

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

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5 Alexandra Correia, Paula Sampaio, Judite Almeida e Célia Pais

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7 Centro de Biologia da Universidade do Minho (CBUM), Departamento de Biologia,

8 4710-057 Braga, Portugal

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13 RUNNING TITLE

14 Identification of *Candida* Clinical Isolates in Portugal

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20 Corresponding author. Mailing address: Célia Pais, Centro de Biologia da

21 Universidade do Minho (CBUM), Departamento de Biologia, Campus de Gualtar,

22 4710-057 Braga, Portugal. Phone: (351) 253-604312. Fax: (351) 253-678980.

23 Email: cpais@bio.uminho.pt

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26 **ABSTRACT**

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

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

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

51 Yeast clinical isolates were obtained from 123 independent patients during the year of 2001 in
52 a Hospital and a Health Center. The yeast strains from the Hospital had been previously
53 isolated at the institution of origin and collected from different body locations. The isolates
54 from the Health Centre were all collected from vaginal exudates and isolated at the
55 Microbiology Laboratory, University of Minho. The type cultures *Candida albicans* PYCC
56 3436 (ATCC 18804), *C. parapsilosis* CBS 604 (ATCC 22019), *C. krusei* PYCC 3343 (ATCC
57 6258), *C. tropicalis* PYCC 3097 (ATCC 750), *C. guilliermondii* PYCC 2730 (ATCC 6260),
58 *C. lusitaniae* PYCC 2705 (ATCC 34449), *C. glabrata* CBS 138 (ATCC 2001), *C.*
59 *dublinsiensis* CBS 7987 (ATCC MYA-646), and *C. dublinsiensis* CBS 7988 were used as
60 reference strains and supplied by the Portuguese Yeast Culture Collection (PYCC), New
61 University of Lisbon, Portugal, except the isolates of *C. parapsilosis*, *C. dublinsiensis* and *C.*
62 *glabrata* that were obtained from Centraalbureau voor Schimmelcultures (CBS), The
63 Netherlands. DNA extraction followed procedures previously described (7) and the
64 oligonucleotide used as a single primer for arbitrary amplification was T3B (5'-AGG TCG
65 CGG GTT CGA ATC C-3'). Amplification reactions were performed according to Thanos *et*
66 *al* (25) without the condensing step of the amplification products. The D1/D2 domain of 26S
67 rDNA was amplified according to the procedures described by Sampaio *et al* (21).
68 Sequencing was performed with a ABI 310 Genetic Analyzer (Applied Biosystems Inc.,
69 Foster City, Calif.) using standard protocols. Forward and reverse sequence alignments were
70 made with MegAlign (DNASar, Inc. Park Street, Madison, USA) and visually corrected.
71 PCR fingerprinting profiles were analysed using the Bionumerics (version 2.0, Applied Maths
72 BVBA, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated using the Dice
73 algorithm and cluster analysis performed by means of the unweighted paired group method
74 using arithmetic averages (UPGMA) (23).

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

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

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

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

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

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

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

132 Of the 177 *Candida* strains 112 isolates were obtained from vaginal swabs, 24 from urine, 23
133 isolated from the upper respiratory system, seven from the anal mucosa and 11 isolates from
134 various sources. The number of strains belonging to each of the species found and their
135 respective clinical origin is shown in Table 1. *C. albicans* was the predominant species
136 (79.0%) followed by *C. tropicalis* (5.6%), *C. glabrata* (4.0%) and *C. parapsilosis* (3.4%)
137 which is in accordance with several reports (6, 4, 8 3, 5). *C. guilliermondii* represented 2.8%,
138 *C. krusei* 2.3%, *C. lusitaniae* 0.6% and the isolates whose identification was not conclusive,
139 *Candida* sp., represented 2.3% of the total. *C. albicans* was present in all types of clinical
140 material except in blood samples and *C. tropicalis* was mainly recovered from the urine and
141 respiratory tract. While *C. albicans* was predominant among vaginal isolates the non-*albicans*
142 species were recovered mainly from other sources. Little is known about the epidemiology of
143 candidiasis in Portugal (17, 15, 22). Our study despite covering a small area and only two
144 medical institutions is a representative survey since patients with different pathologies were
145 included. We show that over 20% of the infections are due to non *albicans* species which may
146 not respond to antifungal agents usually used in treatment requiring more adequate means of
147 therapy. Therefore it is of the utmost importance to identify the species causing infection and
148 this PCR based methodology is simple and can be implemented at relatively low cost for
149 routine identification in hospitals and health centres. This method is more accurate in

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

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

239

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

243

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

246

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

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

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

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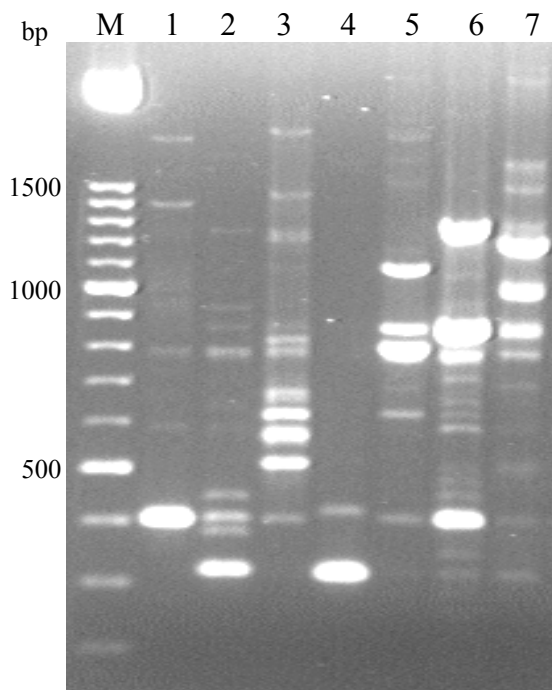
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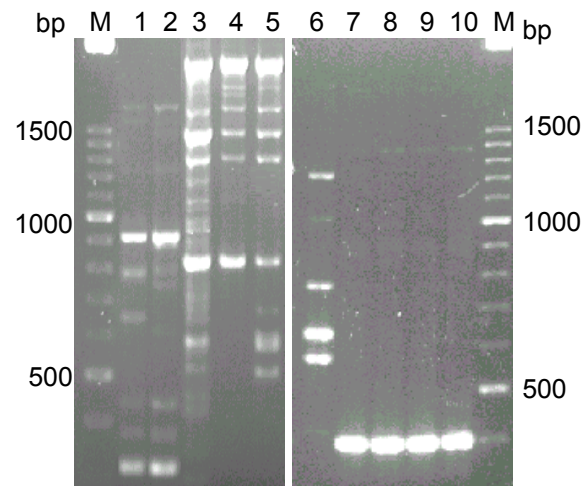
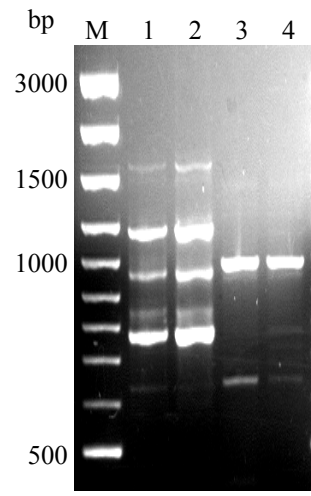


Figure 2



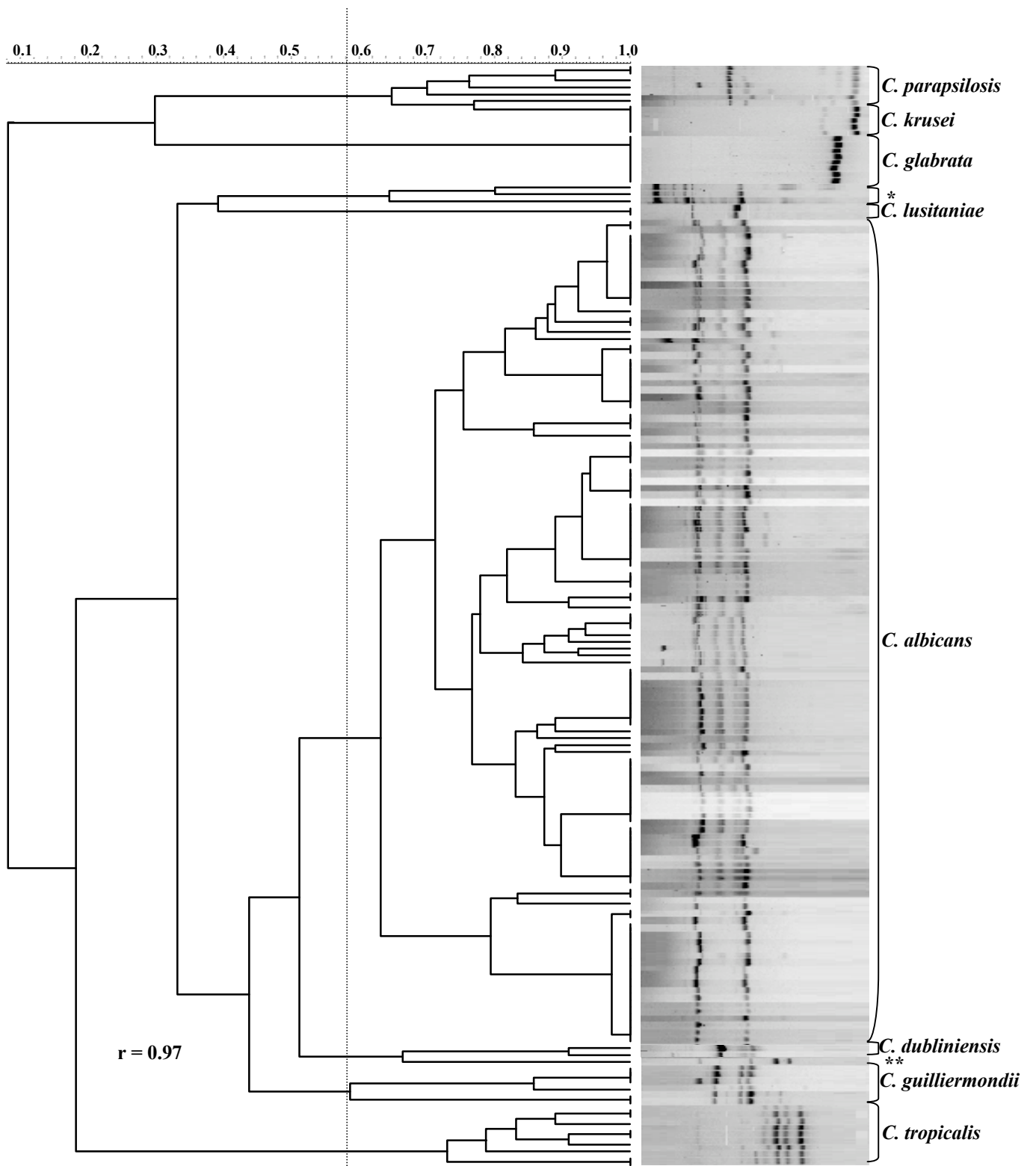


Figure 4