database. PCR primer sets flanking the microsatellite repeats were designed and tested with 20 independent isolates. On the basis of the amplification efficiency, specificity, and observed polymorphism, four of the sequences were selected for strain typing. Two hundred thirty-three independent *C. parapsilosis* sensu stricto isolates were genotyped by using these markers. The polymorphic loci exhibited from 20 to 42 alleles and 39 to 92 genotypes. In a multiplex analysis, 192 genotypes were obtained and the combined discriminatory power of the four microsatellites was 0.99. Reproducibility was demonstrated by submission of subcultures of 4 isolates each, in triplicate, interspersed with unique numbers among a group of 30 isolates for blind testing. Comparison of the genotypes obtained by microsatellite analysis and those obtained by randomly amplified polymorphic DNA analysis, restriction fragment length polymorphism analysis, and internal transcribed sequence grouping was performed and showed that the microsatellite method could distinguish individual isolates; none of the other methods could do that. Related species, *C. orthopsilosis* and *C. metapsilosis*, were not confused with *C. parapsilosis* sensu stricto. These new microsatellites are a valuable tool for use for the differentiation of *C. parapsilosis* sensu stricto strains, vital in epidemiology to answer questions of strain relatedness and determine pathways of transmission.

Species of the genus *Candida* are the agents that are the most frequently implicated in invasive fungal infections, presently ranking as the third most common cause of nosocomial bloodstream infections in the United States (26, 27). In Europe and Latin America, *Candida parapsilosis* is a predominant species found in patients with systemic infections and causes up to 45% of all cases of candidemia (1, 37). This yeast causes several infections in humans, including a variety of life-threatening conditions triggered by bloodstream infections; and it has been associated with catheters, hyperalimentation, premature birth, and contaminated prosthetic devices (23, 40). *C. parapsilosis* is particularly isolated from cultures of blood from ill neonates and children (20, 23), surgical intensive care unit patients (17), as well as hematological patients (7, 31). Unlike other *Candida* species, *C. parapsilosis* has been found on the hands of health care workers, who install and maintain medical devices, suggesting a potential route for transmission (41). The rapid identification of the strains involved in the infection and the elucidation of their patterns of genetic diversity are issues of great medical relevance since such knowledge can contribute to the development of new strategies for the prevention and treatment of the infections.

Physiologically indistinguishable isolates of *C. parapsilosis* have been reported to be genetically heterogeneous. Several reports suggested that *C. parapsilosis* is composed of three distinct groups, groups I, II, and III, on the basis of isoenzyme analysis, random amplification of polymorphic DNA (RAPD) analysis, analysis of the nucleotide sequence of the internal transcribed sequence (ITS) (16, 19, 21, 25), and DNA-DNA hybridization studies (30). Tavanti et al. (36) proposed that the three different *C. parapsilosis* groups be considered different species on the basis of these observations and the degree of sequence variation observed among groups. Group I isolates maintained the former name, *C. parapsilosis* (sensu stricto), and groups II and III included the strains to which the new names *C. orthopsilosis* and *C. metapsilosis*, respectively, have been proposed. The vast majority of clinical isolates belong to *C. parapsilosis* group I, characterized by low levels of nucleotide sequence variation, which constitutes a challenge for the development of genotyping techniques.

The aim of the work described here was to identify and describe new polymorphic microsatellite loci in the genome of the pathogenic yeast *C. parapsilosis* and evaluate their applicability for use as molecular markers for the differentiation of isolates. The performance of this typing method was assessed by comparison of the results to the results obtained by using three previously described typing methods.

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TABLE 1. Microsatellite DNA sequences selected, sequence codes, and primers used for PCR amplification[a]

Microsatellite designation	Accession no.[b]	Primer sequence[c]	Repetitive motif	Expected fragment size (bp)	Dye label
CP1	cpara1131h11.p1k	FWD: 5′-AAAGTGCTACACACGCATCG-3′ REV: 5′-GGCTTGCAATTTCATTTCCT-3′	$(AAG)_{27}$	249	TET
CP4	cpara838b06.q1k	FWD: 5′-CAAATCATCCAGCTTCAAACC-3′ REV: 5′-CATCAAACAAGAATTCGATATCAC-3′	$(AAC)_{29}$	299	HEX
CP6	cpara692b10.q1k	FWD: 5′-CAGGAACAGGACAATGGTGA-3′ REV: 5′-TCTGGAGCCTCTAGGACGTTT-3′	$(AAC)_{48}$	300	FAM
B5[d]		FWD: 5′-AGGTTTGTAGTAGTGTCCCTATGG-3′ REV: 5′-TATCTCTCTCGCCATTTGAACG-3′	$(CA)_{30}$		FAM

[a] The numbers of repeated units and the predicted sizes of the PCR amplification products were calculated according to the sequence of the strain used in the sequencing project.
[b] Code attributed to the sequence in the Sanger Institute.
[c] FWD, forward primer; REV, reverse primer.
[d] Details are provided in reference 16.

MATERIALS AND METHODS

Microsatellite selection and PCR primer design. A search of *C. parapsilosis* genome sequences available in databases from the Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/c_parapsilosis) was performed to identify sequences containing more than 20 tri- or tetramicrosatellite repeat units which were expected to have very high degrees of polymorphism. Eleven microsatellites were selected, and primers specific for the nonvariable flanking regions were designed for locus-specific amplification. Primer3 software (available from http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used for primer design. On the basis of the results of preliminary studies of amplification efficiency (detailed in Results), species specificity, and the polymorphism observed, 3 of the 11 previously selected sequences were selected for further characterization and for application in strain identification. A microsatellite marker, marker B, previously described for use for *C. parapsilosis* strain typing (18), was also included in this study. Marker B was chosen from among the previously described microsatellite markers due to its higher degree of polymorphism. The microsatellite sequences selected and their repetitive motifs, the primers used for PCR amplification, and the expected fragment sizes are presented in Table 1.

Yeast strains and DNA extraction. A total of 236 independent *C. parapsilosis* isolates, including 100 from blood cultures, 9 from the respiratory tract, 27 from skin and nails, 58 from other body sources, 39 from the environment (including hospital air and surfaces), type strain ATCC 22019, and reference strains PNAEQ008-1/2004 and NQ7356/1850 (clinical isolates used for quality control purposes), were selected for use in this study. Most of them were collected from different places in Portugal, but collections of isolates that included those from a variety of geographical origins were also analyzed. These collections were obtained from France, Spain, the United States, and Peru. The type strains of *C. albicans* (ATCC 18804), *C. krusei* (ATCC 6258), *C. tropicalis* (ATCC 750), *C. glabrata* (ATCC 2001), *C. bracarensis* (NCYC D3853), *C. guilliermondii* (ATCC 6260), *C. lusitaniae* (ATCC 34449), *C. dubliniensis* (CBS 7987), *C. orthopsilosis* (ATCC 96139), *C. metapsilosis* (ATCC 96144), and *Lodderomyces elongisporus* (ATCC 22688) were also tested. One isolate of *Aspergillus fumigatus* as well as isolates of the bacteria *Escherichia coli* and *Staphylococcus aureus*, obtained from the culture collection of our laboratory, were also included in this study. All *C. parapsilosis* isolates were identified by using ID 32C strips or Vitek YBC identification cards (bioMérieux, SA, Marcy l'Étoile, France) and by PCR fingerprinting with primer T3B by using the methodology described previously (6). Stock cultures were maintained on Sabouraud glucose agar medium at 4°C. Prior to DNA isolation, yeast cells were grown for 24 h on Sabouraud glucose agar medium at 37°C. DNA extraction was performed by using a High Pure PCR template kit (Roche Diagnostics Corp., Indianapolis, IN), according to the manufacturer's instructions.

PCR amplification conditions. For all microsatellite loci, PCRs were performed with several different strains to evaluate the locus-specific amplification. Loci CP1, CP4, and CP6 (their selection is described in Results) were amplified by using 25 ng of genomic DNA in a 25-μl reaction volume containing 1× PCR buffer, 0.2 mM each four deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.25 μM each primer, and 1 U of *Taq* polymerase (Applied Biosystems, Inc., Foster City, CA). After a 95°C preincubation step for 2 min, PCR amplifications were performed in a total of 28 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 54 to 60°C (depending on the locus) for 30 s, and extension at 72°C for 1 min and with a final extension step of 7 min at 72°C.

Fragment size determination and DNA sequence analysis. Following PCR, the amplification products were diluted 1:10 with distilled water and a 1-μl aliquot was added to 13.7 μl of formamide and 0.3 μl of the internal size standard (GenScan 500 6-carboxytetramethylrhodamine [TAMRA]; Applied Biosystems, Inc.). The samples were denatured at 95°C for 5 min and rapidly chilled on ice. The samples were run on an ABI 310 genetic analyzer (Applied Biosystems). For accurate allele size determination, the forward primers were fluorescently labeled with tetrachloro-6-carboxyfluorescein (TET), 6-carboxyhexafluorescein (HEX), or 6-carboxyfluorescein (FAM) (Table 1) and the sizes of the PCR products were determined by using GeneScan (version 3.7) analysis software. The alleles have been designated by their sizes (in base pairs).

For DNA sequence analysis, only homozygous strains were selected. After PCR amplification, the products were purified with Microspin S-300 HR columns (Pharmacia). The purified products were subjected to a dideoxy cycle sequencing reaction with a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequence analysis was performed on an ABI 310 genetic analyzer.

RFLP, RAPD, and ITS sequencing. RFLP, RAPD, and ITS analyses were performed as described previously (38).

Stability. Three different clinical isolates and the reference strains were grown in 1-liter Erlenmeyer flasks containing 500 ml of Sabouraud medium and incubated at 30°C in an orbital shaker (160 rpm). At the end of the exponential phase, a 1/10 dilution with new medium was made in order to allow the continuation of cell duplication. This procedure went on for 4 weeks, until the completion of about 300 generations. The cells were harvested at the end of approximately 100, 200, and 300 generations; and the DNA was extracted for amplification.

Reproducibility. The reproducibility of the microsatellite method was assessed systematically by including a reference strain as a control in each run. In addition, samples were included in some runs in coded replicates, prepared by coinvestigators not involved in that microsatellite run (blind testing; see Results).

Statistical analysis. Allelic and genotypic frequencies were determined by using ARLEQUIN (version 2.000) software, and the discriminatory power of the markers was calculated as described by Hunter and Gaston (15).

RESULTS

Screening and selection of repeat regions in *C. parapsilosis* genome sequence database. The search of the genomic DNA database for *C. parapsilosis* sequences that was performed provided ~1,000 sequences when AAC, AAG, AAT, CCA, CCG, CTT, CAAA, and AACA queries were used. Selection of the sequences was primarily based on the number of simple units repeated. The criteria defined were that the sequence should be at least 20 repeats, given that these sequences have a higher

TABLE 2. Characteristics of the microsatellite loci selected

Microsatellite designation	No. of alleles	Size range (bp)	No. of repeats	Allele frequency[a]	No. of genotypes	Genotype frequency[a]	DP	% heterozygosity
CP1	20	207–270	14–35	0.002–0.352	39	0.004–0.279	0.85	73.3
CP4	42	240–426	10–72	0.002–0.307	68	0.004–0.27	0.89	21.4
CP6	42	177–375	7–73	0.002–0.176	92	0.004–0.129	0.96	52.3
B5	22	95–153		0.002–0.397	40	0.004–0.33	0.86	30.0
Multiplex					192	0.004–0.047	0.99	

[a] Values are minimums-maximums.

probability of showing greater genetic variability, and they should be located outside known coding regions, since a higher degree of polymorphism is expected in these regions less prone to selective forces (12, 24, 28). Subsequently, another selection was made for sequences that allowed the design of primers with an annealing temperature above 54°C to ensure specificity and good reproducibility. Eleven sequences met our criteria and were selected for locus-specific primer design and amplification in studies with a set of independent *C. parapsilosis* strains. The nomenclature chosen for the new markers was CP, after *C. parapsilosis*, followed by a number that corresponded to the order in which they were studied in the pilot experiments. On the basis of the results of the amplification of DNA from 23 selected *C. parapsilosis* strains, seven markers did not show suitable amplification efficiencies and two markers, although they were chosen from different sequences, turned out to be the same locus. The remaining three, CP1, CP4, and CP6, gave satisfactory results with regard to the selection criteria and were used for further characterization. Table 1 shows the chosen loci and the respective repetitive motifs, the numbers of repetitions, as well as the expected PCR product size, according to the database sequence.

Sequence analysis. Several representative alleles have been sequenced, and sequencing analysis of the amplified fragments revealed that the consensus sequences were in accordance with those originally published (Table 1), confirming the locus-specific amplification and structure of the alleles. A direct correlation between the fragment size and the number of microsatellite repeats was found for all the markers, with the differences in fragment sizes being consistent with the variation in the number of repetitions.

Specificity and stability. These markers were revealed to be species specific, since no amplification products were obtained when the primers and PCR conditions described were used for the screening of other pathogenic *Candida* species, namely, *C. albicans*, *C. krusei*, *C. tropicalis*, *C. glabrata*, *C. bracarensis*, *C. guilliermondii*, *C. lusitaniae*, *C. dubliniensis*, and *C. metapsilosis*. Faint amplification was detected with microsatellite CP6 by analysis with GeneScan software when strains of *C. orthopsilosis* were tested. Raising the PCR annealing temperature to 60°C for the amplification of marker CP6 eliminated the amplicons of the *C. orthopsilosis* strains. This alteration did not interfere with the amplification of the *C. parapsilosis* isolates.

No PCR products were obtained when DNA from *Lodderomyces elongisporus*, a species closely related to and recently described in cases of bloodstream infections misidentified as *C. parapsilosis*, was amplified (22). Likewise, no amplification was obtained with the fungus *Aspergillus fumigatus* or with the bacteria *Escherichia coli* and *Staphylococcus aureus*.

The *in vitro* stability of the microsatellites was followed over 300 generations. For all the strains tested, the genotypes were the same after these generations, suggesting that their expected mutation rate is less than 3.33×10^{-3}. Similar results have previously been reported for *C. albicans* microsatellites (32, 33).

Microsatellite locus analysis. The microsatellites selected were used to type 236 independent *C. parapsilosis* isolates, including clinical isolates from different origins and environmental strains, in order to evaluate their specific amplifications and polymorphisms. One or two PCR fragments per locus were obtained for each strain, and since *C. parapsilosis* is a diploid species (5), each fragment was assigned to an allele. The strains showing two PCR products were typed as heterozygous, while strains presenting a single amplification product were considered homozygous. Less intense stutter bands were frequently present, reflecting polymerase slippage during the PCR, but they did not present any problem with the identification of the correct fragment since they were of a lower intensity. No PCR products were obtained with the new microsatellite markers from three bloodstream isolates assigned to the species *C. parapsilosis*. These isolates were further analyzed by sequencing of the ITS and D1/D2 regions of the 26S rDNA. This showed that two of the isolates were indeed *C. metapsilosis* strains and that the other was a strain of *C. orthopsilosis*. PCR products were obtained for all the other 233 *C. parapsilosis* isolates at all three loci, resulting in 100% typeability.

The analysis of the 233 isolates showed that all the microsatellite loci were polymorphic, presenting between 20 and 42 alleles and from 39 to 92 different genotypes. The size ranges of the alleles as well as the corresponding number of repeats are shown in Table 2. A significant departure from Hardy-Weinberg equilibrium expectations was found ($P < 0.001$), supporting the suggestion that inheritance in *C. parapsilosis* is mainly clonal, as has been inferred by other authors (36).

The discriminatory power (DP) was calculated for each marker according to the Simpson index:

$$\mathrm{DP} = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} nj(nj - 1)$$

where N is the number of strains, s is the total number of different genotypes, and nj is the number of strains of genotype j (15). The results showed that CP6 was the microsatellite with the highest DP value (0.96), while CP1 presented the lowest DP value (0.85). Microsatellite marker B produced 22 different alleles and 40 genotypes, resulting in a discriminatory power of 0.86 (18). The total number of different alleles and genotypes

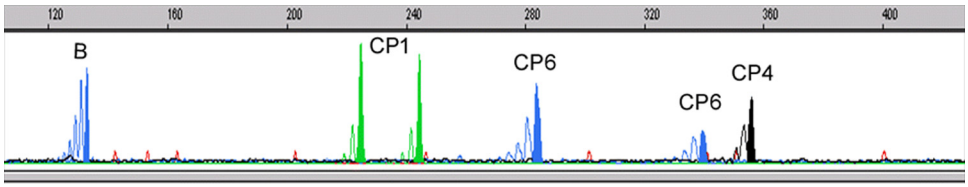

FIG. 1. GenScan profile obtained by multilocus analysis with the markers CP1 (green), CP4 (black), CP6 (blue), and B (blue). The strain analyzed is homozygous for markers CP4 and B and heterozygous for markers CP1 and CP6.

and the respective frequencies obtained for all microsatellite markers are presented in Table 2.

In the multiplex analysis with markers CP1, CP4, CP6, and B, a total of 192 multilocus genotypes were obtained and the combined discriminatory power was 0.99. An example of a multilocus GeneScan profile is shown in Fig. 1. The great majority of the multilocus genotypes were found only once (79%). The most frequently detected multilocus genotype was shared by 11 independent widespread strains, and the 13 most frequently detected multilocus genotype accounted for 21% of the isolates (Table 3).

Comparison of results of different *C. parapsilosis* typing methods. Thirty isolates of the *C. parapsilosis* family typed by ITS sequencing, RFLP analysis, and RAPD analysis were selected for genotyping with the microsatellite markers described above so that the results of the microsatellite marker genotyping method could be compared with those of the other typing methods. Ten isolates of *C. orthopsilosis* and *C. metapsilosis* strains (including one *C. metapsilosis* isolate and one *C. orthopsilosis* isolate, each included in triplicate as a blind reproducibility test) were correctly identified as not *C. parapsilosis* sensu stricto (these would be RAPD groups B and C and RFLP groups other than VII-1). The results of microsatellite genotyping of the isolates determined to be *C. parapsilosis* sensu stricto are shown in Table 4. Fourteen different multilocus genotypes were obtained. Two isolates of *C. parapsilosis* sensu stricto (RAPD group A, RFLP group VII-1) were included in triplicate (isolates IMR013, IMR160, and IMR339 and isolates IMR073, IMR320, and IMR 327, respectively) as a blind reproducibility test, and the isolates in each triplet were correctly identified to be identical.

TABLE 3. Most common multilocus genotypes found among the isolates analyzed and their respective frequencies

Multilocus genotype[a]				No. of isolates with common multilocus genotype	Frequency
CP1	CP4	CP6	B		
222/243	354/354	282/282	129/129	5	0.021
222/243	354/354	282/336	127/127	3	0.013
222/243	354/354	282/336	129/129	11	0.047
222/243	354/354	282/339	129/129	4	0.017
222/243	354/354	321/321	129/129	4	0.017
222/243	354/354	336/336	129/129	5	0.021
222/243	354/354	339/339	129/129	2	0.009
240/243	342/342	285/285	103/103	4	0.017
240/243	360/360	273/321	129/129	2	0.009
240/252	300/300	285/285	147/149	3	0.013
240/252	300/300	285/285	149/149	2	0.009
243/246	354/354	300/318	127/129	2	0.009
243/249	294/294	261/309	131/131	2	0.009

[a] The numbers before and after the slashes indicate the fragment sizes (bp) of the different alleles obtained with the indicated marker.

Thus, from 15 truly unique isolates, 14 different multilocus genotypes were obtained. Two *C. parapsilosis* sensu stricto isolates, isolates IMR649 and IMR013 (and blind replicate isolates of IMR013, isolates IMR160 and IMR339) could not be distinguished by microsatellite typing (nor could they be distinguished by RFLP, RAPD, or ITS1 typing). These two isolates originally came from two different sites (the mouth and the vagina) from patients in two different cities in England (Leeds and London) (although the overall collection studied [36, 38] came from a broad geographic background) in the early 1970s (and were possibly collected in the same year). The two isolates came from two different sets of isolates gathered by the same researcher in both places and presumably came from two different patients. Although one cannot completely exclude the possibility that they were the same patient who may have been involved in two different studies, the best explanation is that two patients were infected with the same genotype, and whether there was any connection between the patients is unknown.

Two unique isolates are special cases. Both had discrepant RAPD results in two laboratories (36, 38), with one laboratory grouping them with *C. parapsilosis* sensu stricto and the other not. These were not RFLP type VII-1, which is generally synonymous with *C. parapsilosis* sensu stricto (38), and were more consistent with *C. orthopsilosis* types, as delineated by RFLP analysis, whereas they were grouped with *C. parapsilosis* sensu stricto by ITS1 sequencing. Thus, it was tentatively concluded (38) either that RFLP analysis can differentiate a few types of *C. parapsilosis* sensu stricto that ITS typing cannot or that these isolates represented some hybrid group sharing some characteristics with *C. parapsilosis* sensu stricto and *C. orthopsilosis*. By microsatellite typing, one of these two isolates was determined not to be *C. parapsilosis* sensu stricto, tentatively grouping the isolate with *C. orthopsilosis* and *C. metapsilosis* (data not shown). The other isolate (isolate IMR082 in Table 4) was determined to be *C. parapsilosis* sensu stricto by microsatellite typing.

DISCUSSION

A variety of strain typing methods have been used for the differentiation of the *C. parapsilosis* family, including electrophoretic karyotype (EK) analysis (9, 35), DNA fingerprinting with probe Cp3-13 (11), RAPD analysis (10, 42), RFLP analysis (38, 39), multilocus sequence typing (MLST) (36), and pyrosequencing (2). van Asbeck et al. (38) compared three of these molecular typing methods, ITS sequencing, RAPD anal-

TABLE 4. Correlation of RLFP, RAPD, and ITS1 typing results with results of microsatellite analysis

Candida parapsilosis isolate	Result by:			Fragment sizes (bp) for microsatellite[d]			
	RFLP analysis	RAPD analysis[a]	ITS1 typing	CP1	CP4	CP6	B
IMR013[b]	VII-1	Ao	I	207/240	300/300	294/294	133/133
IMR160[b]				207/240	300/300	294/294	133/133
IMR339[b]				207/240	300/300	294/294	133/133
IMR649	VII-1	Ao	I	207/240	300/300	294/294	133/133
IMR073[c]	VII-1		I	222/243	354/354	282/339	129/129
IMR320[c]				222/243	354/354	282/339	129/129
IMR327[c]				222/243	354/354	282/339	129/129
IMR048	VII-1	Ao	I	240/243	360/360	297/345	129/151
IMR082	V-3	Ao, Bv*	I	243/243	414/414	297/318	127/127
IMR612	VII-1	Ao	I	222/243	354/354	282/282	129/129
IMR759	VII-1		I	243/246	354/393	309/309	127/127
IMR760	VII-1		I	207/240	300/300	228/333	131/131
IMR761	VII-1	Ao	I	240/240	300/300	276/294	127/127
IMR762	VII-1	Ao	I	222/243	351/354	282/363	129/129
IMR763	VII-1	Ao	I	222/243	354/354	282/321	127/129
IMR764	VII-1	Ao	I	222/243	366/366	321/321	129/131
IMR765	VII-1	Ao	I	222/243	363/363	321/330	129/129
IMR766	VII-1	Ao	I	240/240	300/300	294/294	133/133
IMR767	VII-1	Av	I	240/243	336/336	285/321	105/105

[a] Ao, see reference 33; Bv and Av, see reference 35; *, see text.
[b] Blind replicates.
[c] Blind replicates.
[d] The numbers before and after the slashes indicate the fragment sizes of the different alleles obtained with the indicated marker.

ysis, and RFLP analysis, and indicated that ITS1 typing and RFLP analysis appear to have similar potentials to differentiate *C. parapsilosis* and both have discriminatory abilities superior to the discriminatory ability of RAPD analysis. This inferiority of RAPD analysis was also true for other methods, which subdivide the *C. parapsilosis* sensu lato complex into only three or a few more groups, as ITS sequencing and RFLP analysis are able to subtype RAPD type B/group II/*C. orthopsilosis*. However, the inability of ITS1 typing and RFLP analysis to subtype the dominant group, group I/*C. parapsilosis* sensu stricto, has been an obstacle.

The only previously reported method able to distinguish isolates within the *C. parapsilosis* sensu stricto group is a microsatellite method based on dinucleotide repeats, but the combined DP of 0.97 achieved for all seven loci was not optimal (18). Furthermore, the typing of dinucleotide microsatellites presents limitations for accurate allele identification, even in automatic systems, owing to the high frequency of dinucleotide repeat slippage.

Analysis of microsatellite loci has an advantage over other commonly used typing methods because microsatellite loci behave as codominant markers, evolve rapidly in the genome, and may be able to distinguish between isolates and detect microevolutionary variations (32). The microsatellite genotyping of several yeast species, namely, *Saccharomyces cerevisiae* (14), *C. albicans* (3, 4, 33), *C. krusei* (34), and *C. glabrata* (13), has been described.

In the present report, we described new microsatellite markers for *C. parapsilosis* sensu stricto. In a multilocus analysis, 192 genotypes were found among 233 isolates, resulting in a DP of 0.99, much higher than that published previously. This microsatellite method would therefore be a more robust method for epidemiologic purposes than RFLP analysis, RAPD analysis, MLST, or ITS1 sequencing. None of those methods can distinguish isolates, possibly with rare exceptions (38), coincident with the most common group encountered in patients or the environment, *C. parapsilosis* sensu stricto. The reproducibility of this microsatellite method was demonstrated by the inclusion of replicates in blind testing. Isolates of the related species *C. orthopsilosis*, *C. metapsilosis*, and *L. elongisporus*, isolates of other *Candida* species, and isolates of other fungal and bacterial species were not confused with *C. parapsilosis* sensu stricto.

The high degree of polymorphism observed could be correlated with a high mutation rate, which would eventually limit the use of these markers for strain identification. However, our results demonstrate that these microsatellites are relatively stable in laboratory culture, with the estimated mutation rate being less than 3×10^{-3}. Mutation rates for microsatellites can range from 10^{-2} to 10^{-6}, while point mutations are known to accumulate more slowly, at approximately 10^{-9} mutations per generation (8, 29). Therefore, for outbreak investigations, which usually span short time intervals, we believe that the microsatellites are suitable markers.

In conclusion, these new microsatellites are a valuable tool for use for the differentiation of *C. parapsilosis* strains and for application in studies that must distinguish epidemiologically related isolates, study nosocomial cross-transmission, and study the kinetics of the colonization-to-infection process. This method is easy to perform, discriminatory, and highly reproducible, constituting the ideal method for use in large-scale epidemiological studies. The standardization of the microsatellite typing systems and the creation of public databases that would make microsatellite allele data available worldwide are essential issues that deserve attention.

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REFERENCES

1. **Almirante, B., D. Rodriguez, M. Cuenca-Estrella, M. Almela, F. Sanchez, J. Ayats, C. Alonso-Tarres, J. L. Rodriguez-Tudela, A. Pahissa, and the Barcelona Candidemia Project Study Group.** 2006. Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. J. Clin. Microbiol. **44:**1681–1685.
2. **Borman, A. M., C. J. Linton, D. Oliver, M. D. Palmer, A. Szekely, F. C. Odds, and E. M. Johnson.** 2009. Pyrosequencing analysis of 20 nucleotides of internal transcribed spacer 2 discriminates *Candida parapsilosis*, *Candida metapsilosis*, and *Candida orthopsilosis*. J. Clin. Microbiol. **47:**2307–2310.
3. **Botterel, F., C. Desterke, C. Costa, and S. Bretagne.** 2001. Analysis of microsatellite markers of *Candida albicans* used for rapid typing. J. Clin. Microbiol. **39:**4076–4081.
4. **Bretagne, S., J. M. Costa, C. Besmond, R. Carsique, and R. Calderone.** 1997. Microsatellite polymorphism in the promoter sequence of the elongation factor 3 gene of *Candida albicans* as the basis for a typing system. J. Clin. Microbiol. **35:**1777–1780.
5. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, et al.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature **459:**657–662.
6. **Correia, A., P. Sampaio, J. Almeida, and C. Pais.** 2004. Study of molecular epidemiology of candidiasis in Portugal by PCR fingerprinting of *Candida* clinical isolates. J. Clin. Microbiol. **42:**5899–5903.
7. **Costa-de-Oliveira, S., C. Pina-Vaz, D. Mendonça, and A. G. Rodrigues.** 2008. A first Portuguese epidemiological survey of fungemia in a university hospital. Eur. J. Clin. Microbiol. Infect. Dis. **27:**365–374.
8. **Dallas, J. F.** 1992. Estimation of microsatellite mutation rates in recombinant inbred strains of mouse. Mamm. Genome **3:**452–456.
9. **De Bernardis, F., F. Mondello, R. S. Millán, J. Pontón, and A. Cassone.** 1999. Biotyping and virulence properties of skin isolates of *Candida parapsilosis*. J. Clin. Microbiol. **37:**3481–3486.
10. **Diekema, D. J., S. A. Messer, R. J. Hollis, R. P. Wenzel, and M. A. Pfaller.** 1997. An outbreak of *Candida parapsilosis* prosthetic valve endocarditis. Diagn. Microbiol. Infect. Dis. **29:**147–153.
11. **Enger, L., S. Joly, C. Pujol, P. Simonson, M. A. Pfaller, and D. R. Soll.** 2001. Cloning and characterization of a complex DNA fingerprinting probe for *Candida parapsilosis* J. Clin. Microbiol. **39:**658–669.
12. **Field, D., and C. Wills.** 1998. Abundant microsatellite polymorphism in *Saccharomyces cerevisiae*, and the different distributions of microsatellites in eight prokaryotes and *S. cerevisiae*, result from strong mutation pressures and a variety of selective forces. Proc. Natl. Acad. Sci. U. S. A. **95:**1647–1652.
13. **Foulet, F., N. Nicolas, O. Eloy, F. Botterel, J.-C. Gantier, J.-M. Costa, and S. Bretagne.** 2005. Microsatellite marker analysis as a typing system for *Candida glabrata*. J. Clin. Microbiol. **43:**4574–4579.
14. **Hennequin, C., A. Thierry, G. Richard, G. Lecointre, H. V. Nguyen, C. Gaillardin, and B. Dujon.** 2001. Microsatellite typing as a new tool for identification of *Saccharomyces cerevisiae* strains. J. Clin. Microbiol. **39:**551–559.
15. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
16. **Kato, M., M. Ozeki, A. Kikuchi, and T. Kanbe.** 2001. Phylogenetic relationship and mode of evolution of yeast DNA topoisomerase II gene in the pathogenic *Candida* species. Gene **272:**275–281.
17. **Kuhn, D. M., P. K. Mukherjee, T. A. Clark, C. Pujol, J. Chandra, R. A. Hajjeh, D. W. Warnock, D. R. Soll, and M. A. Ghannoum.** 2004. *Candida parapsilosis* characterization in an outbreak setting. Emerg. Infect. Dis. **10:**1074–1081.
18. **Lasker, B. A., G. Butler, and T. J. Lott.** 2006. Molecular genotyping of *Candida parapsilosis* group I clinical isolates by analysis of polymorphic microsatellite markers. J. Clin. Microbiol. **44:**750–759.
19. **Lehmann, P. F., D. M. Lin, and B. A. Lasker.** 1992. Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. J. Clin. Microbiol. **30:**3249–3254.
20. **Levy, I., I. Rubin, S. Vasishtha, V. Tucci, and S. K. Sood.** 1998. Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. Clin. Infect. Dis. **26:**1086–1088.
21. **Lin, D. M., L. C. Wu, M. G. Rinaldi, and P. F. Lehmann.** 1995. Three distinct genotypes within *Candida parapsilosis* from clinical sources. J. Clin. Microbiol. **33:**1815–1821.
22. **Lockhart, S. R., S. A. Messer, M. A. Pfaller, and D. J. Diekema.** 2008. *Lodderomyces elongisporus* masquerading as *Candida parapsilosis* as a cause of bloodstream infections. J. Clin. Microbiol. **46:**374–376.
23. **Lupetti, A., A. Tavanti, P. Davini, E. Ghelardi, V. Corsini, I. Merusi, A. Boldrini, M. Campa, and S. Senesi.** 2002. Horizontal transmission of *Candida parapsilosis* candidemia in a neonatal intensive care unit. J. Clin. Microbiol. **40:**2363–2369.
24. **Metzgar, D., J. Bytof, and C. Wills.** 2002. Selection against frameshift mutations limits expansion in coding DNA. Genome Res. **10:**72–80.
25. **Nosek, J., L. Tomaska, A. Rycovska, and H. Fukuhara.** 2002. Mitochondrial telomeres as molecular markers for identification of the opportunistic yeast pathogen *Candida parapsilosis*. J. Clin. Microbiol. **40:**1283–1289.
26. **Perlroth, J., B. Choi, and B. Spellberg.** 2007. Nosocomial fungal infections: epidemiology, diagnosis and treatment. Med. Mycol. **45:**321–346.
27. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. **20:**133–163.
28. **Pupko, T., and D. Graur.** 1999. Evolution of microsatellites in the yeast *Saccharomyces cerevisiae*: role of length and number of repeated units. J. Mol. Evol. **48:**313–316.
29. **Richard, G. F., C. Hennequin, A. Thierry, and B. Dujon.** 1999. Trinucleotide repeats and other microsatellites in yeasts. Res. Microbiol. **150:**589–602.
30. **Roy, B., and S. A. Meyer.** 1998. Confirmation of the distinct genotype groups within the form species *Candida parapsilosis*. J. Clin. Microbiol. **36:**216–218.
31. **Sabino, R., C. Veríssimo, J. Brandão, C. Alves, H. Parada, L. Rosado, E. Paixão, Z. Videira, T. Tendeiro, P. Sampaio, and C. Pais.** 5 August 2009. Epidemiology of candidemia in oncology patients: a 6-year survey in a Portuguese central hospital. Med. Mycol. **48:**346–354.
32. **Sampaio, P., L. Gusmão, A. Correia, C. Alves, A. G. Rodrigues, C. Pina-Vaz, A. Amorim, and C. Pais.** 2005. New microsatellite multiplex PCR for *Candida albicans* strain typing reveals microevolutionary changes. J. Clin. Microbiol. **43:**3869–3876.
33. **Sampaio, P., L. Gusmão, C. Alves, C. Pina-Vaz, A. Amorim, and C. Pais.** 2003. Highly polymorphic microsatellite for identification of *Candida albicans* strains. J. Clin. Microbiol. **41:**552–557.
34. **Shemer, R., Z. Weissman, and N. Hashman.** 2001. A highly polymorphic degenerate microsatellite for molecular strain typing of *Candida krusei*. Microbiology **147:**2021–2028.
35. **Shin, J. H., D. H. Shin, J. W. Song, S. J. Kee, S. P. Suh, and D. W. Ryang.** 2001. Electrophoretic karyotype analysis of sequential *Candida parapsilosis* isolates from patients with persistent or recurrent fungemia. J. Clin. Microbiol. **39:**1258–1263.
36. **Tavanti, A., A. D. Davidson, N. A. Gow, M. C. J. Maiden, and F. C. Odds.** 2005. *Candida orthopsilosis* and *Candida metapsilosis* sp. nov. to replace *Candida parapsilosis* groups II and III. J. Clin. Microbiol. **43:**284–292.
37. **Tortorano, A. M., C. Kibbler, J. Peman, H. Bernhardt, L. Klingspor, and R. Grillot.** 2006. Candidemia in Europe: epidemiology and resistance. Int. J. Antimicrob. Agents **27:**359–366.
38. **van Asbeck, E. C., K. V. Clemons, A. N. Markham, and D. A. Stevens.** 2009. Correlation of restriction fragment length polymorphism genotyping with internal transcribed spacer sequence, randomly amplified polymorphic DNA and multilocus sequence groupings for *Candida parapsilosis*. Mycoses **52:**493–498.
39. **van Asbeck, E. C., K. V. Clemons, A. N. Markham, D. A. Stevens, and the Candida Parapsilosis Global Epidemiology Group.** 2008. Molecular epidemiology of the global and temporal diversity of *Candida parapsilosis*. Scand. J. Infect. Dis. **40:**827–834.
40. **van Asbeck, E. C., K. V. Clemons, and D. A. Stevens.** 2009. *Candida parapsilosis*: a review of its epidemiology, pathogenesis, clinical aspects, typing, and antimicrobial susceptibility. Crit. Rev. Microbiol. **35:**283–309.
41. **Wenzel, R., and M. Edmond.** 2001. The impact of hospital-acquired bloodstream infections. Emerg. Infect. Dis. **7:**174–177.
42. **Zancopé-Oliveira, R. M., J. James, P. Derossia, J. L. M. Sampaio, M. M. Muniz, R. K. Li, A. S. A. Nascimento, J. M. Peralta, and E. Reiss.** 2000. Strain characterization of *Candida parapsilosis* fungemia by molecular typing methods. Eur. J. Clin. Microbiol. Infect. Dis. **19:**514–520.