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Original Article

MALDI-TOF typing highlights geographical and fluconazole resistance clusters in *Candida glabrata*

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Abstract

Utilizing matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra for *Candida glabrata* typing would be a cost-effective and easy-to-use alternative to classical DNA-based typing methods. This study aimed to use MALDI-TOF for the typing of *C. glabrata* clinical isolates from various geographical origins and test its capacity to differentiate between fluconazole-sensitive and -resistant strains.

Both microsatellite length polymorphism (MLP) and MALDI-TOF mass spectra of 58 *C. glabrata* isolates originating from Marseilles (France) and Tunis (Tunisia) as well as collection strains from diverse geographic origins were analyzed. The same analysis was conducted on a subset of *C. glabrata* isolates that were either susceptible (MIC $\leq$ 8 mg/l) or resistant (MIC $\geq$ 64 mg/l) to fluconazole.

According to the seminal results, both MALDI-TOF and MLP classifications could highlight *C. glabrata* population structures associated with either geographical dispersal barriers ($p < 10^{-5}$) or the selection of antifungal drug resistance traits ($<10^{-5}$).

In conclusion, MALDI-TOF geographical clustering was congruent with MPL genotyping and highlighted a significant population genetic structure according to fluconazole susceptibility in *C. glabrata*. Furthermore, although MALDI-TOF and MLP resulted in distinct classifications, MALDI-TOF also classified the isolates with respect to their fluconazole susceptibility profile. Further prospective studies are required to evaluate the capacity of MALDI-TOF typing to investigate *C. glabrata* infection outbreaks and predict the antifungal susceptibility profile of clinical laboratory isolates.

Key words: *Candida glabrata*, MALDI-TOF, microsatellites, typing, fluconazole susceptibility, clinical microbiology.

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Introduction

Second only to *Candida albicans*, the emerging human pathogenic yeast *C. glabrata* is characterized by a decreased susceptibility to azole antifungal drugs, especially fluconazole, compared with other *Candida* spp. [1–3]. This haploid yeast has the capacity to develop fluconazole resistance via several mechanisms, including alteration/overexpression of the CgErg11 target enzyme [4] and increased drug efflux triggered by ATP-binding cassette overexpression [5]. Therefore, molecular typing methods have been developed to trace *C. glabrata* strains during outbreak investigations and further understand the spread of fluconazole resistance within this species. Both multilocus locus sequence typing (MLST) [6,7] and microsatellite length polymorphism (MLP) analysis [8,9] are widely applied to trace strains and/or describe the genetic population structure for a number of *Candida* species. High-resolution MLP typing has enabled the tracing of *C. glabrata* strains [8,10]. In contrast, MLST has allowed for grouping of *C. glabrata* isolates in broad subpopulations; however, perhaps due to the highly clonal mode of reproduction of this species, this method displayed insufficient resolution for strain tracing [11,12].

Recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has emerged as a rapid, accurate, simple, and inexpensive method for microorganism identification in the clinical laboratory. Several publications have demonstrated the capacity of MALDI-TOF to differentiate bacterial subspecies [13,14] and detect antibiotic resistance-associated phenotypes [15]. With respect to yeasts, MALDI-TOF has displayed encouraging results for the typing of yeast within the *C. parapsilosis* complex [16,17]. Applying MALDI-TOF mass spectra for *C. glabrata* typing would be cost-effective and easy-to-use compared with DNA-based typing methods. The aim of this study was to apply MALDI-TOF mass spectra for the strain typing of *C. glabrata* clinical isolates derived from different geographical origins and test their capacity to differentiate between fluconazole sensitive and resistant strains.

Methods

Candida glabrata strains

Nine *C. glabrata* BCCM/IHEM reference strains, originating from various geographical areas and collection sites, and 49 clinical isolates collected from either Marseilles (France) or Tunis (Tunisia) university hospital patients, in whom *C. glabrata* had been sequentially isolated, were used in the geographical differentiation analysis (Table 1). Thirty-two unrelated *C. glabrata* clinical strains, which were isolated from different patients, in distinct hospital wards, including 19 fluconazole-resistant and 13 fluconazole-sensitive iso-

Table 1. Detail of the clinical isolates of *Candida glabrata* used in the geographical differentiation analysis.

Identification	Origin	Sampling date	Sampling site
Fr_P1_1	Marseille	11/01/2012	Lower respiratory tract
Fr_P1_2	Marseille	16/01/2012	Lower respiratory tract
Fr_P1_3	Marseille	26/01/2012	Lower respiratory tract
Fr_P1_4	Marseille	07/02/2012	Lower respiratory tract
Fr_P1_5	Marseille	06/06/2012	Lower respiratory tract
Fr_P1_6	Marseille	08/06/2012	Lower respiratory tract
Fr_P2_1	Marseille	07/03/2012	Lower respiratory tract
Fr_P2_2	Marseille	09/03/2012	Lower respiratory tract
Fr_P2_3	Marseille	09/03/2012	Digestive tract
Fr_P2_4	Marseille	09/03/2012	Upper respiratory tract
Fr_P2_5	Marseille	09/03/2012	Skin
Fr_P2_6	Marseille	14/03/2012	Blood
Fr_P2_7	Marseille	15/03/2012	Urine
Fr_P3_1	Marseille	21/05/2012	Urine
Fr_P3_2	Marseille	22/05/2012	Urine
Fr_P3_3	Marseille	23/05/2012	Vagina
Fr_P3_4	Marseille	23/05/2012	Vagina
Fr_P3_5	Marseille	25/05/2012	Vagina
Fr_P3_6	Marseille	08/06/2012	Vagina
Fr_P3_7	Marseille	25/05/2012	Urine
Fr_P4_1	Marseille	01/01/2012	Blood
Fr_P4_2	Marseille	01/01/2012	Blood
Fr_P4_3	Marseille	01/01/2012	Blood
Fr_P4_4	Marseille	02/01/2012	Blood
Fr_P5_1	Marseille	29/06/2012	Blood
Fr_P5_2	Marseille	29/06/2012	Blood
Fr_P5_3	Marseille	29/06/2012	Blood
Tu_P1_1	Tunis	09/01/2011	Urine
Tu_P1_2	Tunis	21/02/2011	Urine
Tu_P1_3	Tunis	26/02/2011	Urine
Tu_P1_4	Tunis	05/03/2011	Urine
Tu_P1_5	Tunis	13/03/2011	Digestive tract
Tu_P1_6	Tunis	09/01/2011	Upper respiratory tract
Tu_P2_1	Tunis	29/06/2012	Urine
Tu_P2_2	Tunis	29/06/2012	Digestive tract
Tu_P2_3	Tunis	14/09/2012	Digestive tract
Tu_P3_1	Tunis	11/06/2012	Digestive tract
Tu_P3_2	Tunis	21/062012	Digestive tract
Tu_P3_3	Tunis	26/06/2012	Upper respiratory tract
Tu_P3_4	Tunis	13/07/2012	Digestive tract
Tu_P4_1	Tunis	27/04/2012	Digestive tract
Tu_P4_2	Tunis	03/09/2012	Digestive tract
Tu_P4_3	Tunis	06/09/2012	Digestive tract
Tu_P4_4	Tunis	21/09/2012	Digestive tract
Tu_P5_1	Tunis	19/06/2011	Urine
Tu_P5_2	Tunis	23/06/2011	Biopsy
Tu_P5_3	Tunis	28/06/2011	Digestive tract
Tu_P6_1	Tunis	13/07/2012	Urine
Tu_P6_2	Tunis	13/07/2012	Digestive tract
Ref_1	French	IHEM22852	Urine
Ref_2	Peru	IHEM16180	Urine
Ref_3	Peru	IHEM16178	Urine
Ref_4	Bolivia	IHEM15478	Vagina
Ref_5	Belgium	IHEM6141	Lower respiratory tract

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Table 1. continued.

Identification	Origin	Sampling date	Sampling site
Ref_6	USA	IHEM9556	Blood
Ref_7	Belgium	IHEM4141	Blood
Ref_8	Belgium	IHEM22819	Blood
Ref_9	Spain	IHEM4210	Vagina

Table 2. The collection of *Candida glabrata* strains used in the Fluconazole susceptibility differentiation analysis. The isolates were classified Fluconazole resistant (R): MIC ≥64 mg/L or Fluconazole sensitive (S): MIC ≤8 mg/l.

Identification	R/S	Fluconazole MIC (mg/l)	Geographical origin
Tu_1_R	R	64	Tunis
Tu_2_R	R	64	Tunis
Tu_3_R	R	128	Tunis
Tu_4_R	R	128	Tunis
Tu_5_R	R	64	Tunis
Tu_6_R	R	64	Tunis
Tu_7_R	R	64	Tunis
Tu_8_S	S	8	Tunis
Tu_9_S	S	8	Tunis
Tu_10_S	S	8	Tunis
Tu_11_S	S	4	Tunis
Fr_1_R	R	256	Marseille
Fr_2_R	R	256	Marseille
Fr_3_R	R	256	Marseille
Fr_4_R	R	256	Marseille
Fr_5_R	R	256	Marseille
Fr_6_R	R	256	Marseille
Fr_7_R	R	256	Marseille
Fr_8_R	R	256	Marseille
Fr_9_R	R	256	Marseille
Fr_10_R	R	256	Marseille
Fr-11_R	R	256	Marseille
Fr_12_R	R	256	Marseille
Fr_13_S	S	4	Marseille
Fr_14_S	S	1	Marseille
Fr_15_S	S	4	Marseille
Fr_16_S	S	4	Marseille
Fr_17_S	S	1,5	Marseille
Fr_18_S	S	4	Marseille
Fr_19_S	S	4	Marseille
Fr_20_S	S	4	Marseille
Fr_21_S	S	4	Marseille

lates, were selected for the fluconazole susceptibility analysis (Table 2). Each strain was cultured on Sabouraud chloramphenicol gentamicin (Oxoid, Dardilly, France) plates at 30°C for 48 h. The *C. glabrata* clinical strains were identified based on the MALDI-TOF MS results, as previously described [18]. All clinical isolates have been deposited in the BCCM/IHEM biomedical fungi and yeasts collection with accession numbers ranging from IHEM25889 to IHEM26007.

Susceptibility testing

Susceptibility to fluconazole was determined according to the minimum inhibitory concentration (MIC) measured via Sensititre™ YeastOne™ (Trek Diagnostic Systems, Ltd., UK) assay as recommended by the manufacturer. *C. glabrata* strains were then classified either fluconazole resistant (MIC ≥64 mg/l) or fluconazole susceptible (MIC ≤8 mg/L) [19].

MALDI-TOF MS typing assay

Proteins were extracted via the conventional extraction method recommended by Bruker Daltonik GmbH (Bremen, Germany), using formic acid and acetonitrile as previously described [18]. The supernatant of an extracted colony was deposited in quadruplicate on the MALDI plate, and MALDI-TOF MS analysis was performed using the Microflex LT™ system (Bruker Daltonik) with the default settings recommended by the manufacturer.

Microsatellite Length Polymorphism typing

DNA was extracted from a colony suspension in 800 μl of lysis buffer (bioMérieux, Craponne, France) using a NucliSENS™ easyMAG™ V2 (bioMérieux) for each isolate. Nine microsatellite loci were analyzed as described by Brisse *et al.* [9].

Population genetics analysis

The MLP typing results were analyzed using Arlequin ver. 3.5 software [20]. In particular, the genetic differentiation of *C. glabrata* strains according to either geographical origin (Marseilles, Tunis or the IHEM strain collection) or fluconazole *in vitro* susceptibility was tested. For this analysis, we assessed the fixation index (F_{ST}), which measures population differentiation due to genetic structure, the corrected average pairwise differences and the exact test of population differentiation based on genotype frequencies, which tests the nonrandom distribution of genotypes within population samples under the panmixia hypothesis [20].

MALDI-TOF phylogenetic tree

The BioNumerics ver. 7.0 software (Applied Maths NV, Sint-Martens-Latem, Belgium) was used to generate an UPGMA dendrogram based on the Pearson correlation coefficient using both microsatellite and MALDI-TOF summary spectra data.

MLP phylogenetic tree

The sequencer trace files of the different microsatellites were imported into BioNumerics version 7.0 (Applied Maths

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NV). After normalization and band detection, a band matching analysis was performed with each band class corresponding to a possible repeat number. The tolerance for matching was set to ±1 bp to the band class position.

Statistical analysis

The strength of agreement between MLP and MALDI-TOF classifications was estimated via Cohen's kappa coefficient. The null hypothesis of random distribution of fluconazole resistance phenotype across MLP or MALDI-TOF clusters was tested using the likelihood ratio test. This analysis was performed with SAS version 9.2 (SAS Institute, Cary, NC, USA) using two-sided tests with alpha <0.05.

Results

Candida glabrata geographical differentiation

The first approach involved analyzing the 58 isolates according to their geographical origin using both MLP and MALDI-TOF assays.

MLP geographical differentiation

The population genetic analysis based on the MLP assay showed a highly significant genetic differentiation ($F_{ST} = 0.35865$, $p < 10^{-5}$) between strains from France and those from Tunisia and the collection strains (Table 3, part A). The differentiation between the isolates from Tunisia and the collection strains ($F_{ST} = 0.20916$, $p < 10^{-5}$) was of lesser magnitude (Table 3, part A). This differentiation is illustrated in the similarity dendrogram (Fig. 1), in which strains of French and Tunisian origin were grouped into separate clusters. The IHEM reference strains were dispersed throughout the dendrogram.

MALDI-TOF geographical differentiation

The MALDI-TOF summary spectra similarity dendrogram (Fig. 1) also separated the strains according to their French or Tunisian origin. The IHEM reference strains were grouped into two distinct clusters. The MALDI-TOF clusters were congruent with the MLP clusters. With the exception a few discrepancies, most of the isolates that were grouped within one MLP cluster were also grouped within the same MALDI-TOF cluster (Fig. 1). The kappa coefficient between MLP and MALDI classifications was 0.3795 (95% CI [0.2329 to 0.5261]), which indicates slight agreement between MLP and MALDI classifications. The null hypothesis of no agreement between the classifications can be rejected ($p < .0001$), which is further supported by the range of the 95% confidence interval, which suggests that the true kappa is greater than zero.

Candida glabrata fluconazole susceptibility differentiation

The strain characteristics and MIC results of the 19 fluconazole-resistant and 13 fluconazole-sensitive isolates are detailed in Table 2.

MLP fluconazole susceptibility differentiation

The MLP-based population genetic analysis showed a significant genetic differentiation ($F_{ST} = 0.22091$, $p < .009$) between *C. glabrata* strains that were either sensitive or resistant to fluconazole (Table 3, part B). This differentiation is highlighted in the MLP similarity dendrogram (Fig. 2, panel A), in which fluconazole sensitive and resistant strains clearly clustered separately. There was a statistically significant ($p = .0044$) association between MLP clusters and fluconazole resistance.

Table 3. Population genetic analysis between *Candida glabrata* strains from different origin and between the resistant and sensible strains to fluconazole showing the genetic differentiation according to the country of origin (A) and fluconazole susceptibility or resistance phenotype (B).

		Population pairwise F_{ST}*		Corrected average pairwise difference		Exact Test of Sample differentiation (*P* value)	
		F_{ST}	*P* value	PXY**	*P* value	Between all pairs	Global test of differentiation
A	Tunisia vs. France	0.35865	10^{-5}	1.77455	10^{-5}	$<10^{-5}$	
	Tunisia vs. IHEM collection	0.06199	0.2959	0.13973	0.31965	0.01538	$<10^{-5}$
	France vs. IHEM collection	0.20916	10^{-5}	1.05340	10^{-5}	$<10^{-5}$	
B	Resistant vs. sensitive	0.22091	0.00901	1.05839	0.00909	$<10^{-5}$	$<10^{-5}$

*F_{ST}: Fixation index. It is a measure of population differentiation due to genetic structure.
**PXY: The average number of pairwise differences among individuals in the sampled populations.

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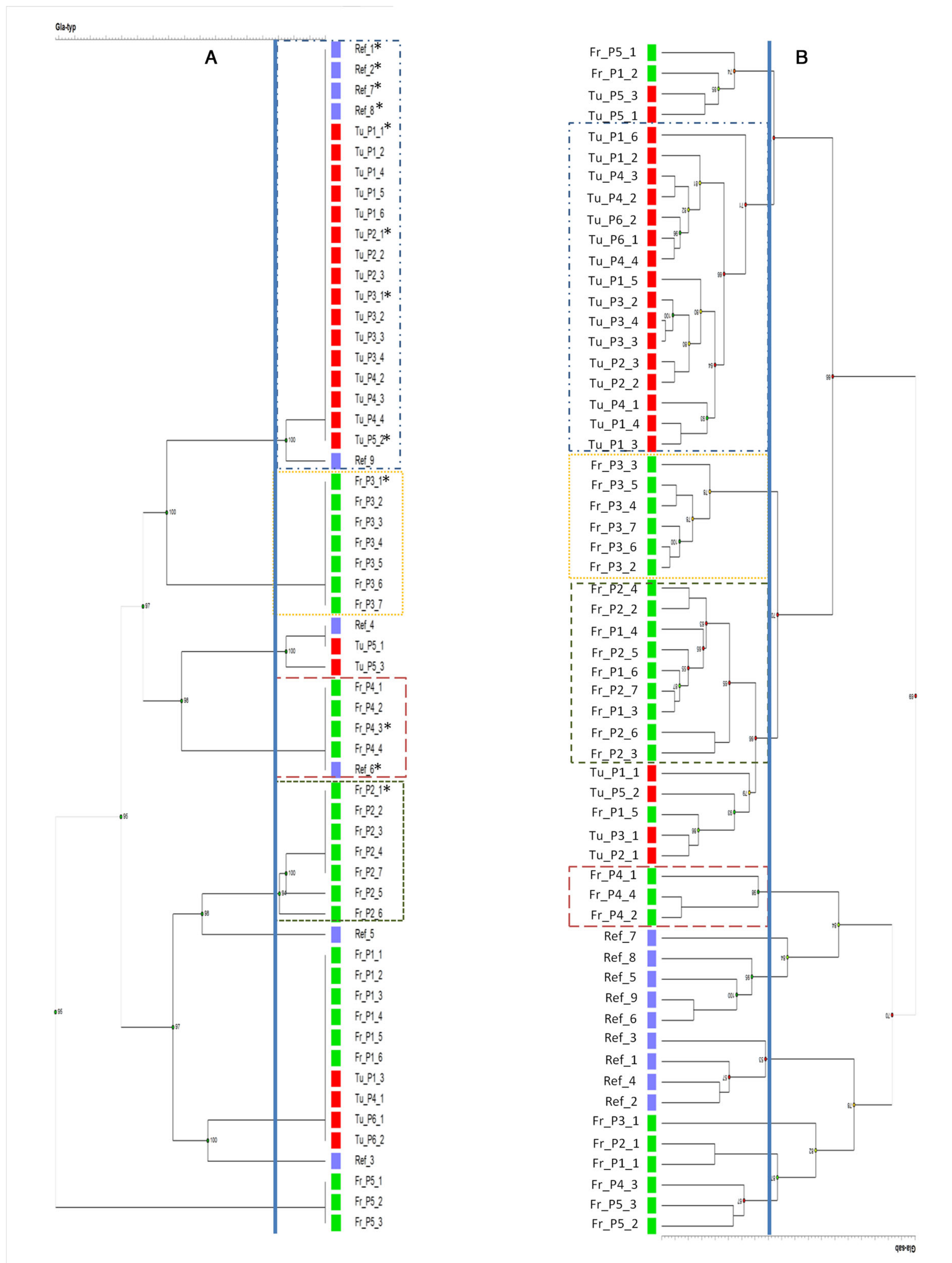

Figure 1. Comparison between microsatellite length polymorphism (MLP) (panel A) and MALDI-TOF (panel B) clusters. In panel A, the main MLP clusters are indicated with various dashed-line squares. The isolates corresponding to the MLP clusters are also indicated in the MALDI-TOF dendrogram with similar style dashed-line squares. The isolates within a MLP cluster that were not present in the corresponding MALDI-TOF cluster are marked with an asterisk. The color of the squares varies according to the origin of the strains. Ref, is for IHEM collection strain; Tu, is for Tunisia; Fr, is for France. The number preceded by the letter "P" identify the patients, either from Tunisia or France. This Figure is reproduced in color in the online version of *Medical Mycology*.

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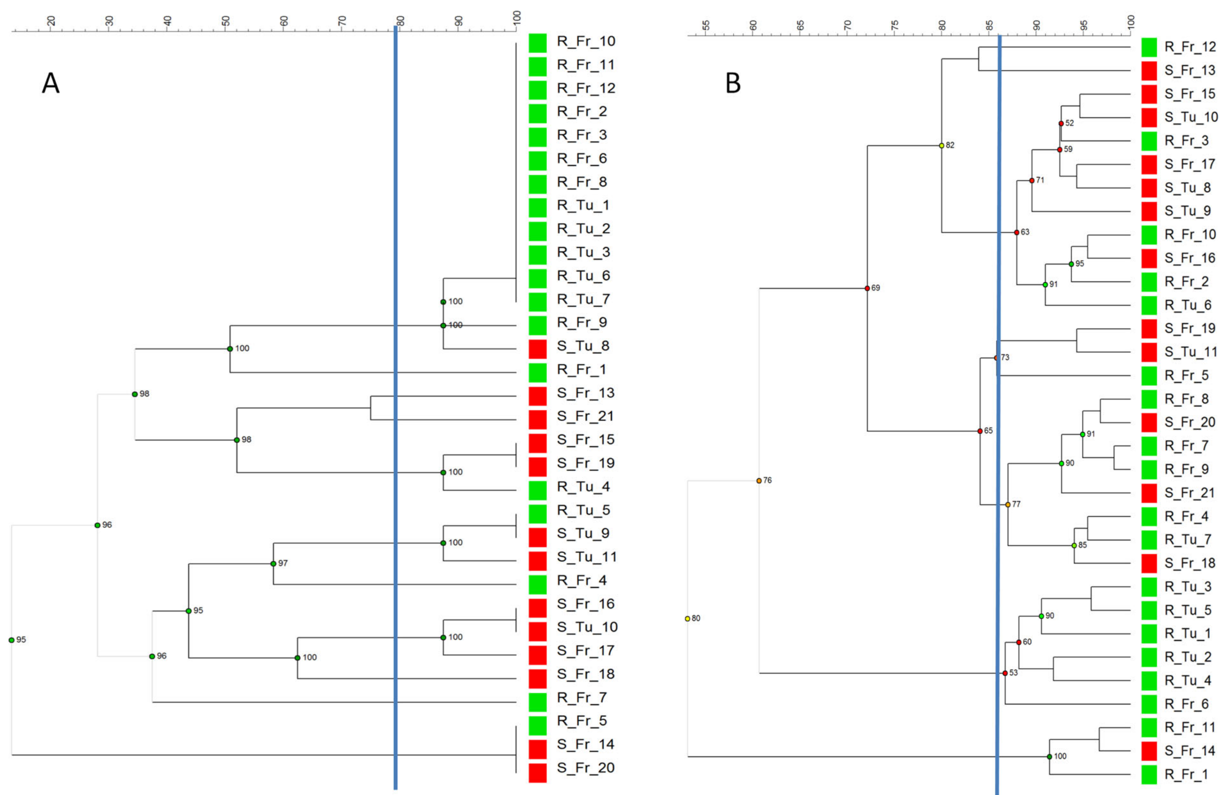

Figure 2. Distribution of fluconazole-resistant (R, light color square) or -sensitive (S, dark color square) *Candida glabrata* strains in microsatellite length polymorphism (panel A) and MALDI-TOF (panel B) dendrograms. Tu is for strains originating from Tunisia; Fr is for strains originating from France. This Figure is reproduced in color in the online version of *Medical Mycology*.

MALDI-TOF fluconazole susceptibility differentiation

The MALDI-TOF summary spectra similarity dendrogram (Fig. 2, panel B) also distinguished the fluconazole sensitive strains from the resistant strains. Of note, this MALDI-TOF classification was independent from both the MLP genotype and geographical origin of the isolates (Fig. 2). There was a statistically significant ($p = .0217$) association between MALDI-TOF clusters and fluconazole resistance.

Discussion

The seminal results described here illustrate that both MALDI-TOF MS and MLP typing were able to highlight *C. glabrata* population structures that were associated with either geographical dispersal barriers or antifungal drug resistance traits. Our observation of a geographical population structure of *C. glabrata* correlates with those of Brisse *et al.* [9] who highlighted, using the same MLP typing scheme, a significant population structure according to the continent of strain origin. Enache-Angoulvant *et al.* [12] have found a significant differentiation between strains involved in blood stream infections compared with those involved in gastrointestinal tract colonization. However, whether strains clustered according to their collection sample was not analyzed in the present study, as this was not an objective.

Several studies have evaluated the capacity of MALDI-TOF MS to rapidly discriminate between isolates within bacterial species. Nagy *et al.* [21] have used MALDI-TOF MS for the rapid identification and discrimination of *Propionibacterium acnes* phylotypes. Kuhns *et al.* [22] have demonstrated the capacity of MALDI-TOF MS to distinguish between typhoid and nontyphoid serotypes of *Salmonella enterica* subspecies *enterica*, while Stephan *et al.* [23] have applied this method for *Yersinia enterocolitica* subtyping. Regarding fungi, Pulcrano *et al.* have found that MALDI-TOF, similar to MLP typing, was useful to investigate the nosocomial transmission of a limited sample set of *C. parapsilosis* strains [16], which is in line with our results.

Interestingly, our MLP findings highlight, for the first time, the genetic differentiation of *C. glabrata* strains with respect to their *in vitro* fluconazole resistance phenotype. This population structure was independent of the geographical origin of the strains, thereby suggesting a selection pressure for resistance in *C. glabrata*, which might be due to the rise in fluconazole prescriptions over the last decades. Although this could not be tested in the present study, it is likely that this epidemiological shift might explain the

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contrast with previous studies that found no significant association between antifungal resistance and *C. glabrata* genotypes [24–26]. This discrepancy may also be associated with the presence of confounding factors [24], the small number of isolates analyzed [25] and/or the insufficient discriminative power of the MLP typing scheme applied [26]. However, the present seminal findings indicating that although MALDI-TOF MS and MLP typing result in dissimilar classifications, MALDI-TOF mass spectra were associated with *in vitro* antifungal susceptibility in *C. glabrata* isolates, which is in line with several studies conducted on the bacterium *Staphyloccocus aureus*. Wolters *et al.* [27] have used MALDI-TOF MS to rapidly identify methicillin-resistant *S. aureus* (MRSA) isolates from various clinical specimens and showed its potential to rapidly type MRSA in infection control. Recently, Wang *et al.* [28] have identified specific biomarkers that differentiated MRSA from MSSA strains. Furthermore, Wybo *et al.* [29] have highlighted approximately 10 distinct MALDI-TOF peaks that could separate meropenem-resistant or -sensitive *Bacteroides fragilis* isolates.

However, sharing the same genotype is probably not the main factor to explain MALDI-TOF mass spectra similarity among *C. glabrata* isolates. Indeed, we observed that many fluconazole-resistant isolates with identical genotypes were grouped into separate MALDI-TOF clusters. In a recent study, we have also observed that MALDI-TOF failed to identify *C. albicans* clades [Dhieb *et al.*, Journal of Mass Spectrometry, *in press*: DOI 10.1002/jms.3538]. This supports that MALDI-TOF is more likely to detect phenotypic differences between isolates of the same species than intrinsic genetic variations which are better detected by nucleic acid-based methods. In addition, MALDI TOF spectra may be more dependent on a variety of technical issues, which is not the case for MLP. Further studies are warranted to gain more insight into the underlying reasons behind the similarity between MALDI-TOF spectra of strains according to their fluconazole susceptibility phenotype.

In conclusion, MALDI-TOF geographical clustering was congruent with MPL genotyping in a set of *C. glabrata* isolates. Moreover, MALDI-TOF classification, as observed with MLP genotyping, highlighted a significant population genetic structure according to fluconazole *in vitro* susceptibility. However, the reason behind the similarities between MALDI-TOF spectra remain unknown, and further studies are required to evaluate the application of MALDI-TOF typing to investigate outbreaks of hospital-acquired *C. glabrata* infection and predict the antifungal susceptibility profile of isolates in the clinical laboratory.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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