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Research Paper

Application of MALDI-TOF MS for requalification of a *Candida* clinical isolates culture collection

Reginaldo Lima-Neto[1,2], Cledir Santos[2], Nelson Lima[2], Paula Sampaio[3], Célia Pais[3], Rejane P. Neves[1]

[1]Department of Mycology, Centre of Biological Sciences, Federal University of Pernambuco, Recife, PE, Brazil.
[2]Micoteca da Universidade do Minho, Centre of Biological Engineering, University of Minho, Braga, Portugal.
[3]Department of Biology, Centre of Molecular and Environmental Biology, University of Minho, Braga, Portugal.

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Abstract

Microbial culture collections underpin biotechnology applications and are important resources for clinical microbiology by supplying reference strains and/or performing microbial identifications as a service. Proteomic profiles by MALDI-TOF MS have been used for *Candida* spp. identification in clinical laboratories and demonstrated to be a fast and reliable technique for the routine identification of pathogenic yeasts. The main aim of this study was to apply MALDI-TOF MS combined with classical phenotypic and molecular approaches to identify *Candida* clinical isolates preserved from 1 up to 52 years in a Brazilian culture collection and assess its value for the identification of yeasts preserved in this type of collections. Forty *Candida* spp. clinical isolates were identified by morphological and biochemical analyses. Identifications were also performed by the new proteomic approach based on MALDI-TOF MS. Results demonstrated 15% discordance when compared with morphological and biochemical analyses. Discordant isolates were analysed by ITS sequencing, which confirmed the MALDI-TOF MS identifications and these strains were renamed in the culture collection catalogue. In conclusion, proteomic profiles by MALDI-TOF MS represents a rapid and reliable method for identifying clinical *Candida* species preserved in culture collections and may present clear benefits when compared with the performance of existing daily routine methods applied at health centres and hospitals.

Key words: BRCs, *Candida*, clinical yeasts, culture collections, MALDI-TOF MS.

Introduction

Culture collections play a key role in microbiology, since they are responsible for gathering and preserving well characterised strains. In addition, culture collections are responsible for *ex situ* preservation of microbial resources and their related information. In fact, each single microbial strain has not great value unless it is very well characterised with the current state of the art applied for each microbial group and the related information available in the appropriate format (Boundy-Mills, 2012).

The scientific community and the end users demand from the culture collections authentic biological materials with reproducible properties that allow them to use the strains to fit their needs: as type strains for taxonomic proposes, reference strains as standards, or even unique strains for research and exploitation of their peculiar properties (Smith, 2012; Simões *et al.*, 2013). The current challenges created by the OECD best practices guidelines for Biological Resource Centres (BRCs) generated an unprecedented wave in culture collections to adopt new practices and methods in order to guarantee high quality operational stan-

Send correspondence to C. Santos. Micoteca da Universidade do Minho, Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: cledir.santos@deb.uminho.pt.

dards and provide authentic materials (OECD, 2007; Schoch *et al.*, 2012).

The historical Brazilian fungal service culture collection University Recife Mycology (URM, www.ufpe.br/micoteca) was established by Prof. Chaves Batista in 1954 and is hosted by the Department of Mycology of the Federal University of Pernambuco (Recife, Brazil). Since the number and clinical impact of severe infections due to yeast species has increased the identification of yeast species with clinical relevance is one among several significant services offered by the URM culture collection. In addition, the sub-set collection of *Candida* species is of relevance due to possibility of supplying reference strains for clinical studies.

Candida species emerged as the major opportunistic pathogens in immunocompromised patients, and currently constitute one of the most common causes of nosocomial infections in intensive care units (Marklein *et al.*, 2009). Currently, this genus comprises more than 160 species, is expanding rapidly and is under a continuous taxonomic revision. Approximately 20 species cause disease in humans, although the number of new reports is increasing (Qian *et al.*, 2008; Putignani *et al.*, 2011). Candidemia is the fourth most common cause of hospital bloodstream infection worldwide being associated with extended hospital stays and high mortality rates among critically ill patients (Medrano *et al.*, 2006). The case/fatality rate is approximately 34% with an estimated 72.8 million worldwide opportunistic *Candida* infections per year (Marklein *et al.*, 2009).

The time required for pathogen identification is an important determinant in the infection-related mortality rates in hospitalised patients. Death rates and costs associated with infectious diseases could significantly be reduced by employing rapid identification techniques (Essendoubi *et al.*, 2005). Inevitably, non-*Candida* yeasts that can act as human pathogens have been misidentified as *Candida* species. Their identification may pose a challenge when the type strains have not yet been included into the diagnostic databases of any semi- or full-automated identification system (Lockhart *et al.*, 2008; Marklein *et al.*, 2009; Putignani *et al.*, 2011). Several reports have addressed the difficulty to identify yeast strains to the species level by conventional methods, since they are highly dependent on variables such as growth medium and temperature (Latouche *et al.*, 1997; Mozina and Raspor, 1997; Correia *et al.*, 2006; Leaw *et al.*, 2006; Qian *et al.*, 2008) and, in some limit cases, the interpretation of the results remain very subjective. This makes both molecular biology and spectral approaches crucial when the mycologist faces an isolate with unusual traits.

Matrix Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) is a physic-chemical technique that has been used for rapid and reliable microbial identification. The spectrum generated is analysed as an individual proteomic profile with the molecular mass ranging from 2000 to 20000 Da, where important ribosomal proteins appear, which is an advantage because they can be readily employed as biomarkers (Santos *et al.*, 2010a, 2011; Passarini *et al.*, 2013). Proteomic profiles by MALDI-TOF MS for rapid identification of intact bacteria were firstly used in 1996 (Holland *et al.*, 1996). More recently MALDI-TOF MS has been used to detect and identify yeasts. It is able to discriminate closely related species, such as *C. glabrata* from *C. bracarensis, C. albicans* from *C. dubliniensis* and, *C. metapsilosis* and *C. orthopsilosis* from *C. parapsilosis,* previously separated only by molecular methods (Sullivan *et al.*, 1995; Tavanti *et al.*, 2005; Correia *et al.*, 2006; van Veen *et al.*, 2010; Jensen and Arendrup, 2011; Marinach-Patrice *et al.*, 2010; Putignani *et al.*, 2011; Santos *et al.*, 2011) or Fourier Transform Infrared (FT-IR) techniques (Santos *et al.*, 2010b).

The purpose of the present study was to apply MALDI-TOF MS combined with classical phenotypic and molecular approaches to identify *Candida* clinical isolates preserved from 1 up to 52 years in a Brazilian culture collection and assess its value for the identification of yeasts preserved in this type of service collections.

Materials and Methods

Strains and growth conditions

All 40 *Candida* isolates analysed in this study were obtained from the URM culture collection (www.ufpe.br/micoteca). This set of yeasts comprises all clinical isolates of this genus available at URM. *Escherichia coli* strain DH5α was used as standard for the MALDI-TOF MS external calibration and was obtained from the Micoteca da Universidade do Minho (MUM, www.micoteca.deb.uminho.pt). *Trichophyton rubrum* MUM 09.09 was incorporated into the analysis as an outgroup. All cultures were preserved at -80 °C and cryovials were thawed, opened and the strains sub-cultured according to the instructions issued by URM and MUM culture collections. Homogenous inocula of yeast cells were grown and maintained on Yeast Extract Peptone Dextrose agar medium (YEPD). *Escherichia coli* cells were grown and maintained on Luria-Bertani agar medium (LB). Incubations were standardised at 20 h and strains were grown aerobically at 37 °C. In order to avoid changes in the protein expression pattern the culture conditions and growth time were standardised as described above. All cultures were checked for purity prior to use and were sub-cultured at least twice prior to MALDI-TOF MS analysis.

Classical phenotypic identification

The isolates were identified by macro- and micromorphology. The colour, shape, topology of each colony and the cell morphology were considered using Sabouraud dextrose agar medium with 2.5% yeast extract (Difco, USA). The induction of sexual reproduction structure using Gorodkowa agar (Difco, USA) was performed. Chlamy-

dospores were induced on bile agar (Difco, USA) and scored as present or absent after 3 days at 25 °C. The isolates were tested biochemically by carbohydrate assimilation and fermentation assays. The production of urease and acetic acid were also assessed using urea and calcium carbonate media (Difco, USA), respectively. The maximum temperature of growth for each isolate was determined according Barnett *et al.* (2000) and De Hoog *et al.* (2000). The isolates which presented ambiguous identification were further analysed by VITEK-2 XL (bioMerieux, Brazil) according to previous reports (Macedo *et al.*, 2009).

MALDI-TOF MS plate preparation

Using a yellow tip a tiny sample (about 10^6 yeasts per sample) was transferred directly from a single colony and spotted onto the 48 well flex target plate (FlexiMass, Shimadzu Biotech, UK). Aliquots of 0.5 μL of 25% formic acid were added and mixed gently with the yeasts. When the liquid medium was almost evaporated 0.5 μL matrix solution (75 mg/mL 2,5-dihydroxybenzoic acid [DHB] in ethanol/water/acetonitrile [1:1:1; v/v/v] with 0.03% trifluoroacetic acid [TFA]) was added and mixed gently. The matrix solution addition was performed without the additional treatment with formic acid for *E. coli*. All solutions were freshly prepared and stored at 5 °C during the work. In order to assess the quality and reproducibility of the spectral data, each yeast isolate was analysed in duplicate. All samples were air dried at room temperature and finally analysed by MALDI-TOF MS.

MALDI-TOF MS data acquisition

The analyses were performed on an Axima LNR system (Kratos Analytical, Shimadzu, UK) equipped with a nitrogen laser (337 nm), where the laser intensity was set just above the threshold for ion production. Twelve defined ribosomal proteins of intact *E. coli* DH5α cells (4365.4, 5096.8, 5381.4, 6241.4, 6255.4, 6316.2, 6411.6, 6856.1, 7158.8, 7274.5, 7872.1, 9742 and 12227.3 Da) were used as external calibrants of the MALDI-TOF MS equipment (Passarini *et al.*, 2013). The mass range from 2000 to 20000 Da was recorded using the linear mode with a delay of 104 ns and an acceleration voltage of +20 kV. Final spectra were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, leading to 1000 laser shots per summed spectrum. The resulting peak lists were exported to the database of the SARAMIS package software (Spectral Archiving and Microbial Identification System, AnagnosTec, Germany, www.anagnostec.eu) where the final identifications were achieved. Identifications by the SARAMIS package are based on the presence or absence of each peak in the spectra.

MALDI-TOF MS clustering analysis

Cluster analyses of the MALDI-TOF MS spectral data were achieved using the SARAMIS database software. In this database peak lists of individual samples were compared to matching SuperSpectra and/or reference spectra generated by the system. For SuperSpectra and reference spectra, SARAMIS uses a point system based on peak list with mass signals weighed according to their specificity. The weighting is based on empirical data from multiple samples of type, reference and well characterised yeast strains. SuperSpectra are consensus spectra containing a pattern of mass signals, which are taxa-specific and allow the identification of strains and cluster analyses of spectra of multiple samples. In present study superspectra for *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *P. kudriavzevii* were used. Reference spectra of *C. albicans* CBD 924, *C. parapsilosis* CBD 1325 and *C. tropicalis* CBD 957 were used as individual empiric spectra of well characterised yeast species. In both cases, the similarity between individual spectra is expressed as a relative or an absolute number of matching mass signals after subjecting the data to a single link agglomerative clustering algorithm. The database included SuperSpectra and/or reference spectra of all the yeasts described herein.

ITS sequencing and sequence analysis

Isolates that presented distinctive identification by the classical and new spectral phenotypic approaches were analysed by ITS sequencing. ITS region was chosen taking into consideration that this region is formally the primary fungal barcode marker recommended by the Consortium for the Barcode of Life (Schoch *et al.*, 2012). Sequence analysis was carried out on the entire ribosomal ITS region (*i.e.*, ITS1/58S rDNA/ITS2), according to White *et al.* (1990). Sequencing was performed with an ABI 310 Genetic Analyser (Applied Biosystems) using standard protocols. Strains were identified at the level of 99.0% sequence similarity or higher after a NCBI blast.

Results

Classical phenotypic identification

Forty yeast isolates from clinical cases were identified using classic phenotypic identification (Table 1). Identities were as follow: 20 *C. albicans*, 5 *C. krusei*, 11 *C. parapsilosis* and 4 *C. tropicalis*. Sexual reproduction was not observed on Gorodkowa agar although, in contrast, chlamydospores were observed from all *C. albicans* isolates. In order to clarify identification difficulties on isolates that presented ambiguity, URM 4990, 4388, 4124, 4818, 4261 and 1150, VITEK-2 XL was used. This method corroborated all previous phenotypic identifications for these 6 isolates.

MALDI-TOF MS identification

Six isolates (15%) identified by MALDI-TOF MS did not correspond to the phenotypic identification (Table 1). The *C. krusei* isolates were classified as *Pichia kudriavzevii*

Table 1 - Phenotypic and MALDI-TOF MS identifications of *Candida* clinical isolates. The highlighted isolates represent discordant identifications.

Strain number (URM)	Classical phenotypic ID[a]	Preservation time (y)	Source	Fermentation[b]						Assimilation[b]										Production		SDA agar result[c]	Morphology[d]	T_{max}	MALDI-TOF MS ID
				D	G	L	M	S	T	C	D	G	L	M	S	St	R	T	X	Urease	Acetic acid				
4990	*C. albicans*	01	Vaginal exudate	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4987	*C. albicans*	01	Vaginal exudate	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4986	*C. albicans*	01	Vaginal exudate	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4820	*C. albicans*	02	Ungual scraping	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4819	*C. albicans*	02	Ungual scraping	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4817	*C. albicans*	02	Ungual scraping	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4609	*C. albicans*	03	Blood	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4606	*C. albicans*	03	Blood	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4388	*C. albicans*	05	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4387	*C. albicans*	05	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4386	*C. albicans*	05	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4385	*C. albicans*	05	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4384	*C. albicans*	05	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4260	*C. albicans*	05	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4127	*C. albicans*	07	Skin of the inguinal area	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4126	*C. albicans*	07	Urine	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4125	*C. albicans*	07	Sputum	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4124	*C. albicans*	07	Oropharyngeal secretion	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
3719	*C. albicans*	10	Tooth swab	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. tropicalis*
3716	*C. albicans*	10	Tooth swab	+	V	-	+	-	V	-	+	+	-	+	+	+	-	+	+	-	-	W/C	PsH, SeH and Csp	45 °C	*C. albicans*
4802	*C. krusei*	02	Unknown	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	W/C	PsH and Bsp	42 °C	*P. kudriavzevii*
4263	*C. krusei*	05	Oropharyngeal secretion	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	W/C	PsH and Bsp	42 °C	*P. kudriavzevii*
1059	*C. krusei*	48	Unknown	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	W/C	PsH and Bsp	42 °C	*P. kudriavzevii*
934	*C. krusei*	49	Appendix biopsy	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	W/C	PsH and Bsp	42 °C	*P. kudriavzevii*
109	*C. krusei*	52	Unknown	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	W/C	PsH and Bsp	42 °C	*P. kudriavzevii*
4984	*C. parapsilosis*	01	Vaginal exudate	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. parapsilosis*
4970	*C. parapsilosis*	01	Vaginal exudate	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. parapsilosis*
4889	*C. parapsilosis*	02	Blood	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. parapsilosis*
4818	*C. parapsilosis*	02	Ungual scraping	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. parapsilosis*
4804	*C. parapsilosis*	02	Unknown	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. parapsilosis*
4608	*C. parapsilosis*	03	Blood	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	Unidentified
4607	*C. parapsilosis*	03	Blood	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. parapsilosis*
4261	*C. parapsilosis*	05	Oropharyngeal secretion	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. tropicalis*
3627	*C. parapsilosis*	12	Sputum	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. albicans*
3624	*C. parapsilosis*	12	Sputum	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. tropicalis*
3621	*C. parapsilosis*	12	Sputum	+	-	-	-	F	V	-	+	+	-	+	+	-	V	+	+	-	-	W/C	PsH and Bsp	40 °C	*C. albicans*
4262	*C. tropicalis*	06	Oropharyngeal secretion	+	+	-	+	V	+	V	+	+	-	+	+	+	+	+	+	-	-	W/C	PsH, SeH and Bsp	40 °C	*C. tropicalis*
1150	*C. tropicalis*	46	Tongue	+	+	-	+	V	+	V	+	+	-	+	+	+	+	+	+	-	-	W/C	PsH, SeH and Bsp	40 °C	*C. tropicalis*
933	*C. tropicalis*	49	Vaginal exudate	+	+	-	+	V	+	V	+	+	-	+	+	+	+	+	+	-	-	W/C	PsH, SeH and Bsp	40 °C	*C. tropicalis*
916	*C. tropicalis*	49	Feces	+	+	-	+	V	+	V	+	+	-	+	+	+	+	+	+	-	-	W/C	PsH, SeH and Bsp	40 °C	*C. tropicalis*

[a]ID, Identification. [b]D, D-gucose; G, D-galactose; L, lactose; M, maltose; S, sucrose; T, α,α-trehalose; C, cellobiose; St, starch; R, ribitol; X, D-xylose; +, presence; -, absence; V, intraspecific variation; F, fermentation after 7 days. [c]SDA agar, Sabouraud dextrose supplemented with 2.5% yeast extract agar; W/C, White to cream colored colonies. [d]PsH, Pseudohyphae; SeH, Septate hyphae; Csp, Chlamydospores; Bsp, Blastospore.

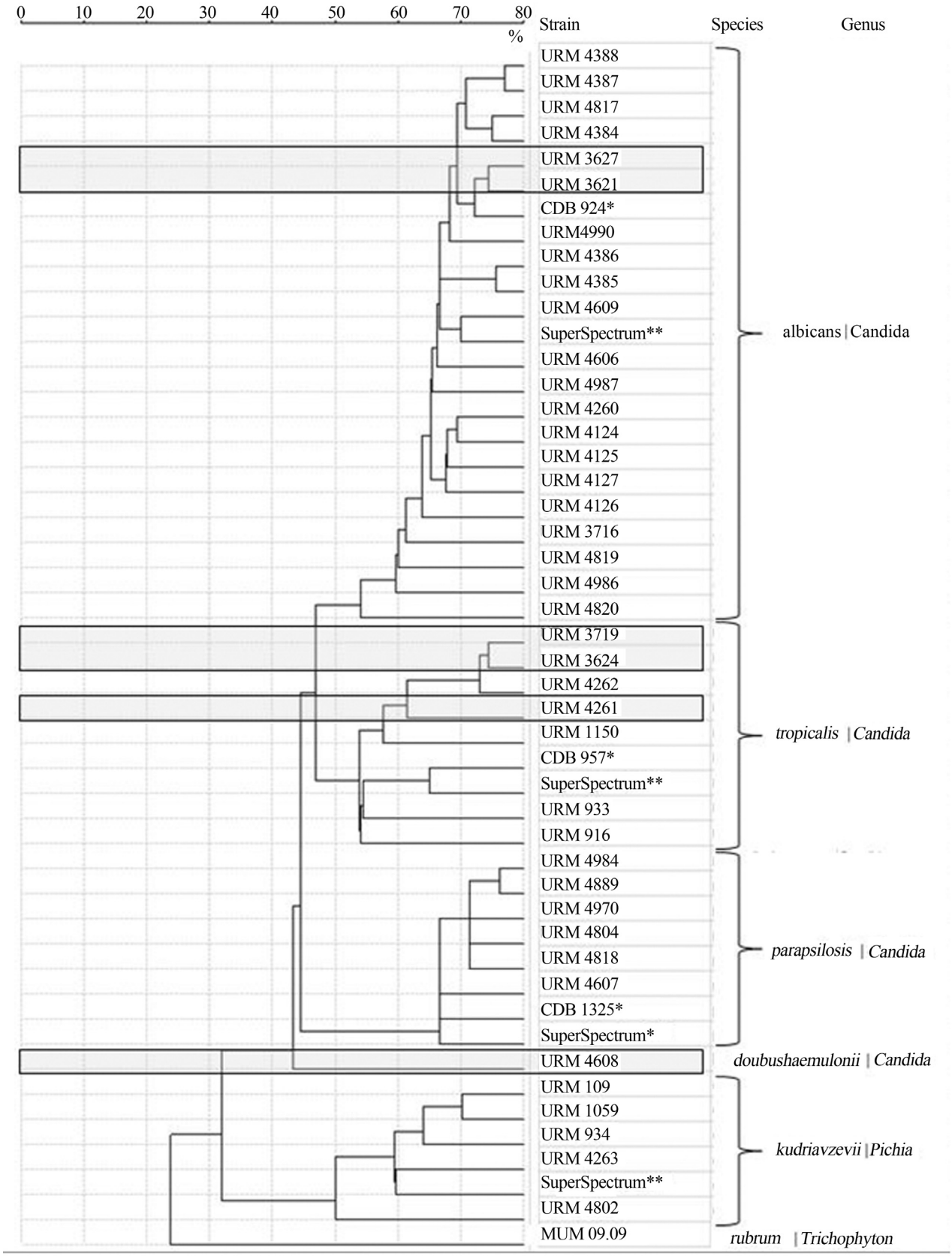

Figure 1 - MALDI-TOF spectra based dendrogram of yeast isolates evaluated in this work. Discordances obtained by MALDI-TOF MS and classical phenotypic analyses are highlighted by grey boxes. CBD reference strains and Superspectra are marked by * and **, respectively. *Trichophyton rubrum* MUM 09.09 was used as outgroup.

(former *Issatchenkia orientalis*) using the SARAMIS software package, which is the basionym teleomorphic name.

Isolates URM 4261, 3627, 3624 and 3621 were identified as *C. parapsilosis* by phenotype. However, URM 4261 and 3624 were *C. tropicalis* and URM 3627 and 3621 were *C. albicans* by MALDI-TOF MS. Isolate URM 4261 was one of the six samples confirmed by VITEK-2 XL as *C. parapsilosis*. This finding corresponds to 16.7% of discordance in the overall isolates analysed by VITEK-2 XL in relation to the spectral phenotypic identification.

URM 4608 was identified as *C. parapsilosis* phenotypically and was recorded as "unidentified" by MALDI-TOF MS, grouping separately on the dendrogram (Figure 1). This separation was confirmed by the molecular biology results presented below.

Finally, URM 3719 was identified as *C. albicans* phenotypically and as *C. tropicalis* by MALDI-TOF MS.

ITS identification

All six isolates that presented discordant results from phenotypic and MALDI-TOF MS identifications were analysed by ITS sequencing. URM 3621 and 3627 isolates were identified as *C. parapsilosis* by phenotype and *C. albican*s by MALDI-TOF MS (Table 1). ITS sequencing confirmed that these clinical yeast strains were *C. albican*s (URM 3627, accession nº KF031307). URM 4261 (accession nº KF031306) and 3624 (accession nº KF031304) were confirmed as *C. tropicalis* by ITS sequencing.

URM 3719 was identified as *C. albicans* by phenotype but grouped with *C. tropicalis* by MALDI-TOF MS (Table 1 and Figure 1), and was confirmed as *C. tropicalis* by ITS analysis (accession nº KF031305).

URM 4608 that was identified as *C. parapsilosis* by phenotype could not be identified by MALDI-TOF MS, grouping separately from the *C. parapsilosis* cluster in the MALDI-TOF MS dendrogram (Figure 1). However, it was identified as *C. doubushaemulonii* by ITS (accession nº KF031310) and when this new taxon information was added to the SARAMIS database, the isolate was recorded as *C. doubushaemulonii* (Figure 1).

Although the good concordance between phenotypical and spectral identification of *Pichia kudriavzevii*, strains URM 1059 and URM 4802 were chosen for additional ITS analysis. The sequencing results confirmed their identifications (accession nº KF031308 and KF031309, respectively).

Discussion

A comparison between the discriminative capability of phenotypic and MALDI-TOF MS characters of 40 clinical yeasts was reported herein. Moreover, discordant isolates were analysed by ITS sequencing which confirmed the MALDI-TOF MS identifications. Phenotype grouped the isolates into four distinct species: *C. albicans*, *C. krusei* (*P. kudriavzevii*), *C. parapsilosis* and *C. tropicalis*. In addition, 6 out of 40 (15%) strains analysed by this method disagreed with the identification performed by MALDI-TOF MS. In line with described by Santos *et al.* (2011) the MALDI-TOF MS results were confirmed strongly by the ITS sequencing analyses. Through these combined approaches five strains were renamed and one additional species was added to the collection: *C. doubushaemulonii*. The current data, when combined with data previously described (Marinach-Patrice *et al.*, 2010; Jensen and Arendrup, 2011; Santos *et al.*, 2011), indicate that MALDI-TOF MS analysis is a powerful technique to identify clinical *Candida* isolates.

Because ribosomal proteins can be easily used as biomarkers in the proteomic-based technique by MALDI-TOF MS the reliability of this methodology is high and can discriminate between closely related *Candida* species. However, due to the biological variability of these yeasts, even for a single species, the availability of an extensive database is required. For example, MALDI-TOF MS generated a distinctive spectrum for *C. doubushaemulonii* URM 4608, separating it from the other isolates in the dendrogram, which is otherwise difficult to do. In this case, the database was not sufficiently extensive to provide straightforward the correct identification due to a complete lack of data related with *C. doubushaemulonii*. As a matter of consequence, the culture collections with high quality biological materials are key elements to feed the MALDI-TOF MS databases with reference spectra. In contrast, with this new approach collections gain the possibility to requalify their holdings renaming misidentified strains.

MALDI-TOF MS is straightforward, rapid and employs partially automated procedures. Results are obtained in approximately 30 sec per sample. Since the time for pathogen identification is an important determinant of infection-related mortality rates of hospitalised patients, this technique is an important tool in the fight to reduce mortalities when the time taken for classical methods is considered.

In summary, the classical phenotypic approaches, including VITEK techniques are very important in the daily routine analyses in health centres and hospitals. However, it is associated with a high degree of misidentifications (Lockhart *et al.*, 2008; Marklein *et al.*, 2009; Putignani *et al.*, 2011) and it would be beneficial for these to be supported by reference strains provided by culture collections and, if available, by MALDI-TOF MS with an appropriate database. MALDI-TOF MS is important to improve the efficiency of the classical phenotypical methods for clinical *Candida* polyphasic identifications even when isolates where preserved for decades in culture collections.

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