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Original Article

Reidentification and antifungal susceptibility profile of *Candida guilliermondii* and *Candida famata* clinical isolates from a culture collection in Argentina

Constanza Giselle Taverna*, Susana Córdoba, Matías Vivot, Wanda Szusz, Walter Vivot, María Eugenia Bosco-Borgeat and Graciela Davel

Departamento Micología, Instituto Nacional de Enfermedades Infecciosas "Dr. Carlos G. Malbrán," Ciudad Autónoma de Buenos Aires, Argentina

*To whom correspondence should be addressed. Constanza Giselle Taverna, MSc. Departamento Micología, Instituto Nacional de Enfermedades Infecciosas "Dr. Carlos G. Malbrán," Ciudad Autónoma de Buenos Aires, Argentina. E-mail: ctaverna@anlis.gov.ar

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Abstract

The aim of this work was to reidentify strains previously identified as *Candida guilliermondii* and *Candida famata* by conventional phenotypic methods conserved in a culture collection from Argentina using ribosomal DNA sequencing, *ACT1* gene sequencing, and matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS). In addition, we performed antifungal susceptibility tests of eight antifungal drugs commonly used in clinical treatment. We identified 68 isolates belonging to the *Candida guilliermondii* species complex (59 *C. guilliermondii*, 8 *C. fermentati,* and 1 *Candida carpophila*), 16 isolates belonging to the *Candida famata* species complex (8 *C. famata*, 6 *Debaryomyces nepalensis*, 1 *Debaryomyces fabryi,* and 1 *Debaryomyces tyrocola*). Although sequencing of ITS region was able to identify *C. guilliermondii* and *D. nepalensis* isolates, sequencing of *ACT1* gene seems to be the most appropriate technique for differentiation between *C. fermentati* and *C. carpophila* and between members of the *C. famata* species complex others than *D. nepalensis*. MALDI-TOF MS has a good potential for the identification of these yeasts, particularly in clinical laboratories since is a rapid and easy to perform technique. Here, we report the first isolation of *D. tyrocola* from a human patient and the first isolation of *D. nepalensis* from lungs and blood of human patients. Finally, correct identification and determination of antifungal susceptibility of those closely related species could be a useful tool for clinicians to choose the most effective antifungal treatment.

Key words: yeasts molecular identification, MALDI-TOF MS, sequencing, *Meyerozyma*, *Debaryomyces*.

Introduction

Candida guilliermondii (*Meyerozyma guilliermondii*) and *Candida famata* (*Debaryomyces hansenii*) are opportunistic human pathogens. While *C. guilliermondii* is considered an emerging infectious yeast, *C. famata* infections are still rare. Identification of *C. guilliermondii* is of clinical interest since this species has shown low *in vitro* susceptibility to fluconazole and higher minimal inhibitory concentrations (MICs) to echinocandins compared with other common *Candida* species.[1–7]

Since the utilization of molecular methods for yeast identification, especially ribosomal DNA sequencing, several studies have demonstrated that these methods are more accurate than conventional phenotypic methods in discriminating closely related species and genetically distant species but with similar phenotypic profiles.[8–10] In this respect, *C. guilliermondii* is frequently misidentified by using conventional phenotypic methods as *C. famata* since they share similar phenotypic profiles, suggesting that *C. famata* is even less frequent as a human pathogen than it was reported before.[11,12,13]

On the other hand, molecular methods have profoundly changed the taxonomy of the yeasts and have contributed to the recognition of cryptic species or species complexes.[14] *Candida*

guilliermondii (*Meyerozyma guilliermondii*) is part of a species complex comprising also *Candida fermentati* (*Meyerozyma caribbica*) and *Candida carpophila*.[15] *Candida famata* (*Debaryomyces hansenii*) is also part of a species complex compromising until now the following species: *C. famata* (*D. hansenii*), *D. fabryi*, *C. flareri* (*D. subglobosus*), *D. macquariensis*, *D. prosopidis*, *D. maramus*, *D. nepalensis*, *D. vietnamensis*, *D. courdetii*, *D. vindobonensis*, and *D. tyrocola*.[16–19]

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) is replacing conventional phenotypic identification methods in clinical laboratories, and it has proven to be a reliable system for identification of common and rare emerging yeast pathogens.[12,20,21]

In light of previous studies that demonstrated that conventional phenotypic identification of *C. guilliermondii* and *C. famata* is not reliable and in view of the new taxonomic changes, the aim of the present work was to reidentify strains previously identified as *C. guilliermondii* and *C. famata* by conventional phenotypic methods conserved in a culture collection using ribosomal DNA sequencing, *ACT1* gene sequencing, and MALDI-TOF MS. In addition, we performed antifungal susceptibility tests of eight antifungal drugs commonly used in clinical treatment.

Methods

Yeast isolates

A total of 84 clinical isolates obtained from individual patients between 1988 and 2015 were included in the study. The isolates are conserved at the culture collection of the Mycology Department of the National Institute of Infectious Diseases "Dr. Carlos G. Malbrán" (DMic), Buenos Aires, Argentina. All isolates had been previously identified by phenotypic conventional methods, including an assessment of growth on 19 carbon and two nitrogen sources by the auxanographic method, the fermentation of six carbohydrates, growth at 35°C and 37°C, urea hydrolysis, and morphological features.[22] Of the total 84 isolates, 61 were previously identified only by conventional techniques as *C. guilliermondii* (n: 23) and *C. famata* (n: 38). Isolates belonging to the period 2012–2015 were also identified as belonging to *Meyerozyma* or *Debaryomyces* genus by sequencing of the ITS1-5.8S-ITS4 regions (ITS) previously by using the methods described below. Strain number, clinical source, and conventional identification are shown in Supplementary Table 1 (S1) and Supplementary Table 2 (S2).

MALDI-TOF MS identification

Isolates were cultured on a plate with Sabouraud dextrose agar (SDA, Britania, Buenos Aires, Argentina) and incubated during 24–48 h at 28°C. In-tube protein extraction was performed by the formic acid/acetonitrile procedure following manufacturer instructions. Finally, 1 μl of the protein extract supernatant with 1 μl of the matrix solution of α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics, Bremen, Germany) was overlaid onto a steel target (Bruker Daltonics). Mass spectrometry was performed on a Microflex LT mass spectrometer (Bruker Daltonics) using the Bruker Daltonics database (BDAL) MBT DB-5627 and using an extended BDAL database with the in-house database "LevDMic" version 1 (C.G. Taverna et. al., unpublished results). This in-house database includes the MSPs of 11 *C. guilliemondii* and 5 *C. fermentati* strains, 5 *C. famata*, 1 *D. nepalensis*, and 1 *D. fabryi*. Results were interpreted based on the log score value of the first best match following manufacturer instructions: $\geq$2.300 < 3.000 highly probable species identification; $\geq$2.000 < 2.300 secure genus identification, probable species identification; $\geq$1.700 < 2.000 probable genus identification; and <1.700 not reliable identification.

DNA extraction PCR amplification and sequencing

DNA extraction was performed by using the Ultraclean Microbial DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, USA) and following manufacturer instructions.

All isolates were first identified by sequencing of ITS with primers ITS1/ITS4 as described before.[9] Isolates identified by sequencing of ITS as *Debaryomyces* spp. were also sequenced in the D1/D2 domain of 26S rDNA (26S) and the *ACT1* gene. Isolates identified by sequencing of ITS as belonging to the *C. guilliermondii* species complex were also sequenced in the *ACT1* gene. Polymerase chain reaction (PCR) amplification of D1/D2 domain of 26S was performed with primers NL1/NL4 as described before.[9] PCR amplification of a partial sequence of the exon 2 of the *ACT1* genewas performed by using the primers described by Fukuda et al.[23], 5´-GGTTGCTGCTTTGGTTAT-3´and 5´-TAGAACCACCAATCCAGA-3´, obtaining a fragment of 1 Kb. Reactions were performed in a volume of 50 μl containing 20 mMTris-HCl (pH 8.4), 50 mMKCl, 2 mM MgCl2, 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Thermo Fisher Scientific, Vilnius, Lithuania), 1 μM each of the primers, 0.5 U Taq DNA polymerase (Invitrogen-Life Technologies; São Paulo, Brazil), and 30 ng of DNA. Amplifications were performed in a Mastercycler epgradient S (Eppendorf; Foster City, CA, USA) using the following parameters: 95°C for 7 min, followed by 30 cycles at 95°C for 1 min, 57.7°C for 2 min, 72°C for 1 min and a final extension at 72°C for 7 min.

PCR products were purified using the PureLink purification kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and were sequenced on both strands by using an ABI Genetic Analyzer 3500 (Applied Biosystems, Foster City, CA, USA). GenBank numbers are shown in S1 and S2.

Antifungal susceptibility tests

Antifungal susceptibility tests were carried out by determining the MICs according to the E.Def 7.3.1 reference document of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[24] The antifungal drugs tested were: amphotericin B, flucytosine, itraconazole (Sigma-Aldrich, Buenos Aires, Argentina), fluconazole, voriconazole, and anidulafungin (Pfizer, Buenos Aires, Argentina), caspofungin and posaconazole (Merck, Buenos Aires, Argentina), and were provided as standard powders of known potency. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains. Amphotericin B MIC end point was defined as the lowest drug concentration that caused a prominent reduction (MIC-0 or 90%) in growth compared with the growth in the drug-free well. Azoles, flucytosine, and equinocandins MIC end points were defined as the lowest drug concentration at which the growth of the isolates was reduced by 50% or more compared with that of the control (MIC 2 or $\geq$ 50%).

To date, no clinical breakpoints have been established to *C. famata* species complex or to *C. guilliermondii* species complex. However, EUCAST has determined the epidemiological cutoff value (ECV) for *C. guilliermondii* to fluconazole (16 mg/l), itraconazole (2 mg/l) and posaconazole (0.25 mg/l)[25–27] (www.eucast.org). ECVs are a useful tool to separate microorganisms with (non-wild type [non-WT]) and without (wild type [WT]) acquired resistance mechanisms to the antimicrobial agent tested.

In absence of clinical breakpoints for these species, categories susceptible/intermediate/resistant were not used and ECVs were considered only for *C. guilliermondii*. Geometric mean, mode, MIC_{50}, MIC_{90}, and range were calculated.

Result

By ITS sequencing, 15 of the 38 isolates previously identified as *C. famata* were assigned to the *C. famata* species complex, and 23 isolates were assigned to the *C. guilliermondii* species complex. On the other hand, by ITS sequencing, all 23 isolates previously identified as *C. guilliermondii* were assigned to the *C. guilliermondii* species complex. Of the remaining 23 isolates identified only by ITS sequencing, 22 belonged to the *C. guilliermondii* species complex and 1 to the *C. famata* species complex. In total, we have 68 isolates belonging to the *C. guilliermondii* species complex and 16 isolates belonging to the *C. famata* species complex.

Identification of *C. guilliermondii* species complex isolates

The performance of all methodologies used to identify the *C. guilliermondii* species complex isolates are shown in Table 1.

Identification by ITS sequencing: 59 isolates were identified as *C. guilliermondii*, 8 as *C. fermentati*, and 1 could not be identified at species level since its sequence had one nucleotide difference with *C. fermentati* and with *C. carpophila* type strains.

Identification by *ACT1* sequencing: 59 isolates were identified as *C. guilliermondii*, 6 as *C. fermentati*, and 1 as *C. carpophila*.

The percent of nucleotide differences between isolates and type strains sequences of members of the *C. guilliermondii* species complex by using ITS and *ACT1* sequences are shown in Table 2. ITS region sequences showed an intraspecies variation of 0–0.2% and interspecies variations of 0.8–1.4% between *C. guilliermondii* and *C. fermentati* or *C. carpophila*; however, interspecies variation between *C. fermentati* and *C. carpophila* was as low as 0.2 to 0.4%. On the other hand, *ACT1* sequences showed a *C. guilliermondii* intraspecies variation of 0 to 0.1% and interspecies variations of 1.7 and 2.0% between *C. guilliermondii* and *C. fermentati* or *C. carpophila*. Interspecies variation between *C. fermentati* and *C. carpophila* was 1.6 to 1.7%.

MALDITOF MS identification by using the BDAL database: of the 59 *C. guilliermondii* isolates, 64.4% (38/59) were correctly identified at species level (score value >2.000), 25.4% (15/59) at genus level (isolates were identified as *C. guilliermondii* with score value >1.700 <2.000) and 10.2% (6/59) were not reliably identified (score value <1.700). Of the 8 *C. fermentati* isolates, 50% (4/8) were identified at genus level (isolates were identified as *C. carpophila* with score values >1.700 <2.000), and the other 50% were not reliably identified. The isolate of *C. carpophila* was correctly identified at species level. To note, BDAL database has no MSP of *C. fermentati*.

MALDITOF MS identification by using the extended database (BDAL + LevDMic): all 59 *C. guilliermondii* isolates were correctly identified at specie level, 87.5% (7/8) of *C. fermentati* isolates were correctly identified at specie level and 12.5% (1/8) was correctly identified at genus level (isolate was identified as *C. fermentati* with score value >1.700 <2.000). The isolate of *C. carpophila* was correctly identified at species level.

Identification of *C. famata* species complex isolates

The performance of all methodologies used to identify the *C. famata* species complex isolates are shown in Table 1.

Identification by ITS sequencing: 6 isolates were identified as *D. nepalensis*. However, the other 10 isolates could not be identified at species level since its sequences had none, one, or two nucleotide difference between members of the *C. famata* species complex others than *D. nepalensis*.

Identification by 26S sequencing: 6 isolates were identified as *D. nepalensis* and the other 10 isolates could not be identified at species level since its sequences had between 0 to 4 nucleotide differences between members of the *C. famata* species complex others than *D. nepalensis*.

Table 1. Performance of all methodologies used for identification of *Candida guilliermondii* and *Candida famata* species complexes isolates.

Species (no. of isolates) based on ID by *ACT1* gene	% (no./total no.) of isolates Conventional ID	ID by sequencing ITS region	ID by sequencing D1/D2 region	ID by sequencing *ACT1* gene	MALDITOF by using the BDAL database	MALDITOF by using the extended database
Candida guilliemondii (59)	47% (18/38) 53% misidentified as *C. famata*	100% (59/59)	ND	100% (59/59)	64.4% (38/59) at species level 25.4% (15/59) at genus level 10.2% (6/59) not reliable ID	100% (59/59) at species level
Candida fermentati (8)	0% (7/7) 100% (7/7) misidentified as *C. guilliermondii* or *C. famata*)	100% (8/8)	ND	100% (8/8)	0% (0/8) at species level 50% (4/8) at genus level as *C. carpophila* 50% (4/8) not reliable ID	87.5% (7/8) at species level 12.5% (1/8) at genus level as *C. fermenati*
Candida carpophila (1)	0% (1/1) 100% (1/1) misidentified as *C. guilliermondii*	0% (0/1)	ND	100% (1/1)	100% (1/1) at species level	100% (1/1) at species level
Candida famata (8)	100% (8/8)	0% (0/8)	0% (0/8)	100% (8/8)	87.5% (7/8) at species level 12.5% (1/8) at genus level	100% (8/8) at species level
Debaryomyces fabryi (1)	0% (1/1) 100% (1/1) misidentified as *C. famata*	0% (0/1)	0% (0/1)	100% (1/1)	0% (1/1) at species level 0% (1/1) at genus level 100% (1/1) not reliable ID	100% (1/1) at species level
Debaryomyces tyrocola (1)	0% (1/1) 100% (1/1) misidentified as *C. famata*	0% (0/1)	0% (0/1)	100% (1/1)	0% (1/1) at species level 0% (1/1) at genus level 100% (1/1) not reliable ID	100% (1/1) misidentified as *C. famata*
Debaryomyces nepalensis (6)	0% (6/6) 100% (6/6) misidentified as *C. famata*	100% (6/6)	100% (6/6)	100% (6/6)	0% (6/6) at species level 0% (6/6) at genus level 100% (6/6) not reliable ID	100% (6/6) at species level

ID, identification.

Table 2. Percent of nucleotide differences between species in the *Candida guilliermondii* species complex by region.

Species	ITS (492 bp)			ACT1 (833 bp)		
	Candida guilliermondii	*Candida fermentati*	*Candida carpophila*	*Candida guilliermondii*	*Candida fermentati*	*Candida carpophila*
Candida guilliermondii	0-0.2	0.8-1.0	1.0-1.4	0-0.1	1.8-2.0	1.7-1.9
Candida fermentati	0.8-1.0	0	0.2-0.4	1.8-2.0	0-0.1	1.6-1.7
Candida carpophila	1.0-1.4	0.2-0.4	0-0.2	1.7-1.9	1.6-1.7	0

Identification by *ACT1* sequencing: 8 isolates were identified as *C. famata*, 6 as *D. nepalensis*, 1 as *D. fabryi*, and 1 as *D. tyrocola*.

The percent of nucleotide differences between isolates and type strains sequences of the *C. famata* species complex by using ITS, 26S, and *ACT1* sequences are shown in Table 3. ITS region sequences showed an intraspecies variation of 0–0.2% and interspecies variations of 0–0.3%. 26S region sequences showed an intraspecies variation of 0% and interspecies variations of 0–0.7%. *ACT1* region sequences showed an intraspecies variation of 0–0.8% and interspecies variations of 1.3–5.9%.

MALDITOF MS identification by using the BDAL database: of the 8 *C. famata* isolates, 87.5% (7/8) were identified at species level and 12.5% (1/8) at genus level (the isolate was identified as *C. famata* with score value >1.700 <2.000). All other isolates were not reliably identified. The BDAL database has only the MSPs of *C. famata* (*D. hansenii*) and *D. etchellsii* in the *Debaryomyces* genus.

MALDITOF MS identification by using the extended database (BDAL + LevDMic): all *C. famata, D. fabryi* and *D. nepalensis* isolates were correctly identified at species level. However, the *D. tyrocola* isolate was misidentified as *C. famata* with a score value >2.000.

Antifungal susceptibility tests

Table 4 and 5 show geometric mean, mode, MIC_{50}, MIC_{90}, and range of all antifungal drugs tested by species complex and species. Two of the 59 *C. guilliermondii* isolates were unable to grow in microdilution plates.

Regarding *C. guilliemondii* species complex, the mode and MIC_{50} of fluconazole and itraconazole for *C. fermentati* isolates were onefold dilution higher than for *C. guilliermondii* isolates. To note, 2/8 *C. fermentati* isolates showed MIC values of 128 mg/l to fluconazole. The MIC_{50} values of voriconazole, posaconazole, flucytosine, anidulafungin, and caspofungin, and the MIC_{90}values of amphotericin B were similar for all species in the complex. Taking into account the ECVs proposed for *C. guilliermondii,* 1/57 isolates was categorized as non-WT to fluconazole (MIC 32 mg/l), 8/57 isolates were categorized as non-WT to posaconazole (MIC values 0.5 mg/l), and all isolates were categorized as WT to itraconazole.

Within *C. famata* species complex, the MIC_{50} of all antifungal drugs for *D. nepalensis* were one- to fourfold dilutions higher than for *C. famata*. However, MIC values of voriconazole, anidulafungin, and caspofungin for both species were low, ranging from 0.015 to 0.13 mg/l.

Discussion

Molecular identification showed that 53% of *C. guilliermondii* isolates had been misidentified as *C. famata* by conventional identification. Similar to other authors' findings, this suggests that human infections due to *C. famata* are less frequent than previously thought.[11,13,28] To note, *C. guilliermondii* species complex isolates were mostly obtained from blood samples or other normally sterile clinical samples (S1). On the other hand, only three *C. famata* species complex isolates were obtained from blood (S2).

Molecular identification of yeasts is commonly based on rDNA sequencing, particularly the ITS region has been proposed as a primary fungal barcode marker by the Consortium for the Barcode of Life (CBOL)[29] and the International Society of Human and Animal Mycology (ISHAM) has created an "ITS reference DNA barcoding database" to achieve a quality controlled standard tool for routine identification of human and animal pathogenic fungi.[30] In this database, the intraspecies variation in most of the species studied was less than 1.5%. However, identification of some taxa could be problematic possibly because the taxa are either insufficiently studied or the ITS region is an inappropriate marker for discrimination between some groups and alternative loci are required for correct identification of these species.[30]

In this study, ITS region sequences showed interspecies variations less than 1.5% between all members in the *C. guilliermondii* species complex. However, identification of *C. guilliermondii* isolates was possible because this species show a very low intraspecies variation (0–0.1%, only one nucleotide difference). Interspecies variation between *C. fermentati* and *C. carpophila* was as low as 0.2 to 0.4% and similar to the intraspecies variations (0–0.2%). On the other hand, *ACT1* sequences showed an intraspecies variation

Table 3. Percent of nucleotide differences between species in the *Candida famata* species complex by region.

	ITS (599 bp) / D1/D2 (551 bp)						*ACT1* (874 bp)					
Species	*Candida famata*	*Debaryomyces vindobonensis*	*Debaryomyces fabryi*	*Debaryomyces prosopidis*	*Debaryomyces subglobosus*	*Debaryomyces tyrocola*	*Candida famata*	*Debaryomyces vindobonensis*	*Debaryomyces fabryi*	*Debaryomyces prosopidis*	*Debaryomyces subglobosus*	*Debaryomyces tyrocola*
Candida famata	0–0.2/0	0.2–0.3/0	0–0.2/0.4	0–0.2/0.4	0–0.2/0.2	0.2–0.3/0–0.4	0–0.8	1.3–1.6	1.7–1.8	4.3–4.6	4.8–5.9	1.7–1.8
Debaryomyces vindobonensis	0.2–0.3/0	nd	0.2/0.4	0.2/0.4	0.2/0.2	0.3/0–0.4	1.3–1.6	nd	3.4	4.3	4.5	1.9
Debaryomyces fabryi	0–0.2/0.4	0.2/0.4	nd	0/0	0/0.2	0.2/0.4–0.5	1.7–1.8	3.4	nd	2.1	2.3	2.9
Debaryomyces prosopidis	0–0.2/0.4	0.2/0.4	0/0	nd	0/0.2	0.2/0.7	4.3–4.6	4.3	2.1	nd	3.3	3.7
Debaryomyces subglobosus	0–0.2/0.2	0.2/0.2	0/0.2	0/0.2	nd	0.2/0.5	4.8–5.9	4.5	2.3	3.3	nd	4.1
Debaryomyces tyrocola	0.2–0.3/0-0.4	0.3/0–0.4	0.2/0.4–0.5	0.2/0.7	0.2/0.5	nd	1.7–1.8	1.9	2.9	3.7	4.1	nd

nd, not determined.

Table 4. Susceptibility tests results of *Candida guilliermondii* species complex isolates.

Species	MIC	MIC (mg/l)							
		AB	FC	FZ	IZ	VZ	ANID	CAS	PZ
Candida guilliermondii species complex $n = 66$	GM	0.23	0.14	3.04	0.11	0.11	0.76	0.42	0.10
	Mode	0.25	0.13	2	0.13	0.06	1	0.5	0.13
	MIC_{50}	0.25	0.13	2	0.13	0.13	1	0.5	0.13
	MIC_{90}	0.5	0.25	8	0.5	0.25	2	1	0.5
	Range	0.03–1	0.13–0.5	0.13–128	0.015–1	0.015–2	0.03–4	0.03–2	0.015–1
Candida guilliermondii $n = 57$	GM	0.23	0.14	2.71	0.10	0.11	0.80	0.42	0.10
	Mode	0.25	0.13	2	0.13	0.06	1	0.5	0.13
	MIC_{50}	0.25	0.13	2	0.13	0.13	1	0.5	0.13
	MIC_{90}	0.5	0.25	8	0.25	0.25	2	1	0.5
	Range	0.03–1	0.13–0.5	0.13–32	0.015–1	0.015–2	0.03–4	0.03–2	0.015–1
	WT* (non-WT)**	NA	NA	56 (1)	57 (0)	NA	NA	NA	49 (8)
Candida fermentati $n = 8$	GM	0.21	0.13	7.33	0.21	0.07	0.49	0.38	0.11
	Mode	0.13	0.13	4	0.25	0.06	1	0.5	0.13
	MIC_{50}	0.25	0.13	4	0.25	0.06	1	0.5	0.13
	MIC_{90}	0.5	0.13	128	0.5	0.25	1	1	0.25
	Range	0.13–0.5	0.13	2–128	0.06–0.5	0.015–0.5	0.03–2	0.13–1	0.13–0.25
Candida carpophila $n = 1$	MIC	0.5	0.5	2	0.25	0.13	1	1	0.25

AB, amphotericin B; FC, fluorocytosine; FZ, fluconazole; IZ, itraconazole; VZ, voriconazole; ANID, anidulafungin; CAS, caspofungin; PZ, posaconazole; MIC, minimal inhibitory concentration; MIC_{50} and MIC_{90}, MIC at which 50% and 90% of isolates are inhibited; GM, geometric mean; *n*, number of isolates;
NA, not available; WT*, number of wild-type isolates; non-WT**, number of non-wild-type isolates.

Table 5. Susceptibility tests results of *Candida famata* species complex isolates.

Species	MIC	MIC (mg/l)							
		AB	FC	FZ	IZ	VZ	ANID	CAS	PZ
Candida famata species complex $n = 16$	GM	0.77	0.41	1.76	0.19	0.05	0.04	0.07	0.06
	MIC_{50}	1	0.5	4	0.25	0.06	0.015	0.13	0.06
	MIC_{90}	1	1	4	0.5	0.13	0.13	0.13	0.25
	Mode	1	0.5	4	0.5	0.06	0.015	0.13	0.015
	Range	0.25–2	0.13–4	0.13–8	0.015–1	0.015–0.13	0.015–0.06	0.015–0.13	0.015–0.13
Candida famata $n = 8$	GM	0.55	0.28	1.10	0.13	0.04	0.02	0.04	0.02
	MIC_{50}	0.5	0.25	1	0.13	0.03	0.015	0.06	0.015
	MIC_{90}	1	1	2	0.25	0.06	0.015	0.13	0.06
	Mode	1	0.5	2	0.13	0.03	0.015	NA	0.015
	Range	0.25–1	0.13–0.5	0.13–4	0.015–0.5	0.015– 0.06	0.015– 0.06	0.015–0.13	0.015–0.13
Debaryomyces nepalensis $n = 6$	GM	1	0.57	4.58	0.57	0.07	0.13	0.15	0.29
	MIC_{50}	1	0.5	4	0.5	0.06	0.13	0.13	0.25
	MIC_{90}	1	1	8	1	0.13	0.13	0.25	0.5
	Mode	1	0.5	4	0.5	0.06	0.13	0.13	0.25
	Range	1	0.5–1	4–8	0.5–1	0.06–0.13	0.06–0.13	0.03–0.25	0.25–0.5
Debaryomyces fabryi $n = 1$	MIC	1	0.13	0.13	0.015	0.015	0.015	0.06	0.015
Debaryomyces tyrocola $n = 1$	MIC	2	4	4	0.13	0.13	0.015	0.13	0.13

AB, amphotericin B; FC, fluorocytosine; FZ, fluconazole; IZ, itraconazole; VZ, voriconazole; ANID, anidulafungin; CAS, caspofungin; PZ, posaconazole; MIC, minimal inhibitory concentration; MIC_{50} and MIC_{90}, MIC at which 50% and 90% of isolates are inhibited; GM, geometric mean; *n*, number of isolates.

of 0 to 0.1% and interspecies variations of 1.7 and 2.0% between members of the *C. guilliermondii* species complex. Interspecies variation between *C. fermentati* and *C. carpophila* was 1.6 to 1.7%. The gap between intraspecies and interspecies variations by *ACT1* sequencing offers a more reliable discrimination between those closely related taxa than by ITS sequencing (Table 2). Similar to the *C. guilliermondii* species complex, differentiation of *C. famata* species complex was clearer by using *ACT1* sequencing than by ITS or 26S sequencing, since the first one offers a better separation of species showing a higher gap between intraspecies and interspecies variations (Table 3).This is in agreement with the previous study of others.[16,31]

MALDI-TOF MS identification is a powerful technique that has demonstrated to be successful in yeast identification, including the discrimination of closely related species.[21,32,33] Other studies have already evaluated the performance of MALDI-TOF MS by using the VITEK MS and MALDI Biotyper platform[12,13,28] in the identification of *C. guilliermondii*, *C. fermentati*, *C. famata,* and *D. fabryi*. However, this is the first study to our knowledge that evaluates the performance of MALDI-TOF MS in the identification of all members of the *C. guilliermondii* species complex and five members of the *C. famata* species complex. In this study, MALDI-TOF MS demonstrated to have potential for the differentiation of *C. guilliermondii* species complex and the differentiation of *C. famata* species complex by using an extended in-house database and the cutoff score values recommended by manufacturer. However, *D. tyrocola* could not be correctly differentiated from *C. famata*. On the other hand, *D. fabryi* was identified by using its own MSP in the database since we had only one isolate available of that species. The addition of MSP of those species could improve the performance of this methodology.

The use of a cutoff score value of 1.700 for species identification has been proposed in several studies[20,34,35]; moreover, in a previous work (C.G. Taverna et. al., unpublished results) we have proven that the use of a cutoff value of 1.700 with our in-house extended database increase the sensibility with a minimal or not impact in the specificity in yeasts identification, and that this score value could be used to correctly identify the yeast species most commonly isolated from human infections. In this study, the use of a cutoff value of 1.700 with the BDAL database would increase the number of *C. guilliermondii* and *C. famata* isolates correctly identified. However, the use of a cutoff value of 1.700 with the extended database would not increase the number of *C. guilliermondii* and *C. famata* isolates correctly identified since all were identified with a score value >2.000. On the other hand, *C. carpophila* would not be correctly differentiated from *C. fermentati* by using a cutoff of 1.700 with any databases (S1).

Maximum growth temperatures (MGTs) has been used in the classification of *Debaryomyces* genus.[16,36] Furthermore, the capacity of growing at or near human body temperature (37°C) is a factor of virulence.[37] In this sense, six *C. famata* and the *D. tyrocola* isolates were unable to grow at 35°C and two *C. famata* isolates grew at 35°C. On the other hand, all six *D. nepalensis* and the *D. fabryi* isolates were able to grown at 37 °C (S2). There are very few reports of infections due to *D. nepalensis*.[12,38] This is the first report of isolation from lungs and blood of *D. nepalensis* in human patients. We observed a possible pseudo-outbreak due to five *D. nepalensis* isolates obtained from bronchial lavage of five patients hospitalized in the same institution during the same period of time. A bronchoscope was presumed to be contaminated and assumed to be the source of the pseudo-outbreak. To our knowledge, this is also the first report of isolation of *D. tyrocola* from a human patient.

Antifungal susceptibility tests showed that fluconazole and posaconazole were the least active antifungal drugs against *C. guilliermondii* species complex; and 1.7% and 14% of *C. guilliermondii* isolates were classified as non-WT to fluconazole and posaconazole, respectively. Amphotericin B, flucytosine, and itraconazole were highly active against *C. guilliermondii* species complex. In addition, a wide range of MIC values were obtained in all the combinations of antifungal/yeast tested. Taking into account that *C. guilliermondii* species complex has a natural reduced susceptibility to echinocandins,[39] our isolates showed relatively low MIC values ranging from 0.015 to 4 mg/l. Our results are in agreement with those of other studies.[1,28,40]

For *C. famata* species complex, data regarding antifungal susceptibility profile is scarce, thus it was a limitation to compare our results with others. In addition, literature available about antifungal susceptibility of these species includes only *C. famata*/*D. hansenii*. Despite that, we observed that *C. famata* MIC values were relatively low for all the antifungals tested; and our findings are in agreement with those obtained by other authors.[41,42] On the other hand, we observed that *D. nepalensis* MIC values of all antifungals tested were higher than those obtained for *D. hansenii*. Similar results were observed by Brilhante et al.[43], who studied *C. famata* and related species isolated from *Scarlet ibises* (*Eudocimusruber*) and reported that all *C. famata* were *in vitro* inhibited by antifungal drugs and 1/8 *D. nepalensis* was not susceptible to fluconazole and voriconazole. To note, although we cannot reach a conclusion about the susceptibility profile of *D. fabryi* and *D. tyrocola*, we observed that MIC values obtained from *D. fabryi* ranged from 0.015 to 1 mg/l similar to those obtained from *C. famata*; meanwhile MIC values obtained from *D. tyrocola* were higher, ranging from 0.015 to 4 mg/l, similar to those observed with *D. nepalensis*.

In conclusion, conventional identification methods are unable to correctly identify the *C. guilliermondii* species complex and *C. famata* species complex. Sequencing of ITS region correctly identifies *C. guilliermondii* and *D. nepalensis* but does not distinguish between *C. fermentati* and *C. carpophila*, and between members of the *C. famata* species complex others than *D.*

nepalensis. *ACT1* gene seems to be the most appropriate technique for differentiation of those closely related species. On the other hand, MALDI-TOF MS has a good potential for the identification of these yeasts, particularly in clinical laboratories since is a rapid and easy technique to perform. However, nowadays databases have no reference spectra for all species in the *C. guilliermondii* and *C. famata* species complexes. The addition of MSPs of those species in databases could improve the performance of MALDI-TOF MS in their identification. Data of antifungal susceptibility tests showed here reinforced the usefulness of *in vitro* test to know the antifungal susceptibility profile of strains circulating in the region. Finally, correct identification and determination of antifungal susceptibility of those closely related species could be a useful tool for clinicians to choose the most effective antifungal treatment.

Supplementary material

Supplementary data are available at *MMYCOl* online.

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Declaration of interest

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

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