Identification of *Candida* Clinical Isolates by PCR fingerprinting: a Contribution to the Study of Molecular Epidemiology of Candidiasis in Portugal

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RUNNING TITLE

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ABSTRACT

PCR fingerprinting was used to type 177 yeast isolates obtained from two Medical Institutions. *Candida albicans* was the predominant species found followed by *C. tropicalis, C. glabrata*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei* which accounted for over 20% of the strains isolated. This survey represents the first study of molecular epidemiology of candidiasis in Portugal.

In the last decade yeasts belonging to the genus *Candida* have emerged as major opportunistic pathogens mainly due to the increase of immunocompromised patients (19, 26, 2, 5). Although *C. albicans* is the most frequent species isolated, other species such as *C. tropicalis*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis* and *C. glabrata* have increasingly been recognized as pathogens with a wide distribution (6, 4). The significant increase in the frequency of candidiasis has promoted the study and development of a variety of molecular based techniques aiming at the replacement of the traditional methods used for the identification and typing of *Candida* clinical isolates. Among the current molecular techniques for genotyping of yeast strains, PCR fingerprinting is in wide use for its high discriminatory power and reproducibility also because it requires very little starting material and is rapid and simple to perform. PCR fingerprinting using the primer named T3B was developed firstly for *Streptococcus* identification (14), but it has been used successfully in the identification of yeast species belonging to the genus *Candida* (25, 1).

The aim of the present work was to study the diversity and distribution of *Candida* species among patients suffering from different pathologies in two medical institutions located in Braga, north of Portugal. Approximately two hundred *Candida* clinical isolates were analysed using a PCR based methodology with primer T3B, representing the first study of molecular epidemiology of candidiasis in this country.

Yeast clinical isolates were obtained from 123 independent patients during the year of 2001 in a Hospital and a Health Center. The yeast strains from the Hospital had been previously isolated at the institution of origin and collected from different body locations. The isolates from the Health Centre were all collected from vaginal exudates and isolated at the Microbiology Laboratory, University of Minho. The type cultures *Candida albicans* PYCC 3436 (ATCC 18804), *C. parapsilosis* CBS 604 (ATCC 22019), *C. krusei* PYCC 3343 (ATCC 6258), *C. tropicalis* PYCC 3097 (ATCC 750), *C. guilliermondii* PYCC 2730 (ATCC 6260), *C. lusitaniae* PYCC 2705 (ATCC 34449), *C. glabrata* CBS 138 (ATCC 2001), *C. dubliniensis* CBS 7987 (ATCC MYA-646), and *C. dubliniensis* CBS 7988 were used as reference strains and supplied by the Portuguese Yeast Culture Collection (PYCC), New University of Lisbon, Portugal, except the isolates of *C. parapsilosis*, *C. dubliniensis* and *C. glabrata* that were obtained from Centraalbureau voor Schimmelcultures (CBS), The Netherlands. DNA extraction followed procedures previously described (7) and the oligonucleotide used as a single primer for arbitrary amplification was T3B (5'-AGG TCG CGG GTT CGA ATC C-3'). Amplification reactions were performed according to Thanos *et al* (25) without the condensing step of the amplification products. The D1/D2 domain of 26S rDNA was amplified according to the procedures described by Sampaio *et al* (21). Sequencing was performed with a ABI 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, Calif.) using standard protocols. Forward and reverse sequence alignments were made with MegAlign (DNAStar, Inc. Park Street, Madison, USA) and visually corrected.

PCR fingerprinting profiles were analysed using the Bionumerics (version 2.0, Applied Maths BVBA, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated using the Dice algorithm and cluster analysis performed by means of the unweighted paired group method using arithmetic averages (UPGMA) (23).

A preliminary characterisation of the 177 yeast strains isolated using a rapid identification kit indicated that they all belonged to the genus *Candida* although several doubts arose regarding species identity. PCR fingerprinting profiles were obtained, with primer T3B, for the type strains of *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis, C. guilliermondii, C. krusei* and *C. lusitaniae,* the most common species found among yeast clinical isolates. Results showed that the different species tested could be clearly distinguished by their amplification patterns since the number and size of the amplification products were characteristic for each species (Fig. 1).

By comparing the PCR profiles of the clinical isolates with those of the reference type strains, all clinical isolates could be identified to the species level, except in the case of four of the strains which did not produce recognisable patterns. Three of them (36M, 65M and 66M) shared identical profiles while 153M presented a different but unique fingerprint. These strains had been preliminarily identified as *C. parapsilosis* and *C. glabrata,* respectively (Fig. 2). Although intraspecies variability was observed, PCR profiles obtained from different strains assigned to the same species were far more similar than those derived from different species. Variability was found for isolates of *C. albicans*, *C. tropicalis*, *C. guilliermondii*, and *C. parapsilosis*, being *C. albicans* the species that exhibited greater diversity. On the opposite, no variability was observed in the profiles obtained for both *C. glabrata* and *C. krusei* isolates. Our results agree with those of Thanos *et al* (25) who used the same methodology to differentiate *Candida* species. These authors used a condensing step of the amplification products before electrophoresis which was not performed in this study and, consequently, variability within a species might have been reduced. However, as our goal was the identification at species level, the methodology with this missing step turned out to be less time consuming and produced easily recognisable profiles maintaining high species discrimination. The high power of discrimination of PCR fingerprinting using primer T3B

allowed the identification of over 98% of the 177 clinical isolates and the detection of misidentifications made by API 32C.

To investigate the presence of *C. dubliniensis* the T3B profiles of two strains, including the type strain, were obtained and compared with the ones found for *C. albicans*. T3B fingerprinting clearly distinguished these closely related species since no similarities were observed between the amplification patterns of the two species (Fig. 3). No isolates of *C. dubliniensis* were found which is not surprising since this yeast species is commonly reported from oral candidiasis, mainly among human immunodeficiency virus-infected individuals (24), not included in this study. Previous reports also refer the differentiation of these two species by PCR fingerprinting (16) but this was the first time that T3B fingerprints have been applied for this purpose.

To evaluate the taxonomic resolution of T3B amplification profiles cluster analysis was applied to the data and the dendrogram presented in Figure 4 was produced showing a very high correspondence between the clusters and the different *Candida* species. The calculated cophenetic correlation coefficient (0.97) indicated that the fit for the cluster analysis was very good. This analysis allowed the distribution of the isolates in seven major clusters corresponding to the species studied. The four isolates that did not produce recognizable T3B patterns grouped separately and the strains that were originally misidentified grouped within the clusters corresponding to their respective T3B profile.

The four strains displaying peculiar banding profiles were further investigated by sequencing the D1/D2 domain of their 26S rDNA. Sequencing results for strains 36M, 65M and 66M (GenBank accession no. AY589574) showed a 100% similarity between them and with strain *Candida* sp. NRRL Y-17456, which appears to be highly related to *C. parapsilosis* but has been referred as a new species (9, 13). Furthermore, their T3B profiles did not match with the ones of the other *C. parapsilosis* strains (Fig. 2). This species remains a source of controversy

since three genetically distinct subtypes (I, II and III) were defined (10, 12, 11, 20) presenting variability enough to justify separate species status for the three subgroups. Strain 153M (GenBank accession no. AY589572) formerly identified as *C. glabrata* also presented differences when compared with sequences available for *C. glabrata* (94% similarity). Despite seeming to be related to *C. glabrata* no conclusive identification was obtained for this strain which is, most probably, a new species based on the high number of nucleotide substitutions observed (9, 18).

Of the 177 *Candida* strains 112 isolates were obtained from vaginal swabs, 24 from urine, 23 isolated from the upper respiratory system, seven from the anal mucosa and 11 isolates from various sources. The number of strains belonging to each of the species found and their respective clinical origin is shown in Table 1. *C. albicans* was the predominant species (79.0%) followed by *C. tropicalis* (5.6%), *C. glabrata* (4.0%) and *C. parapsilosis* (3.4%) which is in accordance with several reports (6, 4, 8 3, 5). *C. guilliermondii* represented 2.8%, *C. krusei* 2.3%, *C. lusitaniae* 0.6% and the isolates whose identification was not conclusive, *Candida* sp., represented 2.3% of the total. *C. albicans* was present in all types of clinical material except in blood samples and *C. tropicalis* was mainly recovered from the urine and respiratory tract. While *C. albicans* was predominant among vaginal isolates the non-*albicans* species were recovered mainly from other sources. Little is known about the epidemiology of candidiasis in Portugal (17, 15, 22). Our study despite covering a small area and only two medical institutions is a representative survey since patients with different pathologies were included. We show that over 20% of the infections are due to non *albicans* species which may not respond to antifungal agents usually used in treatment requiring more adequate means of therapy. Therefore it is of the utmost importance to identify the species causing infection and this PCR based methodology is simple and can be implemented at relatively low cost for routine identification in hospitals and health centres. This method is more accurate in

identifying species of the genus *Candida* than any biochemical approach currently used in Clinical Microbiology laboratories and, consequently, better suited for large epidemiological surveys.

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Fig. 1. PCR profiles obtained with primer T3B for: *Candida glabrata* CBS 138 (1)*; C. parapsilosis* CBS 604 (2)*; C. tropicalis* PYCC 3097 (3)*; C. krusei* PYCC 3440 (4); *C. guilliermondii* PYCC 2730 (5)*; C. lusitaniae* PYCC 2705 (6)*; C. albicans* PYCC 3436 (7); molecular weight marker in bp (M).

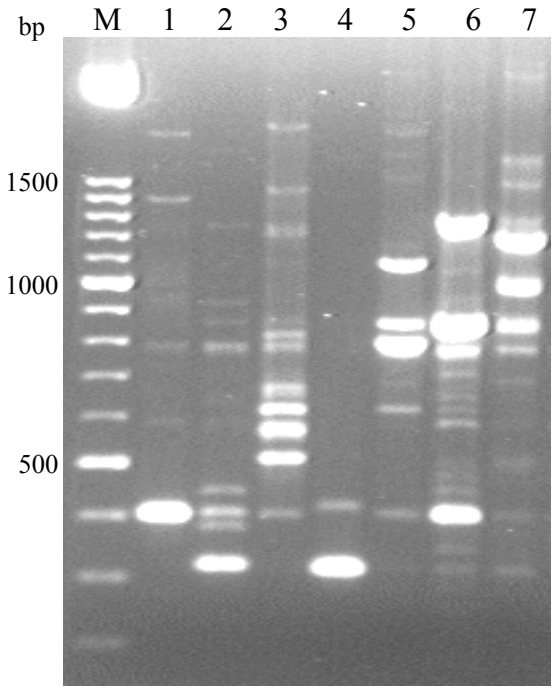
Fig. 2. PCR profiles obtained with primer T3B for *C. parapsilosis* (lanes 1-2); strain 36M (3); strain 65M (4); strain 66M (5); strain 153M (6); *C. glabrata* (lanes 7-10); molecular weight marker in bp (M).

Fig. 3. PCR profiles obtained with primer T3B for *C. albicans* (lanes 1, 2) and *C. dubliniensis* (Lanes 2, 3).

Fig. 4. Dendrogram showing the degree of similarity of T3B fingerprinting profiles among the clinical *Candida* isolates using the Dice coefficient and UPGMA cluster method. The unidentified strains are indicated as follows: * 36M, 65M and 66M; ** 153M. An arbitrary line has been drawn at 0.58 delimitating the major groups. r-cophenetic correlation coefficient.

TABLE 1. Yeast species identified, number of strains of each species, and respective sources of isolation

Species	Number of strains	Body site
C. albicans	19	Urine
	16	Respiratory tract
	2	Peritoneal fluid
	95	Vagina
	6	Anal mucosa
	1	Cateter
	1	Unknown
C. tropicalis	4	Urine
	3	Respiratory tract
	1	Pus
	1	Blood
	1	Vagina
C. parapsilosis	4	Vagina
	1	Blood
	1	Unknown
C. guilliermondii	4	Vagina
	1	Blood
C. glabrata	5	Vagina
	1	Urine
	1	Anal mucosa
C krusei	1	Blood
	1	Unknown
	2	Vagina
C. lusitaniae	1	Respiratory tract
Candida sp.	3	Respiratory tract
	1	Vagina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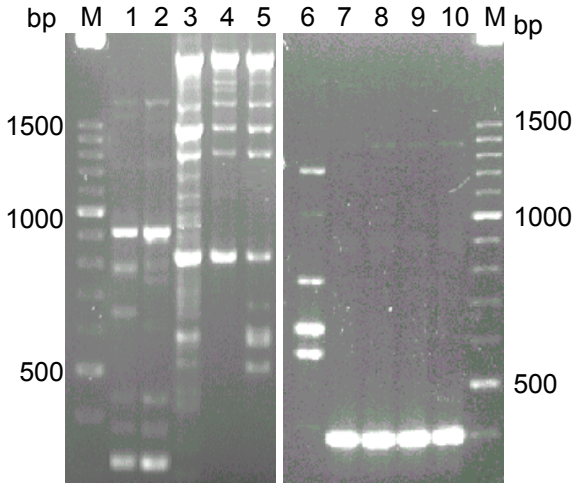
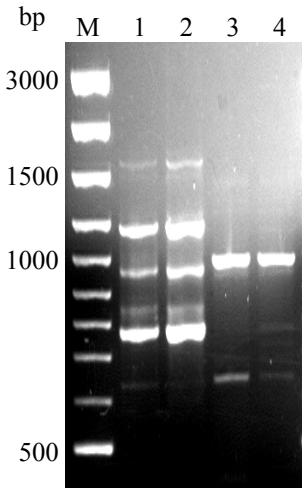


Figure 2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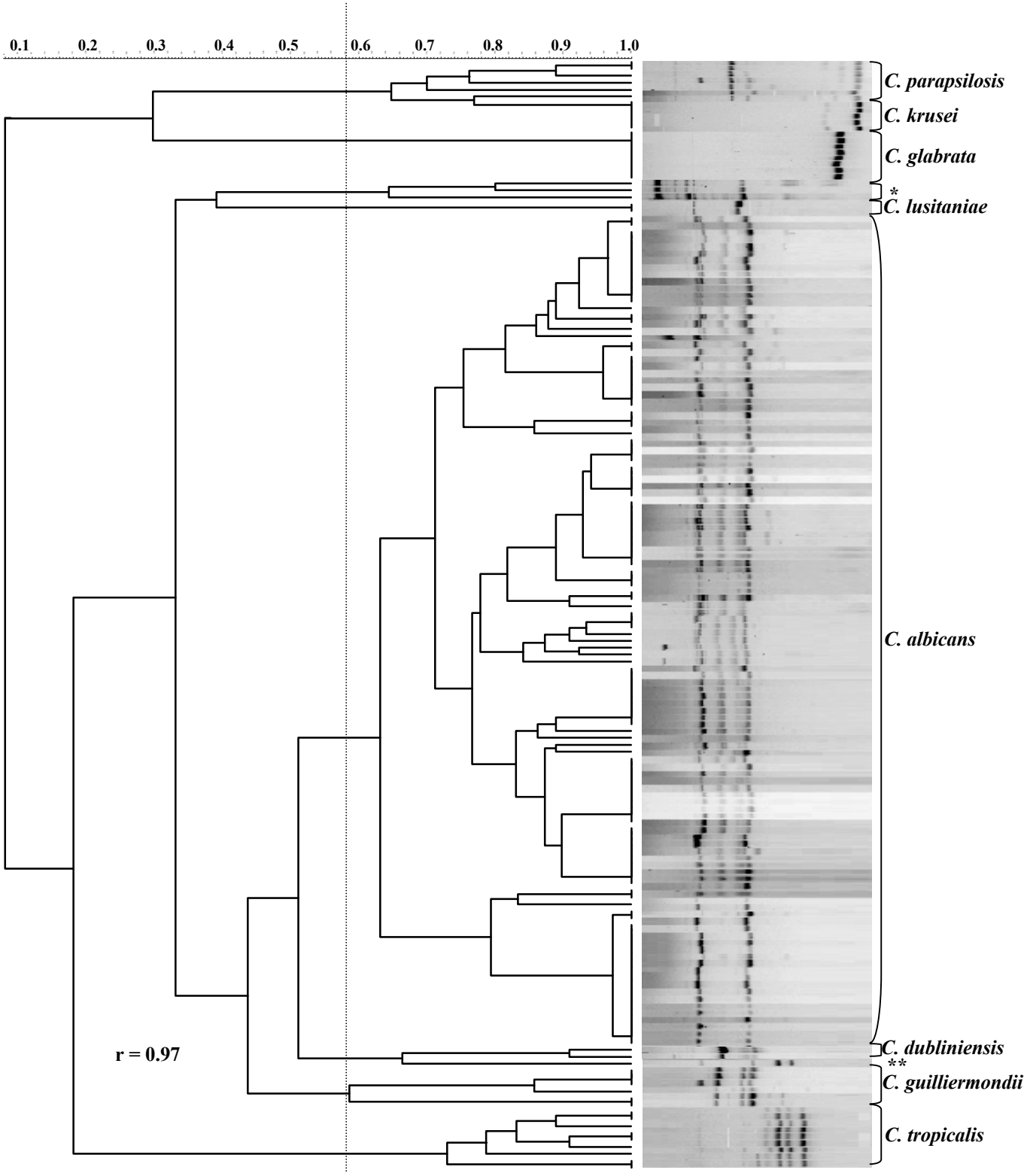
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
C. parapsilosis
C. krusei
C. glabrata
*
C. lusitaniae
C. albicans
C. dubliniensis
**
C. guilliermondii
C. tropicalis
r = 0.97

Figure 4