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ORIGINAL ARTICLE

Identification of pathogenic yeast species by polymerase chain reaction amplification of the *RPS0* gene intron fragment

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Abstract

Aims: This work focuses on the development of a method for the identification of pathogenic yeast. With this aim, we target the nucleotide sequence of the RPS0 gene of pathogenic yeast species with specific PCR primers. PCR analysis was performed with both the genomic DNA, whole cells of clinical isolates of *Candida* species and clinical samples.

Methods and Results: A single pairs of primers, deduced from the nucleotide sequence of the RPS0 gene from pathogenic yeast, were used in PCR analysis performed with both the genomic DNA and whole cells of clinical isolates of *Candida* species and clinical samples. The primers designed are highly specific for their respective species and produce amplicons of the expected sizes and fail to amplify any DNA fragment from the other species tested. The set of primers was tested successfully for the identification of yeast from colonies, blood cultures and clinical samples. These results indicate that genes containing intron sequences may be useful for designing species-specific primers for the identification of fungal strains by PCR. The sensitivity of the method with genomic DNA was evaluated with decreasing DNA concentrations (200 ng to 1 pg) and different cell amounts (10^7–10^5 cells).

Conclusion: The results obtained show that the amplification of RPS0 sequences may be suitable for the identification of pathogenic and other yeast species.

Significance and Impact of the Study: Identification of *Candida* species using molecular approaches with high discriminatory power is important in determining adequate measures for the interruption of transmission of this yeast. The approach described in this work is based on standard technology, and it is specific, sensitive and does not involve complex and expensive equipment. Furthermore, the method developed in this work not only can be used in eight yeast species, but also provides the basis to design primers for other fungi species of clinical, industrial or environmental interest.

Introduction

The frequency and diversity of systemic yeast infections in patients with other serious illnesses has dramatically increased in the last two decades (Pfaller 1996; Okhravi *et al.* 1998; Nucci and Marr 2005; Lai *et al.* 2008; Lass-Flörl 2009) with a mortality rate ranging from 50 to 80%, (Fraser *et al.* 1992). In the absence of pathognomonic signs or symptoms, diagnosis of invasive candidiasis has to be made after isolating and identifying yeast species by morphology and assimilation tests that usually take several days (Velegraki *et al.* 1999). Furthermore,

clinical yeast isolates are sometimes misidentified when automated biochemical systems are used (Dooley *et al.* 1994). Thus, rapid and accurate identification methods of pathogenic fungi at the species level would prove very helpful in clinical terms.

A rapid and accurate diagnosis of an invasive fungal infection is critical for early and appropriate treatment because patient prognosis improves if diagnosis is established early in invasive fungal infection, and adequate antifungal treatment is initiated sooner (Karp *et al.* 1991; Garey *et al.* 2006). Although empirical antifungal therapy may be applied, it is toxic and costly, and may also increase the selection of resistant *Candida* species (Bougnoux *et al.* 1999; Zaragoza *et al.* 2008). Thus, an early and accurate diagnosis of an invasive fungal infection is a matter of utmost importance.

Increasing knowledge of the molecular genetics of *Candida albicans* and of other yeast and fungi species has enabled the use of molecular approaches to improve the diagnosis of yeast and pathogenic fungi infections (Mitchell *et al.* 1994; Sullivan *et al.* 1996; Gottfredsson *et al.* 1998; Reiss *et al.* 1998; Walsh and Chanock 1998). PCR methods are particularly promising because of their simplicity, specificity and sensitivity. Consequently, PCR methods targeting different genes have been described for the identification of *Cryptococcus neoformans* (Tanaka *et al.* 1996), *Aspergillus fumigatus* (Karp *et al.* 1991; Reichard *et al.* 1997) and different yeast species (Buchman *et al.* 1990; Crampin and Matthews 1993; Jordan 1994; Kan 1993; Burgener-Kairuz *et al.* 1994; Dooley *et al.* 1994; Fujita *et al.* 1995; Haynes *et al.* 1995; Prariyachatigul *et al.* 1996; Sullivan *et al.* 1996; van Deventer *et al.* 1996; Wildfeuer *et al.* 1996; Morace *et al.* 1997; Shin *et al.* 1997; Flahaut *et al.* 1998; Okhravi *et al.* 1998; Donnelly *et al.* 1999; Morace *et al.* 1999; Hidalgo *et al.* 2000; Tamura *et al.* 2000; Wahyuningsih *et al.* 2000; E. Lander, B. Birren and C. Cuomo, unpublished data).

Several studies have described probes, restriction fragment length polymorphisms or other methods to identify unique ribosomal DNA (rDNA) sequences (Hopfer *et al.* 1993; Prariyachatigul *et al.* 1996; Kappe *et al.* 1998; Turenne *et al.* 1999; Velegraki *et al.* 1999; Evertsson *et al.* 2000; Kauffman *et al.* 2000; Loeffler *et al.* 2000; Martin *et al.* 2000; Turin *et al.* 2000). The most common approaches have targeted portions of the rDNA of species of yeast (Prariyachatigul *et al.* 1996; Tanaka *et al.* 1996; Elie *et al.* 1998; Flahaut *et al.* 1998; Walsh and Chanock 1998; Evertsson *et al.* 2000; Baquero *et al.* 2001). Although these published PCR methods have proven useful for the identification of fungal species, they normally identify only one species and require a probe hybridization procedure, which is normally time-consuming and expensive.

Here, we describe a sensitive and specific method to rapidly and simultaneously identify the most common pathogenic *Candida* yeast species. The method is based on the use of primers targeted to the yeast *RPS0* gene (intron or exon) to obtain a DNA fragment specific for each yeast species by PCR assay. The *RPS0* gene codes for a protein, which is a component of the translational machinery and is extremely conserved among species (Montero *et al.* 1998); its homology extends to the whole DNA coding sequence, allowing the design of degenerate primers of this gene for amplification purposes, even if the sequence is unknown. However, more yeast species and almost all the fungal species contain one or more introns that completely differ in size and sequence and enable the design of specific primers for the identification of the species.

Materials and methods

Micro-organisms and clinical samples

The reference yeast strains used in this work are listed in Table 1, and the 199 blood culture isolates, stratified by species from the University Hospital 'La Fe' (Valencia, Spain), are presented in Table 2. Isolates were identified by standard microbiological methods and by the VITEK system (bioMerieux, Madrid, Spain). Clinical samples (50 urine and 50 sputum) were obtained from the University Clinic Hospital (Valencia, Spain).

Genomic DNA purification

For DNA extraction, yeasts were routinely grown on Sabouraud dextrose agar plates at 28°C for 24–48 h. A single colony was then grown overnight on YPD broth (1% yeast extract, 2% peptone, 2% dextrose) at 28°C, with shaking at 200 rev min^{-1}. DNA was extracted from cultures by adapting the method described previously (Del Castillo Agudo *et al.* 1995) to yeast; the main modification consisted in the cell disruption method. The yeast cell wall was broken by strong shaking with glass beads. DNA concentrations and *A*260/*A*280 ratios were determined by means of a 'Gene Quant Spectrophotometer' (Pharmacia, Uppsala, Sweden). An *A*260/*A*280 ratio of 1·8–2·1 was considered acceptable.

Rapid DNA extraction procedure for PCR

A loopful of yeast cells were resuspended in 29·7 μl of 10 mmol l^{-1} NaOH, 0·5% (v/v) Tween 20, 0·5% (v/v) Nonidet P-40, and incubated 15 min at 95°C. After treatment, samples were centrifuged, and 10 μl of supernatant was added to the PCR mixture.

Table 1 Reference species, PCR specificity of the primer pairs designed for the different species and expected amplicon sizes. *Candida albicans* (CA), *Candida tropicalis* (CT), *Candida dubliniensis* (CD), *Candida parapsilosis* (CP), *Candida glabrata* (CG), *Candida zeylanoides* (CZ), *Debariomyces hansenii* (DH) and *Pichia guilliermondii* (PG). N, negative; P, positive

Reference species	Primer pair								Amplicon size (bp)
	CA	CT	CG	DH	CD	PG	CP	CZ	
C. albicans (ATCC 1392)	P	N	N	N	N	N	N	N	310
C. albicans (ATCC 5314)	P	N	N	N	N	N	N	N	310
C. tropicalis (CECT 1440)	N	P	N	N	N	N	N	N	147
C. tropicalis (CECT 1005)	N	P	N	N	N	N	N	N	147
Candida boidinii (CECT 1014)	N	N	N	N	N	N	N	N	N
Torulosporora delbruekii (CECT1015)	N	N	N	N	N	N	N	N	N
Candida atlantica (CECT *1016*)	N	N	N	N	N	N	N	N	N
C. glabrata (CECT *1448*)	N	N	P	N	N	N	N	N	406
C. glabrata (CECT *1456*)	N	N	P	N	N	N	N	N	406
C. glabrata (CECT *1021*)	N	N	P	N	N	N	N	N	406
C. glabrata (CECT *10328*)	N	N	P	N	N	N	N	N	406
Pichia guilliermondii (CECT *1437*)	N	N	N	N	N	P	N	N	620
P. guilliermondii (CECT 1021)	N	N	N	N	N	P	N	N	620
P. guilliermondii (CECT 1019)	N	N	N	N	N	P	N	N	620
Pichia jadinii (CECT 1430)	N	N	N	N	N	N	N	N	N
Issatchenkia orientalis (1433)	N	N	N	N	N	N	N	N	N
I. orientalis (10688)	N	N	N	N	N	N	N	N	N
Candida intermedia var. intermedia (CECT 1431)	N	N	N	N	N	N	N	N	N
Kluyveromices marxianus (CECT 1436) (*C.kefir*)	N	N	N	N	N	N	N	N	N
K. marxianus (CECT 1432) (*C.kefir*)	N	N	N	N	N	N	N	N	N
C. zeylanoides var. zeylanoides (CECT 1441)	N	N	N	N	N	N	N	P	66
C. zeylanoides (CECT 1434)	N	N	N	N	N	N	N	P	66
C. parapsilosis (ATCC 22019)	N	N	N	N	N	N	P	N	113
C. parapsilosis (CECT 1449)	N	N	N	N	N	N	P	N	113
Candida mesenterica (CECT 1025)	N	N	N	N	N	N	N	N	N
Candida catenulata (CECT 1428)	N	N	N	N	N	N	N	N	N
Candida maritima (1 CECT 435)	N	N	N	N	N	N	N	N	N
Candida inconspicua (CECT 1980)	N	N	N	N	N	N	N	N	N
Candida sake (CECT 1044)	N	N	N	N	N	N	N	N	N
Kodamaea ohmeri (CECT 1457)	N	N	N	N	N	N	N	N	N
K. ohmeri (CECT 10169)	N	N	N	N	N	N	N	N	N
K. ohmeri (CECT 1022)	N	N	N	N	N	N	N	N	N
C. inconspicua (CECT 1980)	N	N	N	N	N	N	N	N	N
C. dubliniensis (CECT 11455)	N	N	N	N	P	N	N	N	262
C. dubliniensis (NCPF 3649)	N	N	N	N	P	N	N	N	262
D. hansenii (CECT 11370)	N	N	N	P	N	N	N	N	246
D. hansenii (CECT 11364)	N	N	N	P	N	N	N	N	246
D. hansenii (CECT *11957*)	N	N	N	P	N	N	N	N	246
Cryptococcus neoformans (CECT 1697)	N	N	N	N	N	N	N	N	N
Geotrichum candidum	N	N	N	N	N	N	N	N	N
Trichosporum spp.	N	N	N	N	N	N	N	N	N

Purification of DNA from clinical samples

Sputum and urine samples, collected from patients, were used as clinical materials for DNA purification. To reduce the viscosity of the sputum samples, they were mucolyticated with 2% *N*-acetyl-L-cysteine for 30–120 min at room temperature and underwent reciprocal shaking at 30 strokes per min. After treatment, sample solutions were centrifuged at 1200 ***g*** for 15 min at 4°C. Urine samples were centrifuged at 3000 rpm for 15 min, and pellets were suspended in Tris–HCl buffer (pH 7·5). Both pellets were treated as described previously.

PCR assay

The synthetic oligonucleotides used as primers are presented in Tables 2 and 3. One unit of EcoTaq polymerase (Ecogen, Barcelona, Spain) was added to 49·7 μl of a

Table 2 Clinical samples used in this work

Clinical isolates			
Species	Number	Species	Number
Candida albicans	50	*Candida dubliniensis*	25
Candida glabrata	20	*Kodamaea ohmeri*	3
Candida tropicalis	15	*Pichia guilliermondii*	15
Candida zeylanoides	1	*Debariomyces hansenii*	10
Candida parapsilosis	60		

solution consisting of 16·6 mmol l^{-1} $(NH_4)_2SO_4$, 2·5 mmol l^{-1} $MgCl_2$, 67 mmol l^{-1} Tris–HCl (pH 8·8), 0·01% v/v Tween-20, 0·2 mmol l^{-1} each of dATP, dCTP, dGTP, dTTP and, unless otherwise stated, 500 ng of target DNA and 0·3 mmol l^{-1} of each primer were used. DNA was amplified in a PCR thermal cycler (Eppendorf PCR Mastercycler) by running one 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 55°C and 45 s of primer extension at 72°C. Following the last cycle, an additional 10 min incubation at 72°C was carried out to ensure the complete polymerization of any remaining PCR products.

Results

Homology of the Rps0p of fungal species

Having searched the NCBI database (nonredundant protein sequences nr) for a protein homologous to Rps0 of *Candida tropicalis* (Baquero *et al.* 2002) with the BLASTP 2.2.17 (Altschul *et al.* 2005), we confirm the high homology of this protein along the evolutionary tree as we observed a homology ranging from 53% for *Mus musculus*, 59% for *Canis lupus familiaris*, 73% for *Pichia guilliermondii*, 74% for *C. albicans*, 93% for *Candida glabrata* and 94% for *Vanderwaltozyma polyspora*.

The sequences of 25 homologous proteins from 23 fungal species (including the duplicate genes *RPS0A* and *RPS0B* from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) were compared with CLUSTALW at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/) (Fig. 1). Homology of the sequences extended along the full sequence, although it was higher in the 2/3 amino terminal region and less important in the terminal region where insertions/deletions were observed, mainly in relation to the fungi morphology (Fig. 1). The N-terminal region of this gene was highly conserved, allowing the design of universal primers to amplify and sequence the intron of any specific species before defining intron primers to specifically identify these species.

Intron sequences and primer design

Sequences of the *RPS0* introns of eight yeast species (*C. albicans*, *C. tropicalis*, *Candida dubliniensis*, *Candida parapsilosis*, *C. glabrata*, *Candida zeylanoides*, *Debariomyces hansenii* and *P. guilliermondii*) were acquired from genomic databases of a previous work (*C. albicans* and *C. tropicalis*) (Baquero *et al.* 2001, 2002). For *P. guilliermondii*, a yeast species with no intron at the *RPS0* gene, a CLUSTALW analysis of the *RPS0* cDNA from different yeast species was performed to design primers for the identification of this species. The sequences used in this work are presented in Table 3, and the designed primers are shown in Table 4.

Primers specificity

Each pair of primers was used to amplify the total DNA of the different fungal species, and the results are shown in Table 1. Strong specificity (100%) and the expected amplicons size were obtained (Table 1 and Fig. 4).

Notably, despite the high homology of the sequences of this gene among yeast species, even at the nucleotide level, the *P. guilliermondii* exon region primers were highly specific and did not produce amplicons with the other species tested, including *Kodamaea ohmeri* (formerly *Candida guilliermondii* var *membranofaciens*), considered a subspecies of *P. guilliermondii*.

Primers designed for *C. parapsilosis* did not amplify *Candida orthopsilosis* and *Candida metapsilosis* DNA (data not shown).

Sensitivity

The sensitivity of the assay was evaluated by both decreasing DNA concentration (from 200 ng to 0·1 pg per reaction) and cells dilutions (from 10^7 to 5 cells), and the primer sets were able to amplify up to 1 pg of DNA and 10^3 cells (data not shown) per reaction. The results obtained for *C. albicans* and *C. glabrata* DNA are shown in Fig. 3.

Identification of clinical isolates

The clinical isolates, including *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *D. hansenii* and *P. guilliermondii*, which had been previously identified by conventional methods, were identified by amplification with the *RPS0* primers. In a first approach, the DNA from each isolate was probed with the corresponding pair of primers. The amplification results can be seen in Fig. 2. As shown, the identification of *C. albicans*, *C. glabrata*, *C. tropicalis* and *P. guilliermondii* was fully coincident with previous

Table 3 Sequence of the *RPS0* gene intron of the species studied in this work and the partial sequence of the *RPS0* gene from *Pichia guilliermondii*. The sequences used to design the primers are highlighted

Species	Intron/amplicon size (base pairs)	Sequence	References
Candida dubliniensis	357/262 bp	GTATGTTCAACAACATTTTTA**AGTATTGGGAGAGGGAAAGACC**ATTAAGGAAAGGGAAGAGAAAGAGAAAACAGTTTAATCAGAGAC TAGAGATAGATATTCAGCTTAGTTTATTTATTTTTGAGAGGATACCTATAATACAATGTTAGCAATGAATTGGGGAATGTAAGAGATCA GATTTGGGAATTATATGGTATATATACTAGGTTCAACGAATTGGGATATCAAATCAGACTÉATTAAGGAAAGGGAAGAGAAAGAGA**GCAA GAATCGACTTCCCTGT**TTTTTCCTTGATGCCCATCTAAATACTCCTTTATTATGGAATATCGTTTACTAACAATCCTTCATTTTTTTTAG	Baquero Herrera 2003
Candida parapsilosis	276/113 bp	GTATGTTTTTAAGAAATGCATACAGAGGGATTTAATGTGAATTAATGAAATATAGCATGGCATAAACACAACGGAGGAAAGCCATTAAAAA AAC**AGGGATTGCCAATATGCCCA**AAACTCTAGGAAAGGAGATACATTGAATGGGAGTTTGAGAAAAGCTCAAGCCACCCTCAATTGTATT TTAA**CCAAGGATCTACACAATGTCAC**TTGCTATACTCATTCGATTCACATCAACATTTTGGACAATGAAATACTAACAATTTGCTTTTTTTTTAG	Baquero Herrera 2003
Candida zeylanoides	84/66 bp	GTA**TGTGCCCAGTGCCTGCCCTC**CCTCGGGCTCCGGTGAGAATAATTC**TCCGCCAGTTTTTCTGAATCT**CTGCTAACCGTTTAG	Baquero Herrera 2003
Candida tropicalis	336/147 bp	GTATGTTTTTAAAATAATCAAATGAAATGGAGAATAGGATTATTGGGGAAAGAAAAGTGTGTGAAAAAAAAAAATGAGAAGAGTGTAAGAA GATAACTAGGAATAAGAATTGATGGATTGAAACAAATAAATGAGATATAGACAATGTAACAAGATCA**TGATAGT TAGGAAAGATCAGGTG**AATTTATAATATTTGGAATTGACACTTTATCATGGAAATCTAGTGGGAATAATATACTA GAACTTTTTCTTTCAAGAATTTCACCAATATCCTTCTTCATAGATA**CACACACATGGGATATGTT**TTTAATACTAACATTTTTTTTTTTACTTTAG	Baquero *et al.* 2001
Candida glabrata	581/406 bp	AGTACTGAGAGAGAAACCTATAATACAAGTAGTGGATATAATGCTTAAGATGAACGAACTTCTAAGAGTTCTATGAATGAGAAATT**ACATAT GTTTGCTGAAAAGGC**TATGAAATACCACGAATGAGATAATATCGGATAAAAACATGAATCAGTTGGCTTCTTACTGAATAAATTGTAAAGT CATTGTTGGAGTTTTACTTGAAGCCTATCAGGAATGGATATTTTATATGGACAGTAATGCTCAAATTTAATGAAACATTTCCCTAAGATCGAA TGAATGTGGAGATTGAAAAATTTThTATGAAATACCACGAATGAGATAATATCGGATAAAAACATGAATCAGTTGGCTTCTTACTGAATAAA TTGTAAAGTCATTGTTGGAGTTTTAC TATGAAATACCACGAATGAGATAATATCGGATAAAAACATGAATCAGTTGGCTTCTTACTGAATAA TTG**AGAAGTCCTGAACACTAAGAAAAAGT**TACTAACATCATTGTTTCATTTTCTCGACTAAAGCTATTTTCAATTATTGAAGATAATCTTTCA GAGATTGAAATCTTATTTAAAATAG	Dujon *et al.* 2004
Debariomyces hansenii (*Candida famata*)	307/246 bp	GTATGTA**TCGATGAAATGAGACAGTGGTAGGGG**CCAGTGGATCGTACGGGAGTGAAAGTAATAAGAAGGGCCAGAAATCAACTAGAGATG TAAGAATGGTGGATGAGAAGTATGGAGAAGGCCATAGGAGGAGATTGGCGTTGATTTGATAGTTCGGCCGTGGATTCGACTACGTAGATAT AAATCGAAGAAGAGGGATAGAAATAGGCCATAAGGATGGATGAAGÔCCAGTGGATCGTACGGGAGTGAAAGTAA**GCTGCCAGAGCTGT CACCGCATGAA**AATTTGGACTTTTACTAACATTTTATAG	Dujon *et al.* 2004
P. guilliermondii (*Candida guilliermondii var. guilliermondii*) (exon sequence)	0/620 bp	ATGTCATTGCCTGCTTCCTTTGACTTGACTGCTGAAGACGCCAAGTTGTTGTTGGCTGCTAACGTCCA**CTTGGGTTCCAAGAACGTGATT**GTGC ACAACAAACCATACGTTTATAAGACCAGACCCGATGGTGTCAACGTGATCAACATTGGTAAAACATGGGAAAAGATCGTATTGGCTGCTA GAACCATTGCTGCCATCCCAAACGCTGCTGACGTTGCCGTGTGTTCGTCGAGAACCTTTGGTCAAAGAGCTGTGTTGAAATTTGCCGCTCA CACCGGTG1GTGCACAACAAACCATACGTTTATAAGACCAGACCCGATGGTGTCAACGTGATCAACATTGGTAAAACATGGGAAAAGATC GTATTGGCTGCT GTGCACAACAAACCATACGTTTATAAGACCAGACCCGATGGTGTCAACGTGATCAACATTGGTAAAACATGGGAAAAGA TCGTATTGGCTGCTAGAACCATTGCTGCCATCCCAAACGCTGCTGACGTTGCCGTGTGTTCGTCGAGAACCTTTGGTCAAAGAGCTGTGTTG AAATTTGCCGCTCACACCGGTG1GTGCACAACAAACCATACGTTTATAAGACCAGACCCGATGGTGTCAACGTGATCAACATTGGTAAAACAT GGGAAA**TCCAAGGCTGAGGATGCTGAAG**AGGCTCCTGTTGCCGACGCCGAGCCCGACTGGTCCGGAGAGACCGAAGACGTCGACTGGGCC GAGTCCGGTGCCACCCCTGCTGCTGAGGAGGCTGCTGCTTCCAACTGGTAA	E. Lander, B. Birren and C. Cuomo, unpublished data
Candida albicans	389/310 bp	CAACGTCCATTTGGGTGCTAAGAACGTTCAAGTATGTTCAACAATAGCTTTTTAAGTATTTGGGAGAAGGGAAAGGGAAATTTCTCCATTAAGGA AAAGAGAAAGGGTAAGAATATATGAGCAAACAGAGACTAGGGATAGATATTCAGTTTTAATTTTATTGGAAAAATGGGAATAATAAAATGTTGC CAATêCAACGTCCATTTGGGTGCTAAGAACGTTCAAGTATGTTCAACAATAGCTTTTTAAGTATTTGGGAGAAGGGAAAGGGAAATTTCTCCAT TAAG CAACGTCCATTTGGGTGCTAAGAACGTTCAAGTATGTTCAACAATAGCTTTTTAAGTATTTGGGAGAAGGGAAAGGGAAATTTCTCCAT TAAGGAAAAGAGAAAGGGTAAGAATATATGAGCAAACAGAGACTAGGGATAGATATTCAGTTTTAATTTT	Baquero *et al.* 2002

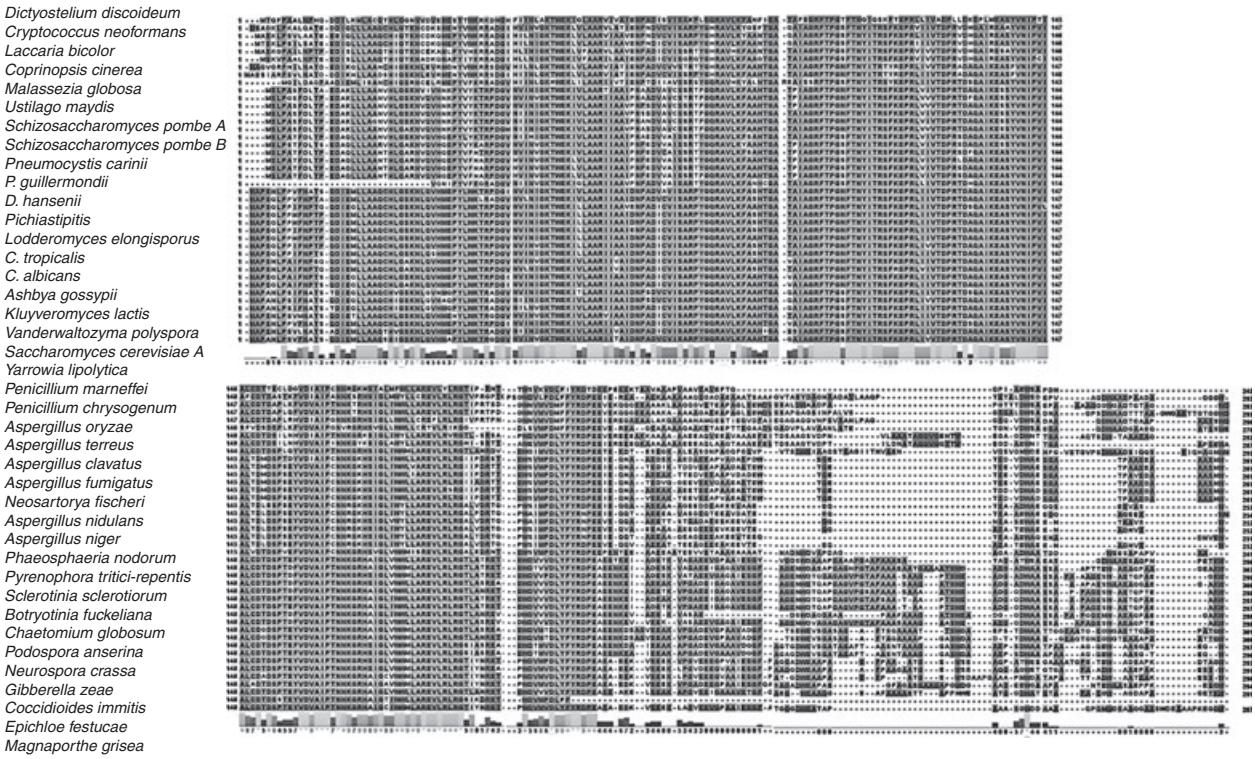

Figure 1 Protein sequence homology of selected fungal Rps0 proteins obtained with the CLUSTALW program. Colours according to the percentage of the residues in each column that agree with the consensus sequence. Protein sequences were obtained after blast *Candida tropicalis* protein sequence against the 'nonredundant protein' database at NCBI. Only the residues that agree with the consensus residue for each column are coloured. Species names are in the same order as the sequences. Drawn with The Jalview Java Alignment Editor (Clamp *et al.* 2004).

identifications. An isolate of *C. parapsilosis*, which was not amplified with the corresponding primers, was identified as *D. hansenii* when probed with the rest of primers. Conversely, three negative *D. hansenii* samples were identified as *C. parapsilosis*, while a sample identified as *C. dubliniensis* was characterized as *C. albicans*. We included *K. ohmerii DNA* as a negative control when testing *P. guilliermondii*.

Table 4 Primers designed for each species. All the primers correspond to the intron region except those for *Pichia guilliermondii* that are defined from the exon areas

Species	Primer	*RPS0* region
Candida albicans	INT1 5′ AAGTATTTGGGAGAAGGGAAAGGG 3′	Intron
	INT2 5′ AAAATGGGCATTAAGGAAAAGAGC 3′	
Candida dubliniensis	CDf 5′AGTATTGGGAGAGGGAAAGACC 3′	Intron
	CDr 5′ ACAGGGAAGTCGATTCTTGC 3′	
Candida glabrata	CGf 5′ ACATATGTTTGCTGAAAAGGC 3′	Intron
	CGr 5′ ACTTTTTCTTAGTGTTCAGGACTTC 3′	
Candida parapsilosis	CPf 5′ AGGGATTGCCAATATGCCCA 3′	Intron
	CPr 5′ GTGACATTGTGTAGATCCTTGG 3′	
Debariomyces hansenii	DHf 5′TCGATGAAATGAGACAGTGGTAGGGG3′	Intron
	DHr 5′ TTCATGCGGTGACAGCTCTGGCAGC3′	
Pichia guilliermondii	PGf 5′CTTGGGTTCCAAGAACGTGATT3′	Exon
	PGr 5′CTTCAGCATTCCTCAGCCTTGGA3′	
Candida zeylanoides	CZf 5′ TGTGCCCAGTGCCTGCCCTC 3′	Intron
	CZr 5′ AGATTCAGAAAAACTGGCCGAG 3′	
Candida tropicalis	CTf 5′TGATAGTTAGGAAAGATCAGGTG3′	Intron
	CTr 5′ AACATATCCCATGTGTGTGT 3′	

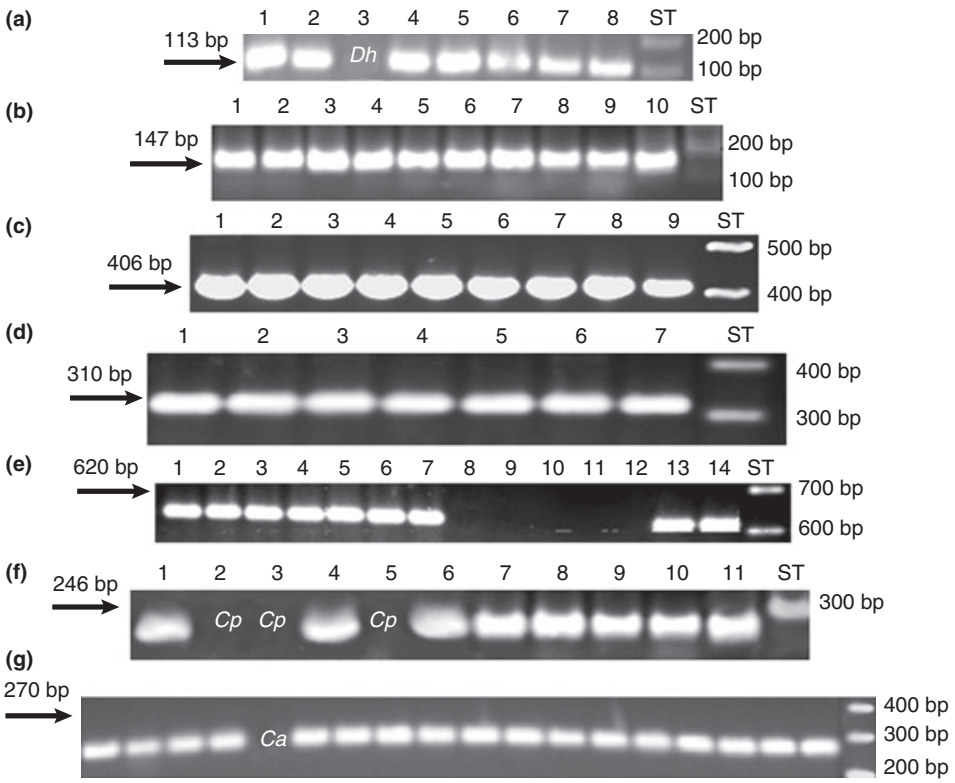

Figure 2 Agarose gel electrophoresis of amplification products from clinical isolates, which have been previously identified by a standard commercial test in Valencian hospitals. (a) *Candida albicans*; negative result in lane 3 identified later by means of our primers as *Debariomyces hansenii* (*Dh*). (b) *Candida tropicalis*. (c) *Candida glabrata*. (d) *C. albicans*. (e) Lanes 1–7 and 13–14 *Pichia guilliermondii*; lanes 8–12 *Kodamaea ohmeri* (formerly *Candida guilliermondii var. membranofaciens)*. (f) *Debariomyces hansenii*; lanes 2, 3 and 5 were identified as *Candida parapsilosis* (Cp). (g) *Candida dubliniensis*; lane 5 was identified as *C. albicans* (*Ca*). ST (100 bp ladder).

Identification from colonies and blood culture

As DNA purification is time-consuming, we evaluated the possibility of shortened the identification time by performing a direct assay from both colonies and blood culture cells. In both cases, cell samples were treated with NaOH, detergents and heat (see Materials and Methods) for DNA extraction. PCR was carried out with 10 μl of cells extract supernatant. The results obtained with the colonies and blood culture cells are shown in Figs 4 and 5, respectively. All the clinical isolates tested by this method were identified correctly, and the time required for identification was cut, as the identification can be carried out in <12 h from a blood culture.

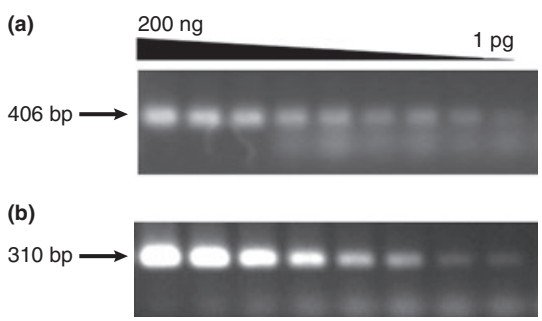

Figure 3 Amplification of different amounts of DNA from *Candida albicans* (a) and *Candida glabrata* (b) with their respective primers.

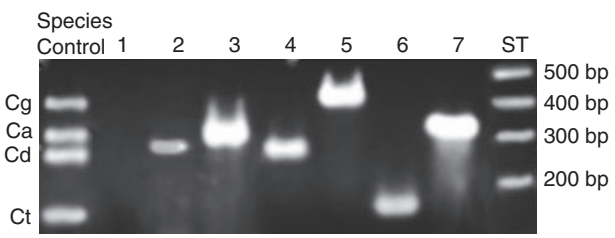

Figure 4 Identification of different yeast species by the direct amplification of colony cells. Species control: amplicons mix from type strains of *Candida glabrata*, *Candida albicans* and *Candida tropicalis*; 1: negative control; rows 2, 3 and 7 *C. albicans* (Ca); 4: *Candida dubliniensis* (Cd); 5: *C. glabrata* (Cg); 6: *C. tropicalis* (Ct); ST (100 bp ladder).

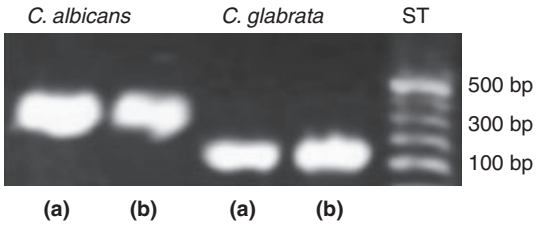

Figure 5 Identification of different yeast species by a direct amplification of blood culture cells from *Candida albicans* and *Candida glabrata*. (a) Artificial blood culture with the type strain (blood culture media inoculated with a previous culture of reference strain); (b) Clinical blood culture. ST (100 bp ladder).

Identification from clinical samples

We continued with the procedure simplification, and we carried out assays with clinical samples by using a pellet obtained after centrifuging urine and sputum samples as a DNA matrix.

Table 5 shows the results obtained by culture or PCR methods. The number of positive samples detected by PCR was higher than that obtained by yeast culture, although re-amplification is recommended if the primary PCR is not clean enough (see Fig 6a,b).

Multiplex assay

We also evaluated the possibility of performing a multiplex assay. For this purpose, we carried out the test using

Table 5 Detection of pathogenic yeast in clinical samples by PCR with the *RPS0* primers for *Candida albicans* (CA), *Candida tropicalis* (CT) and *Candida glabrata* (CG)

No. of samples	No. of positive culture	PCR detections		
		CA	CT	CG
50 Urine	5	8	0	0
50 Sputum	7	12	0	1

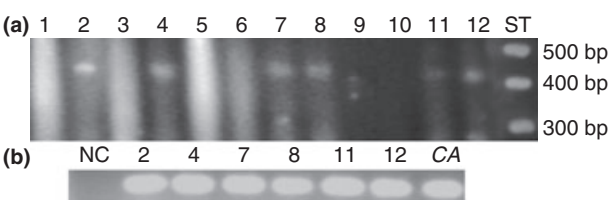

Figure 6 (a) Direct amplification of urine sediments with positive results for *Candida albicans* in rows 2, 4, 7, 8, 11, 12. ST (100 bp ladder); (b) Re-amplification of positive samples. NC, Negative control; CA, *C. albicans* positive control.

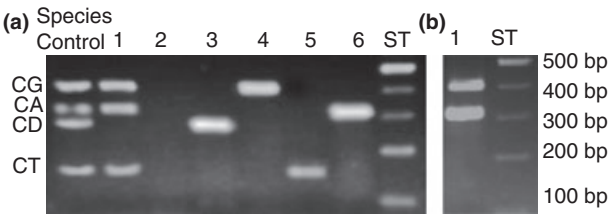

Figure 7 Multiplex amplification with different primer assortments. (a) Species control: amplicons mix from type strains of *Candida glabrata*, *Candida albicans* and *Candida tropicalis*; 1: CG/CA/CT primer assortment and a blend of four yeast species DNA (*Candida dubliniensis*, *C tropicalis*, *C. albicans* and *C. glabrata*); 2: Negative control; 3: CA/CD primer assortment and *C. dubliniensis* DNA; 4: CG/CA primer assortment and *C. glabrata* DNA; 5: CG/CT primer assortment and *C. tropicalis* DNA; CG/CA primer assortment and *C. albicans* DNA. (b) Amplification with the CG/CA primer assortment of a blend of four yeast species DNA (*C. dubliniensis*, *C tropicalis*, *C. albicans* and *C. glabrata*). ST (100 bp ladder).

different assortments of primers (two, three and four pairs of primers) and a blend of genomic DNAs from different species.

Figure 7 shows the results obtained with an assortment of a set of three primers (CAf/CAr;CGf/CGr;CTf/CTr) and a blend of the DNA of four species (*C. dubliniensis* CECT 11455, *C tropicalis* CECT 1440, *C. albicans* ATCC 5314 and *C. glabrata* CECT 1448), or with the DNA of the four species separately (Fig. 7a). The results of amplification with the assortment of two primers (CAf/CAr; CGf/CGr) and the previous blend of DNA are shown in Fig. 7b. A good discrimination is obtained with a three-pair assortment, although the best results were obtained with a two-primer assortment. The use of an assortment of four primers is not recommended as we sometimes observed nonspecific bands.

Discussion

Identification of yeast species is very valuable from both the diagnostic and therapeutic viewpoints (Odds 1988, 1992; Meunier 1989; Bodey 1993). PCR approaches are important in both epidemiological and taxonomic studies. Genes containing intron sequences could prove useful to design specific primers for the identification of yeast strains at the species level. PCR methods to identify *C. albicans* or *C. dubliniensis* isolates based on species-specific primers from the *EFB1*, *RPS0* and *ACT1* intron sequences have been described (Donnelly *et al.* 1999; Maneu *et al.* 2000; Baquero *et al.* 2002). The present study extends the work of Baquero *et al.* (2002) that used *RPS0* intron-based primers to identify *C. albicans* by designing a set of primers for the identification of eight yeast species of relevant clinical interest. These primers

are based on the *RPS0* gene and are mainly derived from intron sequences. However, not all yeasts like *P. guilliermondii* contain an intron in this gene. In this situation, it is possible to design a pair of primers for specific identification purposes based on the less conserved domains of the gene if the whole sequence of the gene is available. With this approach, we designed a pair of primers that identifies *P. guilliermodii* and discriminates this species from *K. ohmeri* (formerly *C. guilliermondii* var *membranofaciens*), a species considered a subspecies of *P. guilliermondii* until recently (formerly *C. guilliermondii* var *guilliermondii*). Furthermore, the primers designed in our study allow the correct identification of species, which have been misidentified by conventional methods, such as *D. hansenii* and *C. albicans*, which are often identified as *C. parapsilosis* and *C. dubliniensis*, respectively.

Work is currently in progress to define primers for other species including *K. ohmerii*, *Isatchenquia orientalis* and the two new species segregated from *C. parapsilosis* (*C. orthopsilosis* and *C. metapsilosis*). According to the results not shown in this work, *K. ohmerii* and *I. orientalis* do not possess an intron, and work is being carried out to sequence the full gene and to design the corresponding set of primers.

According to the previous results, we consider that the PCR primers based on *RPS0* introns are an important tool for the identification of yeasts and fungi of clinical, industrial and environmental interest. Intron sequences, when not present in databases, can be rapidly achieved with the consensus exon primers described in this work. For the few yeast species with no *RPS0* intron, primers from the less conserved regions of the gene can be designed. Alternatively, another gene intron can be eventually used for these few species.

In the last few years, different PCR-based approaches to identify pathogen yeasts have been described. Furthermore, a test based on the real-time PCR amplification of ribosomal genes has been developed by ROCHE; this application is an expensive technique, which requires considerable investment in hardware and consumable goods. The approach described in this work is based on standard technology, and it is specific, sensitive and does not involve complex and expensive equipment, the approach described in this work is based on endpoint PCR, and although specific and sensitive, it does not require the expensive equipment necessary for real-time, quantitative PCR analysis detailed in more recently published methods (Pryce *et al.* 2006; Wise *et al.* 2007; Lau *et al.* 2008; Leake *et al.* 2009) Furthermore, the method developed in this work not only can be used in eight yeast species, but also provides the basis to design primers for other fungi species of clinical, industrial or environmental interest.

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