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Differentiation of *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* by specific PCR amplification of the *RPS0* intron

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ABSTRACT

Although *Candida parapsilosis* is the most prevalent among the 3 species of the **psilosis* group, studies applying DNA-based diagnostic techniques with isolates previously identified as *C. parapsilosis* have revealed that both *C. orthopsilosis* and *C. metapsilosis* account for 0–10% of all these isolates, depending on the geographical area. Differences in the degrees of antifungal susceptibility and virulence have been found, so a more precise identification is required. In a first approach, we reidentified 38 randomly chosen clinical isolates, previously identified as *C. parapsilosis,* using the RPO2 (CA2) RAPD marker. Among them, we reclassified 4 as *C. metapsilosis* and 5 as *C. orthopsilosis*. We previously developed a method to identify different pathogen yeast species, including *C. parapsilosis*, based on the amplification of the *RPS0* gene intron. In this work, we extend this approach to the new **psilosis* species by partially sequencing their *RPS0* gene, including the intron sequence. Based on intron sequences, we designed specific primers capable of identifying *C. orthopsilosis* and *C. metapsilosis* species, and we reidentified species among the initial isolates. These new primers have allowed a specific and rapid identification of *C. orthopsilosis* and *C. metapsilosis*.

Introduction

Although *Candida albicans* is the species most frequently isolated from clinical specimens, other species (*Candida non-albicans*, *CNA*) have increased in the last decade. *CNA* are of particular concern, and some are highly virulent, with reduced susceptibility to antifungal agents, which leads to treatment failure.

C. parapsilosis is an emergent human pathogen (Trofa et al., 2008), often identified as the second most commonly isolated *Candida* species from blood cultures (Pemán et al., 2002, 2005; Tortorano et al., 2004, 2006). In accord with phenotypic and molecular data (Lin et al., 1995) *C. parapsilosis* was divided into 3 groups, I–III. However, recently on the basis of molecular genetic studies, *C. parapsilosis* has been segregated into 3 different species, converting groups II and III into *C. orthopsilosis* and *C. metapsilosis,* respectively; while group I remains as *C. parapsilosis* (Tavanti et al., 2005). Nevertheless, *C. parapsilosis* is responsible for the vast majority of clinical diseases associated with these species. Prior studies with DNA-based diagnostic techniques on isolates previously identified as *C. parapsilosis* have revealed that both *C. orthopsilosis* and *C. metapsilosis* frequency has a geographical variation, with a range between 0% and 10% of all these strains (Chen et al., 2010; Gómez-López et al., 2008; Lockhart et al., 2008a; Silva et al., 2009; Tay et al., 2009). Since there are no commercial systems able to discriminate between these species, clinical reports normally set them together.

According to the studies conducted in vitro with human tissue cultures derived from oral and epidermal epithelium cells, *C. orthopsilosis* isolates cause similar damage to *C. parapsilosis*, while *C. metapsilosis* is less virulent (Gácser et al., 2007). Moreover, antifungal susceptibility differences among the 3 species have been reported (Cantón et al., 2010; Gómez-López et al., 2008; Lockhart et al., 2008a; Tay et al., 2009; van Asbeck et al., 2009) and could affect therapeutic choices.

This genetic variability of *C. parapsilosis* creates the need for a more precise identification method. In this context, different approaches described by Tavanti et al. (2005) based on RAPD (van Asbeck et al., 2008b, 2009), RFLP of *SADH* locus (Mirhendi et al., 2010), sequencing of ribosomal genes (Chen et al., 2010; Gómez-López et al., 2008), and microsatellite analysis (Lasker et al., 2006) have been used to assess the differences between these species. In this work, we describe a specific, sensitive, fast, low-cost method

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Table 1
Yeast isolates and identification by molecular methods. Amplification with specific primers CP1/CP2 for *C. parapsilosis* and amplification with RAPD primer CA2.

Isolate	CP1/CP2	CA2 (RPO2) pattern
ATCC22019	+	*C. parapsilosis*
F109	–	*C. orthopsilosis*
F130	–	*C. metapsilopsis*
F131	–	*C. orthopsilosis*
F132	–	*C. metapsilopsis*
F133	–	*C. metapsilopsis*
F150	–	*C. orthopsilosis*
F151	–	*C. orthopsilosis*
F166	+	*C. parapsilosis*
F167	+	*C. parapsilosis*
F168	+	*C. parapsilosis*
F169	+	*C. parapsilosis*
F170	–	*C. orthopsilosis*
F172	+	*C. parapsilosis*
F174	+	*C. parapsilosis*
F175	+	*C. parapsilosis*
F181	+	*C. parapsilosis*
F182	+	*C. parapsilosis*
F183	+	*C. parapsilosis*
F184	+	*C. parapsilosis*
F259	+	*C. parapsilosis*
F260	+	*C. parapsilosis*
F261	+	*C. parapsilosis*
F262	+	*C. parapsilosis*
F263	+	*C. parapsilosis*
F264	+	*C. parapsilosis*
F265	+	*C. parapsilosis*
F266	+	*C. parapsilosis*
F267	+	*C. parapsilosis*
F268	+	*C. parapsilosis*
F269	+	*C. parapsilosis*
F270	–	*C. metapsilopsis*
F271	+	*C. parapsilosis*
F272	+	*C. parapsilosis*
F273	+	*C. parapsilosis*
F274	+	*C. parapsilosis*
F275	+	*C. parapsilosis*
F276	+	*C. parapsilosis*
F277	+	*C. parapsilosis*

for the identification of these new species, with a potential application in clinical identification.

Materials and methods

Microorganisms

The 38 blood culture isolates listed in Table 1 were obtained from the University Hospital 'La Fe' (Valencia, Spain). The yeast strains used as control in this work, C. *parapsilosis* CECT13009 (AM 2001/0013), *C. metapsilosis* CECT13010 (J960161), and *C. orthopsilosis* CECT13011 (J981226), were originally provided by Dr. Odds, and *Lodderomyces elongisporus*, used as a negative control, was provided by the Spanish Type Culture Collection (CECT10645). The blood culture isolates were previously identified as *C. parapsilosis* by standard microbiological methods and by the VITEK system (bioMerieux, Madrid, Spain). However, some of these isolates were not identified as *C. parapsilosis* by the molecular method based on PCR recently described by our group (García Martínez et al., 2010). *Escherichia coli DH5α* [*F,ϕ80, lac4M15, recA1, endA1, gyrA96, thi-1 (rk⁻, mk⁻), supE44. relA1, deoR, Δ(lacZYA-argF) U16*] (Hanahan, 1983) was the recipient for plasmids.

Yeasts were cultured on either Sabouraud chloramphenicol agar (PRONADISA-CONDA, Torrejón de Ardoz, Spain) or YPD (1% yeast extract, 2% peptone, 2% dextrose) at 28 °C. *E. coli* was grown in LB medium (1% peptone, 0.5% yeast extract, 0.5% NaCl), supplemented with 100 μg/ml ampicillin as required for transformant selection.

Table 2
Oligonucleotides used in this work.

Oligonucleotides	Sequence	Source/ Reference
ISARPS5A	5′-TTGACTTAACTCCAGAAGACGC-3′	This study
ISARPS3B	5′-TCTGTAGAAGTACAAATCTGGCAT-3′	This study
ISARPS5C	5′-ATGTCATTACCAGCTTCATTTGA-3′	This study
ISARPS3C	5′-GCAGTAGCACCAGTGTGAGC-3′	This study
CP1	5′-AGGGATTGCCAATATGCCCA-3′	García Martínez et al. (2010)
CP2	5′-GTGACATTGTGTAGATCCTTGG-3′	"
CM1	5′-AATAGAGGAGATGTTTTATTTGAATTC-3′	This study
CM2	5′-GCAGAATCCGTAAGAACTGGGG-3′	This study
CO1	5′-TTTCAATATGCCTAGAGCCACATTGTG-AATAC-3′	This study
CO2	5′-GCATTAGTTAGTATCGTCTTTTATTAA-ATA-3′	This study

ISARPS: *RPS0* consensus primers; CP: *C. parapsilosis*-specific primers; CM: *C. metapsilosis*-specific primers; CO: *C. orthopsilosis*-specific primers.

DNA extraction

Genomic DNA and rapid DNA extraction procedures were carried out according to García Martínez et al. (2010).

PCR assay

The synthetic oligonucleotides used in this work are presented in Table 2. One unit of EcoTaq DNApolymerase (Ecogen, Spain) was added to 49.7 μl of a solution consisting of 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 67 mM Tris–HCl (pH 8.8), 0.01% vol/vol Tween-20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, and, unless otherwise stated, 50 ng of target DNA and 0.3 mM of each primer were used. DNA was amplified in a PCR thermal cycler (Eppendorf PCR Mastercycler) by running 1 cycle at 95 °C for 3 min, and then 40 cycles as follows: 60 s of denaturation at 94 °C, 30 s of annealing at 63 °C, and 45 s of primer extension at 72 °C. Following the last cycle, additional 10-min incubation at 72 °C was carried out to ensure the complete polymerization of any remaining PCR products.

Cloning and sequencing of RPS0 amplicons

The selected amplicons obtained with consensus ISARPS primers were inserted in the plasmid pTZ57R-T using the 'InsTAclone™ PCR Cloning Kit' (Fermentas, Lithuania), and the resulting plasmids were introduced into *E. coli* DH5α by transformation according to Inoue et al. (1990). Transformed bacteria were selected in S-gal media (Sigma, Missouri, USA). Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly (1979) for a standard analysis or with the plasmid DNA purification kit Wizard Minipreps (Promega, Wisconsin, USA) for insert sequencing.

Sequencing was performed by the dideoxy chain termination method using Taq DNA polymerase, primed with M13 reverse and forward primers with an automated DNA sequencer ABI 3730 (Applied Biosystems, California, USA) at the Sequencing Service of Valencia University. Sequences were analysed with the following software: BLAST (http://blast.ncbi.nlm.nih.gov, Altschul et al., 1990), CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html, Higgins et al., 1994), MUSCLE (http://www.ebi.ac.uk/Tools/muscle, Edgar, 2004), and GENSCAN (Burge, 1998; http://genes.mit.edu/GENSCAN.html).

Results

Molecular identification of C. parapsilosis isolates

The 38 isolates used in this study were first identified based on morphological and physiological criteria as *C. parapsilosis*. The