

Unequivocal Identification of Fungi, Especially *Candida* and Related Species of Medical Interest

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Abstract

Several methodologies have been developed for fungal identification. Nevertheless morphologic and metabolic similarity between species makes correct differentiation difficult. In this paper, we present a Pattern Identification Guide suitable for the identification of 33 species of fungi, especially medically important fungi. The methodology allows discrimination that parallels rDNA sequencing analysis.

Keywords: Fungi identification; *Candida* spp; Molecular methods; Ribotyping method

Introduction

The identification of opportunistic fungi such as *Candida* is especially important in clinical laboratories and medical diagnosis. This step is also important in the study of antifungal susceptibilities and epidemiological surveillance.

Regarding *Candida* and related species, a growing concern is more evident in the public health area where these species have been described as emerging pathogens that can cause opportunistic infections. The immunosuppression caused by HIV (AIDS), medical treatments in which it is necessary to reduce the ability of immune response such as treatment of certain cancers, transplantation, malnutrition, prolonged use of intravenous catheters in several procedures, infant prematurity, increase the susceptibility to fungal infection [1].

A large number of methodologies, especially those based on biochemical assimilation patterns, have been employed to improve and expand our ability to identify fungal species. However, such methods encounter difficulties for those species with similar metabolic characteristics. Biochemical identification of fungi by MALDI-TOF has proven to be accurate and suitable to implement in routine analysis, but requires the equipment of MALDI-TOF [2].

The molecular methods targeting nucleotide sequences of stable RNAs, such as ribosomal RNA, or DNA delivers high precision and sensitivity in the taxonomic identification of microorganisms. This is important when identifying pathogens involved in known and emerging diseases caused by fungi [3].

The search for alternatives to sequencing, but with the same accuracy, led us to explore a methodology that involves PCR amplification of a specific region of the rDNA using primers anchored in conserved regions. With this strategy we were able to amplify a representative rDNA region, 2400 to 3100 base pairs in length, containing substitutions in sufficient quantity to generate a species-specific digestion pattern using enzymes that cut frequently within that region.

In this work, we present a Pattern Identification Guide validated by the analysis of 441 samples that were grouped in 33 restriction patterns that coincide with unique sequence-validated species.

Material and Methods

Phenotypical and biochemical methods

The samples were grown on Sabouraud Glucose Agar (SGA)

supplemented with chloramphenicol (1 mg.ml⁻¹) and CHROMagar *Candida* at 37°C to observe the macroscopic morphological characteristics [4]. Phenotypical tests also included (1) germ tube formation in bovine serum, (2) growth in corn meal agar plus 2% Tween 80 (v/v) and (3) fermentation of carbohydrates [5-7]. The API® 20 C AUX (bioMérieux, Marcy l'Etoile, France) was used as a reference standard for the identification of fungi. This system is commonly used in clinical laboratories.

In silico analysis

We used data from GenBank non-redundant database (nr), EMBL, DDBJ and PDB nucleotide collections. We also sequenced the ribosomal cistron (18S, ITS1, 5.8 S, ITS2 and 28S) for those species that did not have corresponding data in the GenBank database.

The sequences were analyzed, *in silico*, by MapDraw™ for Windows v. 5.00 from DNASTAR® Lasergene® software. This analysis gave us restriction maps that helped in the identification of the most discriminating restriction enzyme (Data not shown).

Total DNA preparation

Due to the large number of samples to analyze, we modified the total yeast DNA extraction protocol described by Philippi et al. for use in microplates, devising a DNA extraction protocol suitable to process 96 samples at a time [8].

The 96-well microplate was loaded with 150 µL of liquid YPD medium containing 0.001% (v/v) Tween 20. The cells were transferred to each well with a 96-pin frog loaded from inocula grown in solid YPD and then incubated at 30°C overnight, without shaking.

After the incubation, the plates were centrifuged for 5 min at 2250 g and 4°C. The supernatant was drained and the cells resuspended in 100

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μ L of lysing buffer (100 mM EDTA, 50 mM Tris-HCl, pH 7.5, 25 mM DTT and 165 g.ml⁻¹ Zymolyase[®]) and then incubated for 30 min at 37°C. Next we added 1% SDS (w/v) final concentration and the plates were kept for 30 min at 65°C.

To remove protein contamination and SDS, 60 μ L of 5 M potassium acetate was added. The plates were kept on ice for 30 min before centrifugation for 25 min at 2250 g and 4°C. After centrifugation, 140 μ L of supernatant was collected and transferred to new plates containing 130 μ L of isopropyl alcohol. The plates were kept on ice for 15 min and then centrifuged for 15 min at 2250 g and 4°C. The supernatant was removed, the pellet washed once with 80% (v/v) ethanol, left to dry at room temperature and redissolved in 30 μ L of TE-RNase buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 10 μ g.ml⁻¹ RNase).

Molecular approach

The GoTaq[®] DNA Polymerase kit (Promega, Madison, WI, USA)

was used to amplify the ribosomal cistron as follow: 1x Colorless GoTaq[®] Reaction Buffer, 0.2 mM dNTPs, 0.2 μ M of each primer, 18SFw2 (5'-GCTTGTCCTCAAAGATTAAGCCATGC)/ Uni-R (5'-GGTCCTGTCTCAAGACG) [9], 0.625 U GoTaq[®] DNA Polymerase, 2 μ L of template and ultrapure water to 25 μ L of final volume. The cycling conditions were set in an Express Thermal Cycler (Hybaid Limited) PCR cycler as follows: 3 min at 95°C followed by 35 cycles of 95°C 30 s, 60°C 30 s and 72°C 2:30 min.

From maps obtained in the *in silico* analysis, we chose the enzyme *DdeI* using the restriction buffer suggested by the manufacturer (New England Biolabs): about 1 μ g of PCR product (about 5 μ L) was digested using 1 X NEB 3 buffer and 2 U of *DdeI* at 37°C 2 h. The digestion pattern was analyzed in 2% agarose gels in 50 x 1 mm wells and stained with 0.5 μ g.ml⁻¹ ethidium bromide.

Detailed information about the tests used for each sample, ATCC

Species	Correct ID n=	Correct ID after extra tests n=	Mis or not-identified n=	Total n=	Correct ID %
<i>Candida glabrata</i>	23	-	0	23	100
<i>Trichosporon inkin</i>	4	-	0	4	100
<i>Pichia norvegensis</i>	3	-	0	3	100
<i>Trichosporon asahii</i>	2	-	0	2	100
<i>Candida albicans</i>	204	10	2	216	99.1
<i>Pichia guilliermondii</i>	6	11	1	18	94.4
<i>Candida dubliniensis</i>	14	-	1	15	93.3
<i>Candida krusei</i>	10	-	1	11	90.9
<i>Candida parapsilosis</i>	28	-	4	32	87.5
<i>Candida tropicalis</i>	28	5	5	38	86.8
<i>Saccharomyces cerevisiae</i>	4	-	1	5	80
<i>Candida pararugosa</i> *	0	-	4	4	0
<i>Pichia anomala</i>	0	-	2	2	0
<i>Candida</i> sp. SK75/76**	0	-	2	2	0
<i>Candida kefyr</i>	0	-	1	1	0
<i>Candida lipolytica</i> *	0	-	1	1	0
<i>Candida</i> sp. L96D**	0	-	1	1	0
<i>Exophiala dermatitidis</i> *	0	-	1	1	0
<i>Geotrichum capitatum</i>	0	-	1	1	0
<i>Ogathaea thermophila</i> *	0	-	1	1	0
Total	326	26	29	381	92.4

* Species not included in API 20C AUX database.

** Misidentified as *C. rugosa*.

Table 1: Species identified by the API 20C AUX.

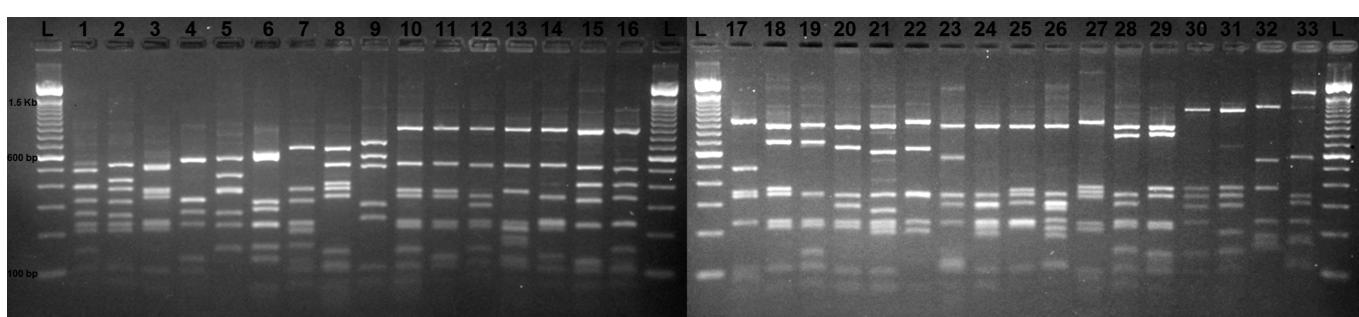


Figure 1: RFLP pattern from 33 different species. Electrophoresis was performed at 90 volts for 60 minutes on a 2% agarose gel visualized by ethidium bromide staining. L, 100 bp DNA Ladder 15628-019 (Invitrogen[™]); 1, *Candida* sp. SK75; 2, *C. rugosa*; 3, *P. guilliermondii*; 4, *C. lusitaniae*; 5, *G. capitatum*; 6, *C. stellata*; 7, *Candida* sp. L96D; 8, *T. mucoides*; 9, *C. lipolytica*; 10, *C. albicans*; 11, *C. dubliniensis*; 12, *C. tropicalis*; 13, *P. anomala*; 14, *D. hansenii*; 15, *C. intermedia*; 16, *O. thermophila*; 17, *P. norvegensis*; 18, *P. subpelliculosa*; 19, *E. endophytica*; 20, *C. parapsilosis*; 21, *C. kefyr*; 22, *C. krusei*; 23, *M. gypseum*; 24, *C. palmoleophila*; 25, *C. zeylanoides*; 26, *P. brasiliensis*; 27, *C. inconspicua*; 28, *S. cerevisiae*; 29, *C. glabrata*; 30, *T. inkin*; 31, *T. asahii*; 32, *C. pararugosa*; 33, *E. dermatitidis*.

strains and isolates used in this work are listed in Supplementary Data (Table 1).

Results

The described procedure for total yeast DNA preparation in a 96-well plate format yielded inhibitor-free material. The PCR product obtained (Figure 2) was abundant and easily digested by *Dde1*.

In the digestion reaction, 5 µl from the PCR was enough for good results, and all patterns matched the *in silico* digestion maps (see Pattern

Identification Guide in supplementary material). We also have tested kits purchased from Promega and Fermentas, for amplification and digestion, and the results were the same as those described here (data not shown). The *Dde1* enzyme worked very well when used directly in the PCR mix after the amplification. Consequently, no purification of the PCR before digestion was necessary (data not shown). The pattern of restriction fragments for each sample was compared with the results of classical tests and API kit results. DNA sequencing was used as the final arbiter whenever there was disagreement or a new electrophoresis pattern emerged (Table 2).

Nº	Strain	Classic identification	API 20C AUX			Sequencing	PCR-RFLP
			Species	%	T		
1.	FCF 14	<i>C. albicans</i>	<i>C. albicans</i> 2	99.5	0.25	<i>C. albicans</i>	<i>C. albicans</i>
2.	FCF 14,1	<i>C. albicans</i>	<i>C. albicans</i> 1	97.2	0.72	<i>C. albicans</i>	<i>C. albicans</i>
3.	ATCC 10231	<i>C. albicans</i>	<i>C. albicans</i> 1	97.2	0.72	<i>C. albicans</i>	<i>C. albicans</i>
4.	LGMG2A	<i>C. glabrata</i>	<i>C. glabrata</i>	99.3	1.0	<i>C. glabrata</i>	<i>C. glabrata</i>
5.	LGMG2B	<i>C. glabrata</i>	<i>C. glabrata</i>	99.3	1.0	<i>C. glabrata</i>	<i>C. glabrata</i>
6.	LGMG2C	<i>C. glabrata</i>	<i>C. glabrata</i>	99.3	1.0	<i>C. glabrata</i>	<i>C. glabrata</i>
7.	LGMG3A	<i>C. krusei</i>	<i>C. krusei/ inconspicua</i>	98.9	0.91	<i>C. krusei</i>	<i>C. krusei</i>
8.	LGMG3B	<i>C. krusei</i>	<i>C. krusei/ inconspicua</i>	98.9	0.91	<i>C. krusei</i>	<i>C. krusei</i>
9.	LGMG3D	<i>C. krusei</i>	<i>C. krusei/ inconspicua</i>	98.9	0.91	<i>C. krusei</i>	<i>C. krusei</i>
10.	ICB06	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>	94.2	0.54	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
11.	LGMG4B	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>	84.3	1.0	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
12.	H585	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>	88.5	0.49	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
13.	LGMG4E	<i>C. famata</i>	<i>P. guilliermondii</i>	60.3	0.83	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
14.	ICB 15	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>	98.9	0.39	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
15.	LGMG5C	<i>C. parapsilosis</i>	<i>C. parapsilosis</i> <i>C. neoformans</i>	-	-	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
16.	LGMG5D	<i>C. parapsilosis</i>	<i>C. tropicalis</i>	91.6	0.61	<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
17.	LGMG7A	<i>C. parapsilosis</i>	<i>C. famata</i>	99.7	0.97	<i>C. tropicalis</i>	<i>C. tropicalis</i>
18.	LGMG7B	<i>C. tropicalis</i>	<i>C. tropicalis</i>	70.2	0.72	<i>C. tropicalis</i>	<i>C. tropicalis</i>
19.	FCF 426	<i>C. tropicalis</i>	<i>C. tropicalis</i>	60.8	0.71	<i>C. tropicalis</i>	<i>C. tropicalis</i>
20.	LGMG 8A	<i>C. glabrata</i>	<i>C. pelliculosa</i>	79.1	0.69	<i>C. subpelliculosa</i>	<i>C. subpelliculosa</i>
21.	LGMG 8B	<i>C. krusei</i>	<i>C. pelliculosa</i>	79.1	0.69	<i>C. subpelliculosa</i>	<i>C. subpelliculosa</i>
22.	004/01	<i>C. albicans</i>	<i>P. norvengensis</i>	96.5	0.74	<i>P. norvengensis</i>	<i>P. norvengensis</i>
23.	004/03	<i>C. albicans</i>	<i>P. norvengensis</i>	96.5	0.74	<i>P. norvengensis</i>	<i>P. norvengensis</i>
24.	LGMG 10C	<i>P. norvengensis</i>	<i>P. norvengensis</i>	96.5	0.74	<i>P. norvengensis</i>	<i>P. norvengensis</i>
25.	LGMG 12A	<i>C. lipolytica</i>	<i>C. krusei</i>	39.9		<i>C. lipolytica</i>	<i>C. lipolytica</i>
26.	L96D	<i>C. rugosa</i>	<i>C. rugosa</i>	98.1	0.72	<i>Candida sp</i>	<i>C. mesorugosa</i>
27.	BMM 35C	Unidentified	Unidentified	-	-	<i>E. dermatitidis</i>	<i>E. dermatitidis</i>
28.	BMM 29A	Unidentified	<i>T. inkin</i>	-	-	<i>T. inkin</i>	<i>T. inkin</i>
29.	BMM 29B	Unidentified	<i>T. inkin</i>	-	-	<i>T. inkin</i>	<i>T. inkin</i>
30.	BMM 29C	Unidentified	<i>T. inkin</i>	-	-	<i>T. inkin</i>	<i>T. inkin</i>
31.	BMM 29D	Unidentified	<i>T. inkin</i>	-	-	<i>T. inkin</i>	<i>T. inkin</i>
32.	BMM30A	<i>C. rugosa</i>	<i>C. rugosa</i>	97.7	0.38	<i>C. pararugosa</i>	<i>C. pararugosa</i>
33.	BMM30B	<i>C. rugosa</i>	<i>C. rugosa</i>	79.8	0.71	<i>C. pararugosa</i>	<i>C. pararugosa</i>
34.	BMM30C	<i>C. rugosa</i>	<i>C. rugosa</i>	79.8	0.71	<i>C. pararugosa</i>	<i>C. pararugosa</i>
35.	BMM30D	<i>C. rugosa</i>	<i>C. rugosa</i>	97.7	0.38	<i>C. pararugosa</i>	<i>C. pararugosa</i>
36.	SK 75	<i>C. rugosa</i>	<i>C. rugosa</i>	97.1	0.93	<i>Candida sp</i>	<i>C. metarugosa</i>
37.	SK 76	<i>C. rugosa</i>	<i>C. rugosa</i>	97.1	0.93	<i>Candida sp</i>	<i>C. metarugosa</i>
38.	ATCC 62894	<i>C. famata</i>	<i>P. guilliermondii</i> <i>C. famata</i>	60.3 39.6	0.83 39.6	<i>C. palmoleophila</i>	<i>C. palmoleophila</i>
39.	LGMG 1D	<i>C. krusei</i>				<i>C. albicans</i>	<i>C. albicans</i>
40.	ICB 58	<i>C. albicans</i>				<i>C. albicans</i>	<i>C. albicans</i>
41.	ATCC 14053	<i>C. albicans</i>				<i>C. albicans</i>	<i>C. albicans</i>
42.	UNESP 15D	<i>C. albicans</i>				<i>C. albicans</i>	<i>C. albicans</i>
43.	LGMG 1H	<i>P. guilliermondii</i>				<i>C. albicans</i>	<i>C. albicans</i>
44.	LGMG 1J	<i>C. albicans</i>				<i>C. albicans</i>	<i>C. albicans</i>

45.	LGMG 1K	<i>C. albicans</i>				<i>C. albicans</i>	<i>C. albicans</i>
46.	LGMG 2E	<i>C. krusei</i>				<i>C. glabrata</i>	<i>C. glabrata</i>
47.	ATCC 2001	<i>C. glabrata</i>				<i>C. glabrata</i>	<i>C. glabrata</i>
48.	LGMG 4B	<i>P. guilliermondii</i>				<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
49.	H1020	<i>P. guilliermondii</i>				<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
50.	ATCC 6260	<i>P. guilliermondii</i>				<i>P. guilliermondii</i>	<i>P. guilliermondii</i>
51.	LGMG 5A	<i>C. albicans</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
52.	LGMG 5E	<i>C. krusei</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
53.	LGMG 5F	<i>P. guilliermondii</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
54.	LGMG 5G	<i>C. tropicalis</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
55.	LGMG 5H	<i>P. guilliermondii</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
56.	LGMG 5I	<i>C. krusei</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
57.	LGMG 5J	<i>C. krusei</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
58.	LGMG 5K	<i>C. albicans</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
59.	LGMG 5L	<i>P. guilliermondii</i>				<i>C. parapsilosis</i>	<i>C. parapsilosis</i>
60.	LGMG 6 ^a	<i>C. tropicalis</i>				<i>C. lusitaniae</i>	<i>C. lusitaniae</i>
61.	LGMG 7D	<i>C. tropicalis</i>				<i>C. tropicalis</i>	<i>C. tropicalis</i>
62.	LGMG 7E	<i>C. albicans</i>				<i>C. tropicalis</i>	<i>C. tropicalis</i>
63.	ATCC 750	<i>C. tropicalis</i>				<i>C. tropicalis</i>	<i>C. tropicalis</i>
64.	ATCC 40197	<i>P. anomala</i>				<i>P. anomala</i>	<i>P. anomala</i>
65.	ATCC 40050	<i>C. inconspicua</i>				<i>C. inconspicua</i>	<i>C. inconspicua</i>
66.	ATCC	<i>C. intermedia</i>				<i>C. intermedia</i>	<i>C. intermédia</i>
67.	ATCC 4156	<i>C. zeylanoides</i>				<i>C. zeylanoides</i>	<i>C. zeylanoides</i>
68.	ATCC 40139	<i>C. stellata</i>				<i>C. stellata</i>	<i>C. stellata</i>
69.	ATCC 36239	<i>D. hansenii</i>				<i>D. hansenii</i>	<i>D. hansenii</i>
70.	Mg 27	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
71.	80SP	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
72.	USP474	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
73.	DR 25	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
74.	169SP	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
75.	RO349	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
76.	MEG13	<i>M. gypseum</i>				<i>M. gypseum</i>	<i>M. gypseum</i>
77.	CD33		<i>C. dubliniensis</i>	99.9	0.69	<i>C. dubliniensis</i>	<i>C. dubliniensis</i>
78.	CD36		<i>C. dubliniensis</i>	99.9	0.69	<i>C. dubliniensis</i>	<i>C. dubliniensis</i>
79.	USP777		<i>C. famata</i>	99.9	0.72	<i>C. dubliniensis</i>	<i>C. dubliniensis</i>
80.	ATCC778157		<i>C. dubliniensis</i>	99.9	0.69	<i>C. dubliniensis</i>	<i>C. dubliniensis</i>
81.	08D		<i>C. sphaerica</i>	99.2	0.66	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
82.	21A		<i>S. cerevisiae</i>	98.7	1.00	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
83.	21B		<i>S. cerevisiae</i>	93.5	0.88	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
84.	21C		<i>S. cerevisiae</i>	99.9	0.94	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
85.	21D		<i>S. cerevisiae</i>	93.5	0.88	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
86.	SK 18		<i>S. cerevisiae</i>	81.2	0.92	<i>C. kefyr</i>	<i>C. kefyr</i>
87.	SK 19		<i>H. polymorpha</i>	-	-	<i>O. thermophila</i>	<i>O. thermophila</i>
88.	SK 21		<i>T. asahii</i>	99.9	0.9	<i>T. asahii</i>	<i>T. asahii</i>
89.	SK 92		<i>T. asahii</i>	99.9	0.9	<i>T. asahii</i>	<i>T. asahii</i>
90.	1A	<i>C. albicans</i>	<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
91.	1B	<i>C. albicans</i>	<i>P. guilliermondii</i>	84.3	1.0		<i>P. guilliermondii</i>
92.	1C	<i>C. albicans</i>	<i>P. guilliermondii</i>	84.3	1.0		<i>P. guilliermondii</i>
93.	1D	<i>C. albicans</i>	<i>P. guilliermondii</i>	84.3	1.0		<i>P. guilliermondii</i>
94.	1X	<i>C. albicans</i>	<i>C. albicans</i>	97.4	1.0		<i>C. albicans</i>
95.	3A	<i>C. glabrata</i>	<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
96.	3B	<i>C. glabrata</i>	<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
97.	3D	<i>C. glabrata</i>	<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
98.	5E	<i>C. Krusei</i>	<i>C. norvegensis</i> <i>G. capitatum</i>	43.8 31.7	1.0 1.0		<i>C. Krusei</i>
99.	5G	<i>C. Krusei</i>	<i>C. krusei/</i> <i>inconspicua</i>	98.9	0.91		<i>C. Krusei</i>
100.	4A	<i>P. guilliermondii</i>	<i>P. guilliermondii</i>	84.3	1.0		<i>P. guilliermondii</i>
101.	4B	<i>P. guilliermondii</i>	<i>C. famata</i> <i>P. guilliermondii</i>	62.6 37.2	0.65 0.55		<i>C. parapsilosis</i>
102.	4C	<i>P. guilliermondii</i>	<i>C. parapsilosis</i>	96.1	0.54		<i>C. parapsilosis</i>

103.	4D	<i>P. guilliermondii</i>	<i>C. albicans</i>	97.4	1.0		<i>C. albicans</i>
104.	6A	<i>C. parapsilosis</i>	<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
105.	6B	<i>C. parapsilosis</i>	<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
106.	6C	<i>C. parapsilosis</i>	<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
107.	6N	<i>C. parapsilosis</i>	<i>C. glabrata</i>	99.3	1.00		<i>C. glabrata</i>
108.	7A	<i>C. tropicalis</i>	<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
109.	7C	<i>C. tropicalis</i>	<i>C. albicans</i>	68.8	0.65		<i>C. albicans</i>
110.	7Q	<i>C. tropicalis</i>	<i>C. famata</i> <i>C. laurentii</i>	84.8 8.6	0.68 0.52		<i>C. tropicalis</i>
111.	7X	<i>C. tropicalis</i>	<i>C. famata</i> <i>C. laurentii</i>	84.8 8.6	0.68 0.52		<i>C. tropicalis</i>
112.	7Y	<i>C. tropicalis</i>	<i>C. famata</i>	95.9	0.60		<i>C. tropicalis</i>
113.	8D	<i>C. tropicalis</i>	<i>C. famata</i>	98.2	0.92		<i>C. tropicalis</i>
114.	SK 001		<i>C. albicans</i>	-	-		<i>C. albicans</i>
115.	SK 002		<i>C. parapsilosis</i>	93.6	0.57		<i>C. parapsilosis</i>
116.	SK 003		<i>C. albicans</i>	-	-		<i>C. albicans</i>
117.	SK 004		<i>C. parapsilosis</i>	99.9	1.0		<i>C. parapsilosis</i>
118.	SK 005		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
119.	SK 006		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
120.	SK 007		<i>C. parapsilosis</i>	99.9	0.68		<i>C. parapsilosis</i>
121.	SK 008		<i>C. albicans</i>	-	-		<i>C. albicans</i>
122.	SK 009		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
123.	SK 010		<i>C. parapsilosis</i>	99.9	1.0		<i>C. parapsilosis</i>
124.	SK 011		<i>C. albicans</i>	-	-		<i>C. albicans</i>
125.	SK 012		<i>C. albicans</i>	-	-		<i>C. albicans</i>
126.	SK 013		<i>C. albicans</i>	-	-		<i>C. albicans</i>
127.	SK 014		<i>C. albicans</i>	-	-		<i>C. albicans</i>
128.	SK 015		<i>C. albicans</i>	-	-		<i>C. albicans</i>
129.	SK 016		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
130.	SK 017		<i>C. norvegensis</i> <i>G. capitatum</i>	43.8 41.4	1.0 1.0		<i>G. capitatum</i>
131.	SK 020		<i>C. albicans</i>	-	-		<i>C. albicans</i>
132.	SK 022		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
133.	SK 023		<i>C. albicans</i>	-	-		<i>C. albicans</i>
134.	SK 024		<i>C. albicans</i>	-	-		<i>C. albicans</i>
135.	SK 025		<i>C. albicans</i>	-	-		<i>C. albicans</i>
136.	SK 026		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
137.	SK 027		<i>C. albicans</i>	-	-		<i>C. albicans</i>
138.	SK 029		<i>C. albicans</i>	-	-		<i>C. albicans</i>
139.	SK 030		<i>C. albicans</i>	-	-		<i>C. albicans</i>
140.	SK 031		<i>C. albicans</i>	-	-		<i>C. albicans</i>
141.	SK 032		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
142.	SK 033		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
143.	SK 034		<i>C. albicans</i>	-	-		<i>C. albicans</i>
144.	SK 035		<i>C. albicans</i>	-	-		<i>C. albicans</i>
145.	SK 036		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
146.	SK 037		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
147.	SK 038		<i>C. parapsilosis</i>	99.9	0.86		<i>C. parapsilosis</i>
148.	SK 039		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
149.	SK 040		<i>C. glabrata</i>	99.3	1.0		<i>P. guilliermondii</i>
150.	SK 041		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
151.	SK 042		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
152.	SK 043		<i>P. guilliermondii</i>	84.3	1.00		<i>P. guilliermondii</i>
153.	SK 044		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
154.	SK 045		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
155.	SK 046		<i>C. albicans</i>	-	-		<i>C. albicans</i>
156.	SK 047		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
157.	SK 048		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
158.	SK 049		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
159.	SK 050		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
160.	SK 051		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>

161.	SK 052		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
162.	SK 053		<i>C. parapsilosis</i>	99.9	0.86		<i>C. parapsilosis</i>
163.	SK 054		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
164.	SK 055		<i>C. albicans</i>	-	-		<i>C. albicans</i>
165.	SK 056		<i>C. albicans</i>	-	-		<i>C. albicans</i>
166.	SK 057		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
167.	SK 058		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
168.	SK 059		<i>C. parapsilosis</i>	99.9	0.86		<i>C. parapsilosis</i>
169.	SK 060		<i>C. albicans</i>	-	-		<i>C. albicans</i>
170.	SK 061		<i>C. albicans</i>	-	-		<i>C. albicans</i>
171.	SK 062		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
172.	SK 063		<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
173.	SK 064		<i>C. albicans</i>	-	-		<i>C. albicans</i>
174.	SK 065		<i>C. albicans</i>	-	-		<i>C. albicans</i>
175.	SK 066		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
176.	SK 067		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
177.	SK 068		<i>C. albicans</i>	-	-		<i>C. albicans</i>
178.	SK 069		<i>C. albicans</i>	-	-		<i>C. albicans</i>
179.	SK 070		<i>C. albicans</i>	-	-		<i>C. albicans</i>
180.	SK 071		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
181.	SK 072		<i>C. albicans</i>	-	-		<i>C. albicans</i>
182.	SK 073		<i>C. albicans</i>	-	-		<i>C. albicans</i>
183.	SK 074		<i>C. albicans</i>	-	-		<i>C. albicans</i>
184.	SK 077		<i>C. albicans</i>	-	-		<i>C. albicans</i>
185.	SK 078		<i>C. albicans</i>	-	-		<i>C. albicans</i>
186.	SK 079		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
187.	SK 080		<i>C. parapsilosis</i>	99.9	0.82		<i>C. parapsilosis</i>
188.	SK 081		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
189.	SK 082		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
190.	SK 083		<i>C. tropicalis</i>	88.9	0.89		<i>C. tropicalis</i>
191.	SK 084		<i>P. guilliermondii</i>	99.7	0.99		<i>P. guilliermondii</i>
192.	SK 085		<i>C. albicans</i>	-	-		<i>C. albicans</i>
193.	SK 086		<i>C. albicans</i>	-	-		<i>C. albicans</i>
194.	SK 087		<i>C. albicans</i>	-	-		<i>C. albicans</i>
195.	SK 088		<i>C. krusei/ Inconspicua</i>	96.2	1.00		<i>C. krusei</i>
196.	SK 089		<i>C. parapsilosis</i>	99.9	0.86		<i>C. parapsilosis</i>
197.	SK 090		<i>C. parapsilosis</i>	99.9	0.86		<i>C. parapsilosis</i>
198.	SK 091		<i>C. albicans</i>	-	-		<i>C. albicans</i>
199.	SK 093		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
200.	SK 094		<i>C. albicans</i>	-	-		<i>C. albicans</i>
201.	SK 095		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
202.	SK 096		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
203.	SK 097		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
204.	SK 098		<i>C. parapsilosis</i>	99.9	0.82		<i>C. parapsilosis</i>
205.	SK 099		<i>C. parapsilosis</i>	99.9	0.82		<i>C. parapsilosis</i>
206.	SK 100		<i>C. parapsilosis</i>	99.9	1.00		<i>C. parapsilosis</i>
207.	SK 101		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
208.	SK 102		<i>C. parapsilosis</i>	99.9	0.68		<i>C. parapsilosis</i>
209.	SK 103		<i>C. tropicalis</i>	95.7	1.0		<i>C. tropicalis</i>
210.	SK 104		<i>C. albicans</i>	-	-		<i>C. albicans</i>
211.	SK 105		<i>C. albicans</i>	-	-		<i>C. albicans</i>
212.	01 ANR A		<i>C. albicans</i>	79.2	0.16		<i>C. albicans</i>
213.	01 ANR C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
214.	02ANR A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
215.	02ANR B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
216.	02ANR C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
217.	02ANR D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
218.	04 ANP A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
219.	04 ANP B		<i>C. albicans</i>	88.9	0.4		<i>C. albicans</i>
220.	04 ANP C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>

221.	04 ANP D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
222.	06 ANP A		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
223.	06 ANP B		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
224.	06 ANP C		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
225.	06 ANP D		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
226.	07 ANP A		<i>C. albicans</i>	98	0.58		<i>C. albicans</i>
227.	07 ANP B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
228.	07 ANP C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
229.	08 (1BN) A		<i>C. parapsilosis</i>	99.9	1.0		<i>C. parapsilosis</i>
230.	08 (1BN) C		<i>C. parapsilosis</i>	92.9	0.72		<i>C. parapsilosis</i>
231.	9 AN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
232.	10 (2BN) A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
233.	10 (2BN) B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
234.	10 (2BN) C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
235.	10 (2BN) D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
236.	11ANBN A		<i>C. krusei/ inconspicua</i>	98.9	0.91		<i>C. Krusei</i>
237.	11ANBN B		<i>C. krusei/ inconspicua</i>	98.9	0.91		<i>C. Krusei</i>
238.	11 ANBN C		<i>C. tropicalis</i>	93.1	0.56		<i>C. tropicalis</i>
239.	11 ANBN D		<i>C. albicans</i>	79.2	0.16		<i>C. albicans</i>
240.	11 ANBN E		<i>C. tropicalis</i>	70.2	0.72		<i>C. tropicalis</i>
241.	12AN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
242.	12AN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
243.	12AN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
244.	12AN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
245.	12AN E		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
246.	12AN F		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
247.	12AN G		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
248.	12AN H		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
249.	13 ANP A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
250.	13 ANP B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
251.	13 ANP C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
252.	13 ANP D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
253.	14ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
254.	14ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
255.	14 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
256.	14 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
257.	14 ANBN E		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
258.	16 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
259.	16 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
260.	16 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
261.	17 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
262.	17 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
263.	17 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
264.	19 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
265.	19 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
266.	19 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
267.	20 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
268.	20 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
269.	20 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
270.	20 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
271.	21 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
272.	21 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
273.	21 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
274.	21 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
275.	21 ANBN E		<i>C. tropicalis</i>	93.1	0.56		<i>C. tropicalis</i>
276.	21 ANBN F		<i>C. tropicalis</i>	93.1	0.56		<i>C. tropicalis</i>
277.	23 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
278.	23 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
279.	23 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
280.	23 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
281.	25 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>

282.	25 ANBN B		<i>C. albicans</i>	77.8	0.59		<i>C. albicans</i>
283.	25 ANBN C		<i>C. albicans</i>	77.8	0.59		<i>C. albicans</i>
284.	25 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
285.	25 ANBN E		<i>C. albicans</i>	98	0.58		<i>C. albicans</i>
286.	25 ANBN F		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
287.	26 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
288.	26 ANBN B		<i>C. albicans</i>	77.8	0.59		<i>C. albicans</i>
289.	26 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
290.	26 ANBN E		<i>C. albicans</i>	98	0.58		<i>C. albicans</i>
291.	26 ANBN F		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
292.	28 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
293.	28 ANBN B		<i>C. dubliniensis</i>	99.9	0.69		<i>C. dubliniensis</i>
294.	28 ANBN C		<i>C. dubliniensis</i>	99.9	0.69		<i>C. dubliniensis</i>
295.	28 ANBN D		<i>C. dubliniensis</i>	67.7	0.36		<i>C. dubliniensis</i>
296.	29 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
297.	29 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
298.	29 ANBN C		<i>C. albicans</i>	88.5	0.46		<i>C. albicans</i>
299.	29 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
300.	30 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
301.	30 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
302.	30 ANBN E		<i>C. parapsilosis</i>	98.5	0.61		<i>C. parapsilosis</i>
303.	31 ANBN A		<i>C. dubliniensis</i>	67.7	0.36		<i>C. dubliniensis</i>
304.	31 ANBN B		<i>C. dubliniensis</i>	99.9	0.69		<i>C. dubliniensis</i>
305.	31 ANBN C		<i>C. dubliniensis</i>	99.9	0.69		<i>C. dubliniensis</i>
306.	32 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
307.	32 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
308.	32 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
309.	32 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
310.	34 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
311.	34 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
312.	35 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
313.	35 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
314.	36 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
315.	36 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
316.	36 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
317.	37 ANBN A		<i>C. famata</i>	96	0.51		<i>C. albicans</i>
318.	37 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
319.	37 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
320.	39 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
321.	39 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
322.	39 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
323.	39 ANBN D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
324.	39 ANBN E		<i>C. albicans</i>	93.9	0.59		<i>C. albicans</i>
325.	42 ANBN A		<i>C. parapsilosis</i>	98.9	0.39		<i>C. parapsilosis</i>
326.	42 ANBN C		<i>C. parapsilosis</i>	92.9	0.72		<i>C. parapsilosis</i>
327.	45 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
328.	45 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
329.	45 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
330.	46 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
331.	46 ANBN B		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
332.	46 ANBN C		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
333.	48 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
334.	48 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
335.	49 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
336.	49 ANBN B		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
337.	50 ANBN A		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
338.	50 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
339.	50 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
340.	52 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
341.	52 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
342.	52 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>

343.	53 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
344.	53 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
345.	53 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
346.	55 ANBN A		<i>C. dubliniensis</i>	99	0.33		<i>C. dubliniensis</i>
347.	55 ANBN B		<i>C. dubliniensis</i>	---	----		<i>C. dubliniensis</i>
348.	56 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
349.	57 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
350.	57 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
351.	57 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
352.	58 ANBN A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
353.	58 ANBN B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
354.	58 ANBN C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
355.	01 Cont a		<i>C. dubliniensis</i>	91.9	0.47		<i>C. dubliniensis</i>
356.	07 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
357.	07 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
358.	07 Cont C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
359.	07 Cont D		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
360.	10 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
361.	10 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
362.	10 Cont C		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
363.	10 Cont D		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
364.	12 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
365.	12 Cont B		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
366.	15 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
367.	17 Cont A		<i>C. glabrata</i>	99.3	0.5		<i>C. glabrata</i>
368.	17 Cont B		<i>C. glabrata</i>	99.3	0.5		<i>C. glabrata</i>
369.	19 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
370.	19 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
371.	19 Cont C		<i>C. albicans</i>	98.2	0.87		<i>C. albicans</i>
372.	21 Cont A		<i>C. krusei</i>	98.9	0.41		<i>C. krusei</i>
373.	21 Cont A		<i>C. krusei</i>	98.9	0.41		<i>C. krusei</i>
374.	21 Cont C		<i>C. dubliniensis</i>				<i>C. dubliniensis</i>
375.	21 Cont D		<i>C. dubliniensis</i>	99.9	0.66		<i>C. dubliniensis</i>
376.	23 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
377.	23 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
378.	24 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
379.	24 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
380.	25 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
381.	25 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
382.	26 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
383.	28 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
384.	28 Cont B		<i>C. tropicalis</i>	79.2	0.88		<i>C. albicans</i>
385.	31 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
386.	31 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
387.	33 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
388.	33 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
389.	35 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
390.	35 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
391.	36 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
392.	37 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
393.	37 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
394.	39 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
395.	40 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
396.	40 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
397.	42 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
398.	42 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
399.	43 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
400.	45 Cont A		<i>P. guilliermondii</i>	60.3	0.83		<i>P. guilliermondii</i>
401.	46 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
402.	46 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
403.	48 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>

404.	50 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
405.	50 Cont B		<i>C. albicans</i>	59.3	0.37		<i>C. albicans</i>
406.	51 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
407.	51 Cont B		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
408.	52 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
409.	52 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
410.	54 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
411.	54 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
412.	56 Cont A		<i>C. glabrata</i>	99.3	1		<i>C. glabrata</i>
413.	58 Cont A		<i>C. albicans</i>	97.2	0.72		<i>C. albicans</i>
414.	Nufab 3		<i>C. glabrata</i>	29.2	0.9		<i>C. glabrata</i>
415.	Nufab 5		<i>C. glabrata</i>	99.3	1.0		<i>C. glabrata</i>
416.	Nufab 6		<i>C. glabrata</i>	15.0	0.4		<i>C. glabrata</i>
417.	Nufab 6A1		<i>C. tropicalis</i>	-	-		<i>C. parapsilosis</i>
418.	Nufab 11		<i>C. krusei</i>	98.9	0.91		<i>C. krusei</i>
419.	Nufab 13		<i>C. tropicalis</i>	29.2	0.6		<i>C. tropicalis</i>
420.	ATCC 157		<i>C. tropicalis</i>	29.8	0.43		<i>C. tropicalis</i>
421.	LBM 001	<i>C. parapsilosis</i>					<i>C. parapsilosis</i>
422.	LBM 003	<i>P. subpelliculosa</i>					<i>P. subpelliculosa</i>
423.	LBM 004	<i>T. asahii</i>					<i>T. asahii</i>
424.	LBM 005	<i>P. subpelliculosa</i>					<i>P. subpelliculosa</i>
425.	ATCC 30070	<i>C. glabrata</i>					<i>C. glabrata</i>
426.	USP 8987	<i>C. dubliniensis</i>					<i>C. dubliniensis</i>
427.	USP 7988	<i>C. dubliniensis</i>					<i>C. dubliniensis</i>
428.	222/02	<i>C. krusei</i>					<i>C. albicans</i>
429.	227/02	<i>C. albicans</i>					<i>C. albicans</i>
430.	212/02	<i>C. glabrata</i>					<i>C. glabrata</i>
431.	215/02	<i>C. glabrata</i>					<i>C. glabrata</i>
432.	218/02	<i>C. glabrata</i>					<i>C. glabrata</i>
433.	192/02	<i>C. glabrata</i>					<i>C. glabrata</i>
434.	42 ANBN B	<i>P. guilliermondii</i>					<i>P. guilliermondii</i>
435.	ATCC 4135	<i>C. kefyr</i>					<i>C. kefyr</i>
436.	ATCC 10663	<i>G. capitatum</i>					<i>G. capitatum</i>
437.	YPH 499	<i>S. cerevisiae</i>					<i>S. cerevisiae</i>
438.	ATCC	<i>T. mucoides</i>					<i>T. mucoides</i>
439.	ATCC 40038	<i>C. parapsilosis</i>					<i>C. parapsilosis</i>
440.	ATCC 40146	<i>C. lusitaniae</i>					<i>C. lusitaniae</i>
441.	ATCC40199	<i>C. lipolytica</i>					<i>C. lipolytica</i>

- Red letters was used to highlight the differences found among methods used.
- % - Designates the similarity between the unknown and the standard sample used when the test was Assembled.
- T - Confiability index; maximum value is 1.

Table 2: Comparison between different methods with the strains tested.

Using the PCR-RFLP method we were able so far to establish 33 distinct patterns, each one corresponding to a specific DNA sequence (Figure 1). *C. albicans* and *C. dubliniensis*, two well known closely related species, have distinct patterns (Figure 1, lanes 11 and 12). Basically, the difference is due to an additional restriction site at position 2168 bp of the amplicon in *C. dubliniensis* (Figure 3). In addition, *P. guilliermondii*, *D. hansenii*, and *C. palmoleophila* were easily differentiated (Figure 1, lanes 3, 14 and 24, respectively).

The digestion *in silico* with rDNA sequences available in databases from species that we did not process such as *Aspergillus fumigatus*, *Aspergillus nidulans*, *Candida fructos*, *Cryptococcus neoformans*, *Mucor racemosus*, *Pichia myanmaensis* and *Rhizopus oryzae* indicates that the PCR-RFLP method would also differentiate between these species (Figure 4).

Over several years we have collected and identified 441 samples with the RFLP method (Supplementary Data Table 1). 121 Species were also identified by classical methods. The identification of 62% of

these was confirmed by the PCR-RFLP assay. The API 20C AUX kit was used in 352 samples and 92.4% were confirmed by PCR-RFLP assay. Twenty-nine isolates were misidentified API methods and one isolate, BMM 35C, could not be identified by classical or API methods, and we identified it by sequencing as *Exophiala dermatitidis* (Table 1). Its digestion pattern is also specific and unique in our sample.

Discussion

Problems of misidentification with fungi, especially *Candida* species, have been reported in many studies especially those using the molecular tools to validate the phenotypic findings. In addition, the usual diagnosis based on the classical methodology is time-consuming and can lead to an imprecise identification. The industry has introduced methods using patterns of carbon assimilation that considerably improved results; the API 20C system is one of such methods. However, on average, 10% of isolates cannot be identified or are misidentified (manufacturer's information).

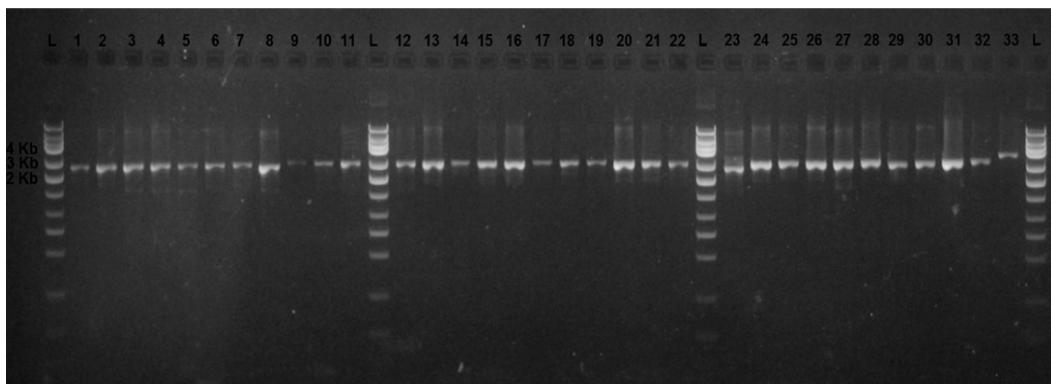


Figure 2: Ethidium bromide staining of a 1% agarose gel. A representative figure of amplicons is shown. L, 1Kb DNA Marker (Axxygen). Numbers in parenthesis represent the expected size of amplicon for each species: 1, *G. capitatum* (2464 bp); 2, *C. lipolytica* (2508 bp); 3, *Candida* sp. L96D (2593 bp); 4, *Candida* sp. SK75 (2601 bp); 5, *C. rugosa* (2595 bp); 6, *C. intermedia* (2647 bp); 7, *C. stellata* (2662 bp); 8, *C. lusitaniae* (2631 bp); 9, *C. pararugosa* (2679 bp); 10, *C. inconspicua* (2767 bp); 11, *C. krusei* (2804 bp); 12, *P. norvegensis* (2812 bp); 13, *C. parapsilosis* (2841 bp); 14, *C. tropicalis* (2843 bp); 15, *C. albicans* (2858 bp); 16, *C. dubliniensis* (2860 bp); 17, *T. mucoides* (2889 bp); 18, *T. inkin* (2901 bp); 19, *E. endophytica* (2918); 20, *P. guilliermondii* (2944 bp); 21, *P. anomala* (2951 bp); 22, *M. gypseum* (2953 bp); 23, *C. zeylanoides* (2960 bp); 24, *P. subpelluculosa* (2965 bp); 25, *C. palmioleophila* (2981 bp); 26, *D. hansenii* (2975 bp); 27, *P. brasiliensis* (2986 bp); 28, *C. thermophila* (3064 bp); 29, *T. asahii* (2904 bp); 30, *C. kefyr* (3027 bp); 31, *S. cerevisiae* (3175 bp); 32, *C. glabrata* (3224 bp); 33, *E. dermatitidis* (3966 bp).

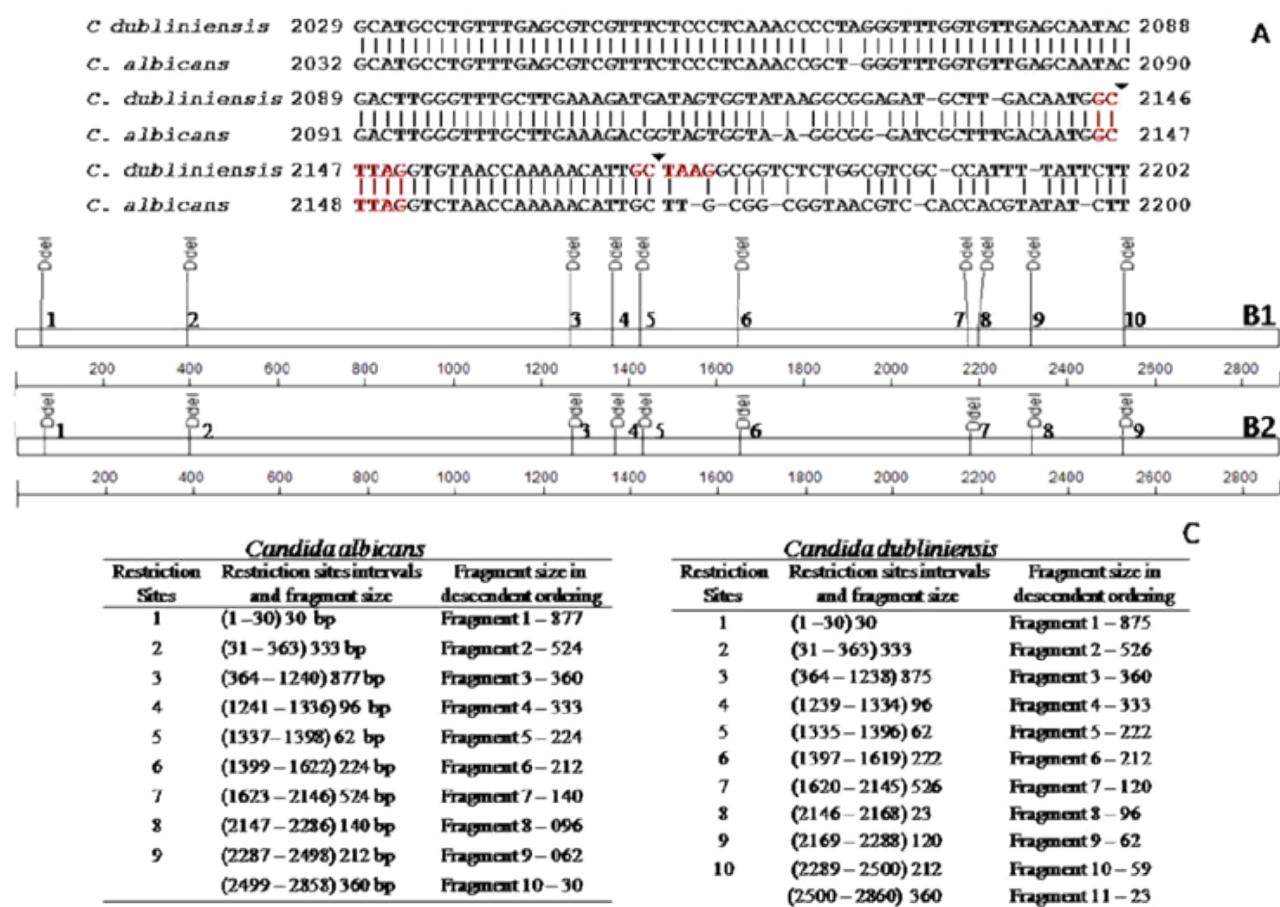


Figure 3: *C. albicans* and *C. dubliniensis* *in silico* comparison

A, BLAST alignment of nucleotide sequences: arrows show the position of restriction site to *Dde1* enzyme, red letters represent the complete sequence recognized by the enzyme.

B, Restrictionmaps to *Dde1* enzyme digestion predicted by MapDraw™.

B1, *C. dubliniensis* and B2, *C. albicans*; number near of each site was used to correspond the position with tables below. C, number of restriction sites, position, expected size and expected pattern for each species are showed here. *C. dubliniensis* has an additional restriction site (nº 8) at position 2168 bp.

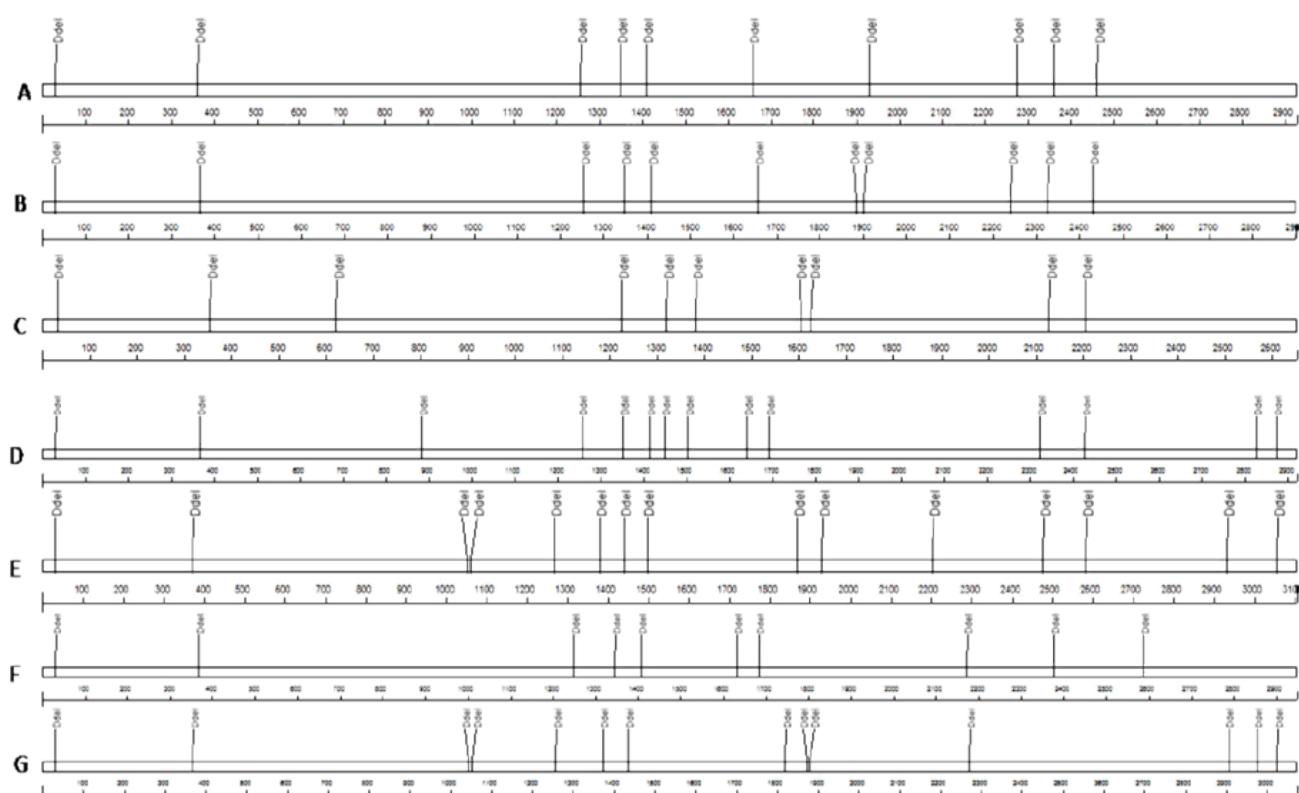


Figure 4: Restriction maps to DdeI enzyme digestion predicted by MapDraw™. The analyze *in silico* for 8 species is showed here: A, *Aspergillus fumigatus*; B, *Aspergillus nidulans*; C, *Candida fructos*; D, *Cryptococcus neoformans*; E, *Mucor racemosus*; F, *Pichia myanmaensis*; G, *Rhizopus oryzae*.

In this research we developed an alternative identification method for fungi, especially *Candida* species, based on the RFLP pattern of a specific DNA fragment. We compared the identification results obtained by this method of 441 samples with classical methods, the API 20C system and sequencing.

In our experience, the API 20C system resulted in 92.4% accurate identification of species. Complementary testing was frequently required for isolates identified as *C. famata* (after 72 h). These tests identified often mislabeled as *C. albicans*, *C. tropicalis* or *P. guilliermondii*. Limitations found for this system were the impossibility of identifying *C. lipolytica* and *E. dermatitidis* and also discriminating (1) *C. krusei* and *C. inconspicua*; (2) *D. hansenii* and *C. palmoleophila*; (3) *P. anomala* and *P. subpelliculosa*; (4) *C. rugosa*, *C. pararugosa*, *Candida* sp. SK75 and *Candida* sp. L96D.

The problems we experienced have been described by others using similar methodologies such as Vitek Yeast Biochemical Card and API Candida system or ID32C, purchased from bioMériaux, France [10-12]. It should be noted that the accuracy of these systems was better for common yeasts than for rare yeasts [13].

For *P. guilliermondii* and *D. hansenii* (teleomorphic phase of *C. famata*) the chance of misidentification is greater due to the similarity in carbon assimilation [11]. We must note that the discrimination of *D. hansenii* and *C. palmoleophila* is difficult even by sequencing since their 18S rRNA similarity is 98%. *C. famata* ATCC 62894 was misidentified in the ATCC collection; we have tested this strain and *D. hansenii* ATCC 36239 by PCR-RFLP and have found different patterns. The sequence of ITS 2 region identified ATCC 62894 as *C. palmoleophila*.

The sequencing of selected parts of well-known regions of the genome of these species as the repeat unit that encodes the genes for ribosomal 18S, 5.8 S, 28S and 5S including the spacer regions ITS1 and ITS2 have been used as the gold standard identification method [14]. Yet, DNA sequencing is not fast and inexpensive enough yet for routine laboratory use. The coupling of PCR amplification and RFLP has several advantages such as the possibility of obtaining results using minimal amounts of the original sample or partially degraded material; the choice of specific regions of the genome defined by primers; homogeneity in the patterns of amplification and subsequent restriction and also simultaneous manipulation of a large number of samples. Although the PCR-RFLP assay has been used before, the authors investigated just 6 species, a coverage much restricted from that presented here [15]. In addition, works that use RFLP to identify fungi are focused in the ITS region. This region generally vary from 400 to 600 bp in length in fungi and this fact limits its use after digestion by restriction endonucleases, because the fragments generated are too short and similar in length. The difference in the present work is the length of the amplicon that is bigger, to about 3000 bp. The restriction fragments span from 60 bp to 1000 bp with good pattern of differentiation on agarose gels.

The coverage of this method is certainly greater than the 33 validated patterns. We tested species far distant from *Candida* genus such as *E. dermatitidis*, *E. endophytica* and *Trichosporon* spp and the method presented here worked very well. In addition, the *in silico* analysis has corroborated these findings: other genera, families, order, classes and phyla can be amplified by the primers, and the putative amplicons result in discriminating patterns when digested by the *DdeI* restriction endonuclease.

The results presented here are promising and could overcome the limitations found in some methods such as API system and ITS region RFLP-ribotyping. The PCR-RFLP method so far provided the same accuracy as DNA sequencing.

Our future goals for using this method include expanding the database and automating the identification of the RFLP patterns using gel analysis software such as BioNumerics^{*} (Applied Maths NV).

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