ORIGINAL ARTICLE

J Mycol Infect 2026; 31(1): 25-38
pISSN:3058-423X, eISSN:3058-4302
https://dx.doi.org/10.17966/JMI.2026.31.1.25

JMI

Comparative Evaluation of MALDI-TOF MS and ITS Barcoding for the Identification of Clinical and Environmental Yeast Isolates

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Background: ITS barcoding is the "gold standard" method for fungal identification. However, it is associated with high costs, requires specialized expertise, and entails lengthy turnaround times. MALDI-TOF MS represents a more rapid and lower-cost alternative, but it is generally less sensitive and less specific than ITS barcoding.

Objective: This study aimed to investigate and assess MALDI-TOF MS and ITS barcoding for identifying yeast strains isolated from patients and domestic environments.

Methods: A total of 115 yeast strains, including 89 pathogenic and 26 environmental, were identified using MALDI-TOF MS and ITS barcoding with phylogenetic analyses. The identification results from the two methods were compared and assessed.

Results: ITS barcoding identified 115 strains, including ascomycetous yeasts (5 *Candida* species and 9 non-*Candida* species) and basidiomycetous yeasts (5 species and 1 species complex). In contrast, MALDI-TOF MS identified 74.78% (86/115) of the isolates, with an accuracy of 96.51% (83/86). The MS showed substantial agreement with ITS barcoding ($\kappa = 0.65$, $p < 0.001$) when all isolates were included, and this agreement strengthened ($\kappa = 0.76$, $p < 0.001$) when the analysis was restricted to species represented in the MS database. Furthermore, almost perfect agreement was observed ($\kappa = 0.81$, $p < 0.001$) for common pathogenic *Candida* species, achieving 100% identification rate and accuracy for *C. albicans* and *C. tropicalis*.

Conclusion: ITS barcoding remains the gold standard method for yeast identification. MALDI-TOF MS is a viable option for routine clinical use, but it requires database expansion and technical improvements for uncommon species or species complexes.

Key Words: DNA barcoding, ITS, MALDI-TOF MS, Yeast identification

Received: December 10, 2025 **Revised:** December 31, 2025 **Accepted:** January 13, 2026

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INTRODUCTION

Fungal identification using conventional methods in routine practice often faces challenges, such as the need for cultivation, being time-consuming, and low specificity. With the introduction of antifungal stewardship programs and routine antifungal susceptibility testing for managing fungal infections, precise identification of fungal pathogens has become increasingly important. Identification using DNA barcoding markers has been recognized as another "gold standard" method for fungal identification; however, this method entails high costs, complex protocols, and long turnaround times. The internal transcribed spacer (ITS) region of ribosomal DNA has been widely adopted as the primary DNA barcode for fungal identification due to several key attributes[1,2], including the availability of comprehensive reference databases with type specimens to discriminate most species, standardized amplification protocols, and a phylogenetic framework that allows evolutionary interpretation. These features collectively establish ITS sequencing as the current reference standard for definitive fungal identification, particularly for species that cannot be reliably distinguished by phenotypic methods. Since MALDI-TOF MS was developed[3,4], many studies have applied the MS to fungal identification, especially for yeast pathogens in routine diagnostics[5-11], to overcome DNA barcoding disadvantages. MALDI-TOF MS identifies microorganisms by analyzing their protein profiles, generating unique mass spectra patterns that serve as molecular fingerprints for each species. This technique has gained popularity in clinical microbiology due to its rapid turnaround time (minutes vs. days) and low operational costs[12,13].

In the present study, we compared and assessed two molecular methods, MALDI-TOF MS and DNA barcoding using ITS, to identify yeast strains isolated from patients and domestic environments.

MATERIALS AND METHODS

1. Collection of yeast strains

One hundred and fifteen yeast strains were collected from 2022 to 2025. Of these 89 strains (strains F1-F54 and B2-B57) were isolated from clinical specimens of patients who were admitted at the Srinagarind Hospital, Khon Kaen, Thailand. The remaining 26 strains (strains E3-E39) were collected from household environments within the Khon Kaen municipal area. The colonial morphology of each strain was examined using Sabouraud dextrose agar and CHROMagar (Brilliance™ *Candida* Agar, Oxoid, UK), while micromorphology was done using lactophenol cotton blue and/or nigrosin staining.

2. Identification by MALDI-TOF MS

All yeast strains were identified using MALDI-TOF MS (MALDI Biotyper®, autoflex® maX mass, Bruker Daltonics, Bremen, DE). The method was performed according to the manufacturer's protocol. Sample preparation followed the manufacturer's formic acid extraction protocol. Briefly, yeast cells were smeared onto a ground steel MALDI target plate (MTP 384), air-dried, overlaid with 1 μl of 70% formic acid and dried, then overlaid with 1 μl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution and dried again. The steel plate was examined to obtain mass spectral profiles using an Autoflex maX mass spectrometer. Each yeast strain profile was identified using Bruker's MALDI Biotyper® Compass software version 4.1.100 with the MBT 8468 Species/Entry List Revision C (March 2019), and identifications were accepted according to the manufacturer's criteria.

3. PCR sequencing of ITS

All strains were DNA-extracted using a DNA extraction kit (Vivantis Technologies Sdn Bhd., Selangor Darul Ehsan, Malaysia). The DNAs were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA). The ITS sequence in the ribosomal gene was amplified by PCR using the forward primers ITS1 (TCCGTAGGTGAACCTGCGG), V9G (TTACGTCCCTGCCCTTTGTA)[14], or ITS5 (TCCTCCGCTTATTGATATGC)[15] and the reverse primers including ITS4 (GGAAGTAAAAGTCGTAACAAGG)[15] or LS266 (GCATTCCCAAACAACTCGACTC)[16]. Different primer combinations were used to optimize amplification success across diverse yeast taxa. The primer pair ITS1/ITS4 was primarily used, while alternative primer pairs (V9G/LS266 or ITS5/ITS4) were employed when amplification failed or produced weak products. The PCR reaction contained 10 μM of each primer, 12.5 μl of KOD One® PCR Master Mix (TOYOBO, JP), and >10 ng genomic fungal DNA in 25 μl of total volume reaction. The PCR conditions started at 95℃ for 5 min, followed by 35 cycles of denaturation at 95℃ for 35 sec, annealing at 48℃ for 30 sec, and extension at 72℃ for 30 sec. Amplification was performed using a Veriti® Thermal Cycler (Applied Biosystems, CA, USA). The amplification products were examined with 2% gel electrophoresis and submitted to Sanger's sequencing.

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4. Identification by ITS barcoding

The ITS sequences obtained from Sanger's sequencing were edited using BIOEDIT v.7.2.5[17] and blasted against the type materials in GenBank using megablast in BLAST® blastn[18]. The datasets, including the sequences of the tested strains and those retrieved from megablast, were prepared using BIOEDIT v.7.2.5 and aligned with the algorithms, specifically ClustalW in BIOEDIT v.7.2.5, Multiple Alignment using Fast Fourier Transform (MAFFT)[19], or MUltiple Sequence Comparison by Log-Expectation (MUSCLE)[20] via the EMBI-EBI web server[21]. Phylogenetic analyses for species identification were performed using MEGA11[22]. The best model and appropriate phylogenetic algorithms were inferred to reconstruct a phylogenetic tree and to estimate percentage similarity. Species identification was considered valid when the test strain formed a monophyletic clade with the type species in the phylogenetic tree and/or showed sequence similarity with the type specimen across the ITS region.

5. Assessment of MALDI-TOF MS for yeast identification

With ITS barcoding serving as the reference method, the identification rate and accuracy of MALDI-TOF MS were calculated using the following formulas:

$$\% \text{ Identification rate} = \frac{\text{Total number of identifications using MALDI-TOF MS x 100}}{\text{Total number of identifications using ITS barcoding}}$$

$$\% \text{ Accuracy} = \frac{\text{Number of correct identifications using MALDI-TOF MS x 100}}{\text{Total number of identifications using MALDI-TOF MS}}$$

Agreement between the two methods was assessed using Cohen's kappa coefficient, calculated for two scenarios: (1) all isolates and (2) isolates of species represented in the MALDI-TOF MS database. Statistical analysis was performed using SPSS version 29.0 (IBM Corp., Armonk, NY, USA). The Cohen's kappa (κ) coefficients were interpreted based on the criteria established by Landis and Koch[23]: ≤ 0 (no agreement), 0.01-0.20 (slight), 0.21-0.40 (fair), 0.41-0.60 (moderate), 0.61-0.80 (substantial), and 0.81-1.00 (almost perfect agreement).

RESULTS

1. Identification by MALDI-TOF MS

MALDI-TOF MS identified 86 strains belonging to 11 species (number of strains), including *C. albicans* (46), *C. tropicalis* (22), *C. metapsilosis* (2), *C. parapsilosis* (1), *Meyerozyma guilliermondii* (syn. *Candida guilliermondii*) (1), *Nakaseomyces glabratus* (syn. *Candida glabrata*) (7), *Pichia kudriavzevii* (syn. *Candida krusei*) (2), *Diutina rugosa* (syn. *Candida rugosa*) (1), *Kodamaea ohmeri* (1), *Cystobasidium minutum* (syn. *Rhodotorula minuta*) (1), and *Rhodotorula mucilaginosa* (1). However, the MS failed to identify 29 strains. All results are shown in Table 1.

2. Identification by ITS barcoding

All 115 yeast strains were identified using phylogenetic analysis of their ITS sequences in comparison with the type materials. Species identification was determined based on monophyletic clustering and/or the highest sequence similarity with the type species, as shown in Fig. 1. Of the 115 strains, 102 were classified as ascomycetous yeasts belonging to 14 species, whereas 13 strains were classified as basidiomycetous yeasts, comprising 5 species and 1 species complex. The ascomycetous species (number of strains) included *Candida albicans* (46), *C. tropicalis* (22), *Nakaseomyces glabratus* (8), *C. orthopsilosis* (6), *C. metapsilosis* (4), *Pichia kudriavzevii* (4), *Wickerhamomyces sydowiorum* (3), *C. parapsilosis* (2), *Debaryomyces nepalensis* (2), *Meyerozyma caribbica* (1), *Kodamaea ohmeri* (1), *Diutina mesorugosa* (1), *Trichomonascus ciferrii* (syn. *Candida ciferrii*) (1), and *Yarrowia lipolytica* (1). The basidiomycetous species (number of strains) included *Papiliotrema rajasthanensis* (5), *Rhodotorula mucilaginosa* (3), *Kwoniella heveanensis* (2), *Cystobasidium benthicum* (1), *Cystobasidium minutum* (1), and *Naganishia albidosimilis-liquefaciens* (syn. *Cryptococcus albidosimilis* and *C. liquefaciens*) (1). *Candida albicans*, *C. tropicalis*, *C. parapsilosis* complex, and *Nakaseomyces glabratus* were the predominant species isolated from patients' specimens, whereas basidiomycetous species predominated in environmental specimens. All details are presented in Table 1.

3. Assessment of MALDI-TOF MS for yeast identification

MALDI-TOF MS and ITS barcoding results are compared in Table 1. In addition to the 29 unidentified strains, the MS misidentified three strains as *Candida parapsilosis*, *Diutina*

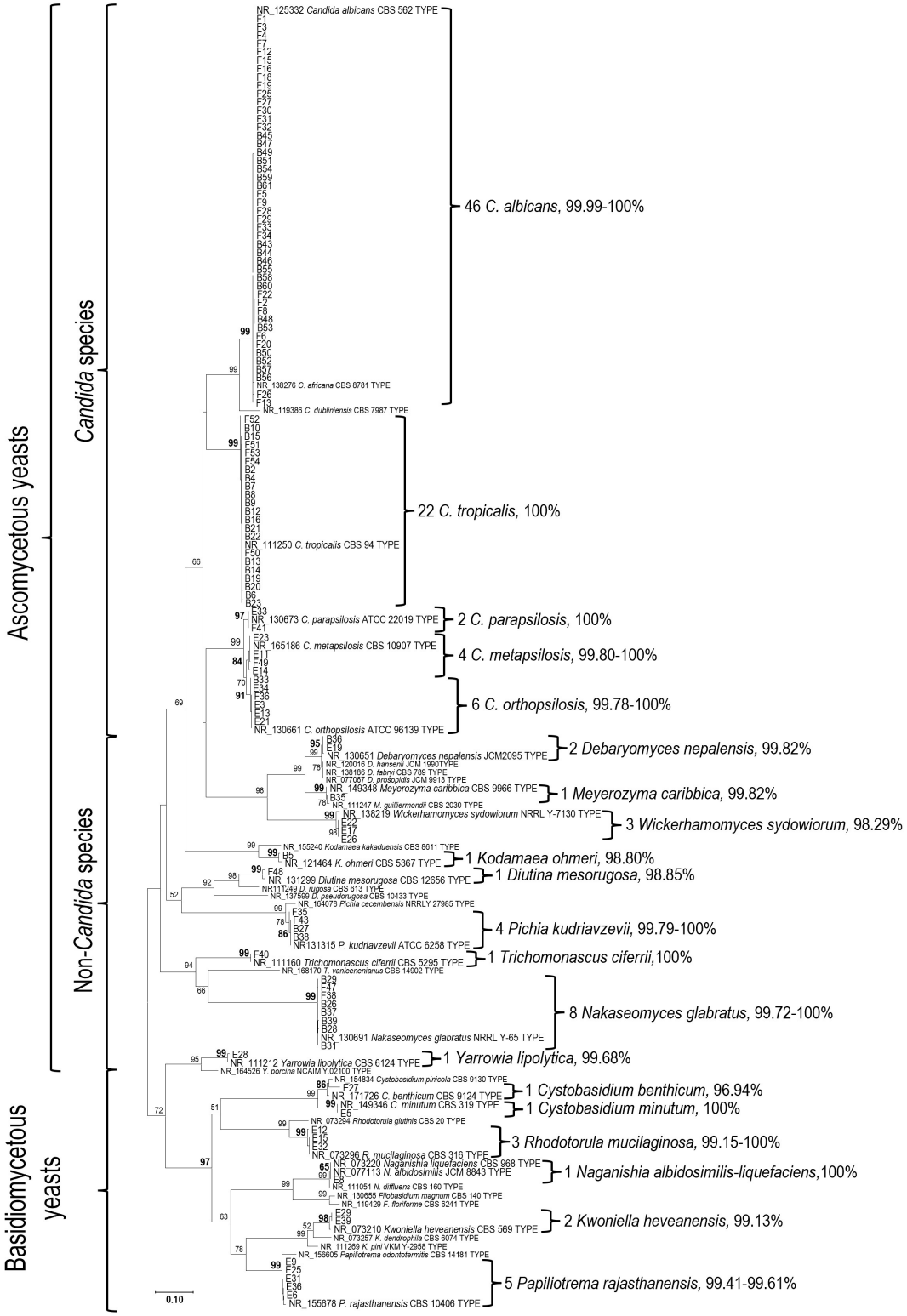

Fig. 1. Phylogenetic tree of 115 yeast strains and 41 type materials according to a dataset of 156 ITS sequences. The dataset was aligned to 951 positions by MAFFT and analyzed in MEGA11 by the Neighbor-joining method with the Tamura 3-parameter + G model having 1,000 bootstrap replications. Number on the nodes = % bootstrap support, ≥50% nodes shown. Number behind identified species = % similarity to the type-material

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Table 1. Identification using MALDI-TOF MS and ITS-barcoding

No.	Strain	Source	Maldi-TOF MS	ITS barcoding
1	E3	Refrigerator	No identification possible	*Candida orthopsilosis*
2	E5	Electric fan	*Cystobasidium minutum*	*Cystobasidium minutum*
3	E6	Refrigerator	No identification possible	*Papiliotrema rajasthanensis*
4	E8	Air conditioner	No identification possible	*Naganishia albidosimilis-liquefaciens*
5	E9	Air conditioner	No identification possible	*Papiliotrema rajasthanensis*
6	E11	Floor	No identification possible	*Candida metapsilosis*
7	E12	Floor	No identification possible	*Rhodotorula mucilaginosa*
8	E13	Lid of trash box	No identification possible	*Candida orthopsilosis*
9	E14	Air purifier	*Candida metapsilosis*	*Candida metapsilosis*
10	E15	Refrigerator	No identification possible	*Rhodotorula mucilaginosa*
11	E17	Bathroom	No identification possible	*Wickerhamomyces sydowiorum*
12	E19	Air conditioner	No identification possible	*Debaryomyces nepalensis*
13	E21	Office area	No identification possible	*Candida orthopsilosis*
14	E22	Bathroom	No identification possible	*Wickerhamomyces sydowiorum*
15	E23	Electric fan	No identification possible	*Candida metapsilosis*
16	E25	Glass window	No identification possible	*Papiliotrema rajasthanensis*
17	E26	Bathroom	No identification possible	*Wickerhamomyces sydowiorum*
18	E27	Air conditioner	No identification possible	*Cystobasidium benthicum*
19	E28	Floor	No identification possible	*Yarrowia lipolytica*
20	E29	Door	No identification possible	*Kwoniella heveanensis*
21	E31	Table	No identification possible	*Papiliotrema rajasthanensis*
22	E32	Bathroom	*Rhodotorula mucilaginosa*	*Rhodotorula mucilaginosa*
23	E33	Air conditioner	*Candida parapsilosis*	*Candida parapsilosis*
24	E34	Floor	No identification possible	*Candida orthopsilosis*
25	E36	Glass window	No identification possible	*Papiliotrema rajasthanensis*
26	E39	Door	No identification possible	*Kwoniella heveanensis*
27	F1	Patient's fluid	*Candida albicans*	*Candida albicans*
28	F2	Patient's fluid	*Candida albicans*	*Candida albicans*
29	F3	Patient's fluid	*Candida albicans*	*Candida albicans*
30	F4	Patient's fluid	*Candida albicans*	*Candida albicans*
31	F5	Patient's fluid	*Candida albicans*	*Candida albicans*
32	F6	Patient's fluid	*Candida albicans*	*Candida albicans*
33	F7	Patient's fluid	*Candida albicans*	*Candida albicans*
34	F8	Patient's fluid	*Candida albicans*	*Candida albicans*

Table 1. Identification using MALDI-TOF MS and ITS-barcoding (Continued)

No.	Strain	Source	Maldi-TOF MS	ITS barcoding
35	F9	Patient's fluid	*Candida albicans*	*Candida albicans*
36	F12	Patient's fluid	*Candida albicans*	*Candida albicans*
37	F13	Patient's fluid	*Candida albicans*	*Candida albicans*
38	F15	Patient's fluid	*Candida albicans*	*Candida albicans*
39	F16	Patient's fluid	*Candida albicans*	*Candida albicans*
40	F18	Patient's fluid	*Candida albicans*	*Candida albicans*
41	F19	Patient's fluid	*Candida albicans*	*Candida albicans*
42	F20	Patient's fluid	*Candida albicans*	*Candida albicans*
43	F22	Patient's fluid	*Candida albicans*	*Candida albicans*
44	F25	Patient's fluid	*Candida albicans*	*Candida albicans*
45	F26	Patient's fluid	*Candida albicans*	*Candida albicans*
46	F27	Patient's fluid	*Candida albicans*	*Candida albicans*
47	F28	Patient's fluid	*Candida albicans*	*Candida albicans*
48	F29	Patient's fluid	*Candida albicans*	*Candida albicans*
49	F30	Patient's fluid	*Candida albicans*	*Candida albicans*
50	F31	Patient's fluid	*Candida albicans*	*Candida albicans*
51	F32	Patient's fluid	*Candida albicans*	*Candida albicans*
52	F33	Patient's fluid	*Candida albicans*	*Candida albicans*
53	F34	Patient's fluid	*Candida albicans*	*Candida albicans*
54	F35	Patient's fluid	No identification possible	*Pichia kudriavzevii*
55	F36	Patient's fluid	No identification possible	*Candida orthopsilosis*
56	F38	Patient's fluid	No identification possible	*Nakaseomyces glabratus*
57	F40	Patient's fluid	No identification possible	*Trichomonascus ciferrii*
58	F41	Patient's fluid	No identification possible	*Candida parapsilosis*
59	F43	Patient's fluid	*Pichia kudriavzevii*	*Pichia kudriavzevii*
60	F47	Patient's fluid	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
61	F48	Patient's fluid	*Diutina rugosa*	*Diutina mesorugosa*
62	F49	Patient's fluid	*Candida metapsilosis*	*Candida metapsilosis*
63	F50	Patient's fluid	*Candida tropicalis*	*Candida tropicalis*
64	F51	Patient's fluid	*Candida tropicalis*	*Candida tropicalis*
65	F52	Patient's fluid	*Candida tropicalis*	*Candida tropicalis*
66	F53	Patient's fluid	*Candida tropicalis*	*Candida tropicalis*
67	F54	Thrombus	*Candida tropicalis*	*Candida tropicalis*
68	B2	Hemoculture	*Candida tropicalis*	*Candida tropicalis*

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Table 1. Identification using MALDI-TOF MS and ITS-barcoding (Continued)

No.	Strain	Source	Maldi-TOF MS	ITS barcoding
69	B4	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
70	B5	Hemoculture	*Kodamaea ohmeri*	*Kodamaea ohmeri*
71	B6	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
72	B7	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
73	B8	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
74	B9	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
75	B10	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
76	B12	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
77	B13	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
78	B14	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
79	B15	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
80	B16	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
81	B19	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
82	B20	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
83	B21	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
84	B22	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
85	B23	Hemoculture	*Candida tropicalis*	*Candida tropicalis*
86	B26	Hemoculture	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
87	B27	Hemoculture	No identification possible	*Pichia kudriavzevii*
88	B28	Hemoculture	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
89	B29	Hemoculture	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
90	B31	Hemoculture	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
91	B33	Hemoculture	*Candida parapsilosis*	*Candida orthopsilosis*
92	B35	Hemoculture	*Meyerozyma guilliermondii*	*Meyerozyma caribbica*
93	B36	Hemoculture	No identification possible	*Debaryomyces nepalensis*
94	B37	Hemoculture	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
95	B38	Hemoculture	*Pichia kudriazevii*	*Pichia kudriazevii*
96	B39	Hemoculture	*Nakaseomyces glabratus*	*Nakaseomyces glabratus*
97	B43	Hemoculture	*Candida albicans*	*Candida albicans*
98	B44	Hemoculture	*Candida albicans*	*Candida albicans*
99	B45	Hemoculture	*Candida albicans*	*Candida albicans*
100	B46	Hemoculture	*Candida albicans*	*Candida albicans*
101	B47	Hemoculture	*Candida albicans*	*Candida albicans*
102	B48	Hemoculture	*Candida albicans*	*Candida albicans*

Table 1. Identification using MALDI-TOF MS and ITS-barcoding (Continued)

No.	Strain	Source	Maldi-TOF MS	ITS barcoding
103	B49	Hemoculture	*Candida albicans*	*Candida albicans*
104	B50	Hemoculture	*Candida albicans*	*Candida albicans*
105	B51	Hemoculture	*Candida albicans*	*Candida albicans*
106	B52	Hemoculture	*Candida albicans*	*Candida albicans*
107	B53	Hemoculture	*Candida albicans*	*Candida albicans*
108	B54	Hemoculture	*Candida albicans*	*Candida albicans*
109	B55	Hemoculture	*Candida albicans*	*Candida albicans*
110	B56	Hemoculture	*Candida albicans*	*Candida albicans*
111	B57	Hemoculture	*Candida albicans*	*Candida albicans*
112	B58	Hemoculture	*Candida albicans*	*Candida albicans*
113	B59	Hemoculture	*Candida albicans*	*Candida albicans*
114	B60	Hemoculture	*Candida albicans*	*Candida albicans*
115	B61	Hemoculture	*Candida albicans*	*Candida albicans*

Table 2. The strain numbers of species identified with ITS barcoding and MALDI-TOF MS assessment

Cluster/ Group		Species	Source‡	ITS	MALDI-TOF MS					Cohen's kappa
			B/F/E		Right Ident.	Mis. Ident.	Not Ident.	Ident. (%)	Accur. (%)	
Ascomycetous yeasts	*Candida* species	*Candida albicans*	19/27/-	46	46	0		100	100	
		C. tropicalis	17/5/-	22	22	0		100	100	
		C. parapsilosis	-/1/1	2	1	0	1	50	100	
		C. metapsilosis	-/1/3	4	2	0	2	50	100	
		C. orthopsilosis	1/1/4	6	0	1	5	16.66	0	
		Total number of each source	**37/35/8**							
		MALDI-TOF assessment								
		Only species in MS-database		**80**	**71**	**1**	**8**	**90.00**	**98.61**	**0.81§**
		All species		**80**	**71**	**1**	**8**	**90.00**	**98.61**	**0.81§**
	Non-*Candida* species	*Meyerozyma caribbica**	1/-/-	1	0	1	0	100	0	
		*Debaryomyces nepalensis**	1/-/1	2	0	0	2	0	–	
		*Wickerhamomyces sydowiorum**	-/-/3	3	0	0	3	0	–	
		Kodamaea ohmeri	1/-/-	1	1	0	0	100	100	
		*Diutina mesorugosa**	-/1/-	1	0	1	0	100	0	
		Pichia kudriavzevii	2/2/-	4	2	0	2	50	100	
		Trichomonascus ciferrii	-/1/-	1	0	0	1	0	–	

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Table 2. The strain numbers of species identified with ITS barcoding and MALDI-TOF MS assessment (Continued)

Cluster/ Group		Species	Source‡	ITS	MALDI-TOF MS					Cohen's kappa
			B/F/E		Right Ident.	Mis. Ident.	Not Ident.	Ident. (%)	Accur. (%)	
Ascomycetous yeasts	Non-*Candida* species	*Nakaseomyces glabatus*	6/2/-	8	7	0	1	87.50	100	
		Yarrowia lipolytica	-/-/1	1	0	0	1	0	–	
		Total number of each source	**11/6/5**							
		MALDI-TOF assessment								
		Only species in MS-database		**15**	**10**	**0**	**5**	**66.66**	**100**	**0.53§**
		All species		**22**	**10**	**2**	**10**	**54.54**	**83.33**	**0.37§**
Basidiomycetous yeasts		*Cystobasidium benthicum*⋆	-/-/1	1	0	0	1	0	–	
		Cystobasidium minutum	-/-/1	1	1	0	0	100	100	
		Rhodotorula mucilaginosa	-/-/3	3	1	0	2	33.33	100	
		Naganishia albidosimilis-liquefaciens†	-/-/1	1	0	0	1	0	–	
		Kwoniella heveanensis⋆	-/-/2	2	0	0	2	0	–	
		Papiliotrema rajasthanensis⋆	-/-/5	5	0	0	5	0	–	
		Total number of each source	**-/-/13**							
		MALDI-TOF assessment								
		Only species in MS-database		**5**	**2**	**0**	**3**	**40**	**100**	**0.28‖**
		All species		**13**	**2**	**0**	**11**	**15.38**	**100**	**0.13§**
		Total number of each source	**48/41/26**							
		Total MALDI-TOF assessment								
		Only species in MS-database		**100**	**83**	**1**	**16**	**84**	**98.81**	**0.76§**
		All species		**115**	**83**	**3**	**29**	**74.78**	**96.51**	**0.65§**

⋆ = Not in MS-database, † = species complex, ‡ = Source of strain: B (Blood)/F (Fluid&other)/E (Domestic environment)
§ = $p < 0.001$, ‖ = $p < 0.05$

rugosa, and *Meyerozyma guilliermondii*; these were subsequently identified as *C. orthopsilosis*, *D. mesorugosa*, and *M. caribbica*, respectively, based on ITS sequencing. Using ITS barcoding as the reference method, the overall identification rate for MALDI-TOF MS was 74.78%, with an overall accuracy of 96.51%. The MS system demonstrated the highest identification rate for *Candida* species (100%), whereas the lowest rate was observed for basidiomycetous species (15.38%). Specifically, accuracy values were 98.61% for *Candida* species, 83.33% for non-*Candida* species, and 100% for basidiomycetous species. Cohen's kappa analysis demonstrated substantial agreement between ITS barcoding and MALDI-TOF MS across all isolates (n = 115, κ = 0.65, $p < 0.001$). This agreement strengthened when the analysis was restricted to isolates of species represented in the MALDI-TOF MS database (n = 100, κ = 0.76, $p < 0.001$). Moreover, almost perfect agreement (κ = 0.81, $p < 0.001$) was achieved for common pathogenic *Candida* species, including *C. albicans*, *C. tropicalis*, and the *C. parapsilosis* complex. Detailed results are shown in Table 2.

DISCUSSION

Invasive fungal infections, particularly candidemia and invasive aspergillosis, are associated with mortality rates of

20-40% and 30-95%, respectively, depending on the patient population and timeliness of appropriate therapy[24,25]. Early identification has been shown to improve survival outcomes[26]. Recent stewardship studies have demonstrated that rapid diagnosis can reduce inappropriate antifungal use and shorten hospital stays[27].

In our study, DNA barcoding using ITS yielded satisfactory results, confirming its role as another "gold standard" method for yeast identification. The well-supported phylogenetic species clusters and/or the minimal genetic distances to type strains serve as the criteria for sequence-based identification, as previously mentioned[28,29]. DNA barcoding represents a highly robust methodology for species identification, as its procedures align with the foundational protocols established in contemporary yeast taxonomy through molecular phylogenetic analysis. However, to ensure precise identification, DNA barcoding necessitates the integration of molecular techniques and deep taxonomic knowledge. While BLAST® blastn[18] appears to be an efficient tool for identifying DNA barcoding sequences, results can sometimes be difficult to interpret without sufficient expertise. Although modern fungal taxonomy relies on the genealogical species concept, challenges persist regarding taxonomic non-consensus and species complexes. Assigning a single barcoding marker often proves problematic for species defined via multigene analysis or those with ambiguous classifications. Therefore, phylogenetic analysis, including comparisons with type materials, is essential to achieve accurate identification. Our study utilized a straightforward phylogenetic approach using ITS, a method rarely incorporated into MALDI-TOF assessment studies. This approach successfully identified nearly all pathogenic species at both the species and species complex levels. Notably, ITS cannot differentiate two basidiomycetous species, *Naganishia albidosimilis* and *N. liquefaciens* (syn. *Cryptococcus albidosimilis* and *C. liquefaciens*), due to their identical ITS sequences. However, these species can be distinguished through phylogenetic analysis of an alternative marker, the D1/D2 domain of the LSU rDNA[30]. Despite its high resolution, DNA barcoding remains limited by long turnaround times and high operational costs, which are significant drawbacks for routine clinical use.

Across all species investigated, MALDI-TOF MS showed substantial agreement with ITS barcoding (κ = 0.65), with an identification rate of 74.78% and a high accuracy of 96.51%. However, the system failed to identify seven species not represented in its database, highlighting a primary limitation of MALDI-TOF MS. These species included *Meyerozyma caribbica*, *Debaryomyces nepalensis*, *Wickerhamomyces sydowiorum*, *Diutina mesorugosa*, *Cystobasidium benthicum*, *Kwoniella heveanensis*, and *Papiliotrema rajasthanensis*. When the analysis was restricted to species represented in the database, the MS demonstrated higher agreement with ITS barcoding (κ = 0.76), with the identification rate and accuracy reaching 84% and 98.91%, respectively. Identification rates for uncommon yeasts and molds typically range from 65% to 85%, depending on the instrument and database[10]. Although the MS database used in this study included many species (Table 2), it still failed to identify certain strains. This may be attributed to factors such as the partial extraction method used for the specimens[31] or strain variations in mass spectral profiles[32].

The overall identification accuracy in our study was highly satisfactory at 96.51%, reaching 98.81% when restricted to species present in the database. Such high accuracy (often > 95%) is consistent with findings for common yeast identification using either the Bruker MALDI Biotyper or the bioMérieux VITEK MS/MS[33-35]. MALDI-TOF MS, when combined with curated libraries or purpose-built datasets, has been shown to successfully differentiate species complexes, including *C. parapsilosis*, *C. guilliermondii*, *C. glabrata*, and *Cryptococcus neoformans/gattii*[8-11].

However, in this study, the MS failed to differentiate the species within the *C. parapsilosis* complex and the *Meyerozyma guilliermondii* (syn. *Candida guilliermondii*) complex. In addition to technical limitations, incomplete databases, and variations in mass spectral profiles, the close genetic relationships among these species could be a plausible explanation for identification failure. MALDI-TOF MS identifies organisms based on ribosomal protein mass spectra, which are highly conserved among closely related species[36]. When species share more than 98% sequence similarity, discriminatory protein peaks may be subtle or absent, resulting in misidentification or non-identification. This phenomenon has been documented in other fungal complexes, including *Aspergillus niger*, *A. flavus*, and the *A. nidulans* complex[37], as well as the *Cryptococcus gattii* complex[11]. Similarly, the close phylogenetic relationship between *C. albicans* and *C. africana* poses significant challenges for MALDI-TOF MS[38]. Database expansion alone may not fully resolve this issue. Species complexes with minimal protein-level divergence may require supplementary identification methods, such as ITS sequencing or multilocus sequence typing, for definitive differentiation[39].

From a clinical perspective, these misidentifications may have limited therapeutic impact. Species within the *C. parapsilosis* complex generally exhibit similar antifungal susceptibility profiles, with minimal differences in minimum inhibitory concentration distributions for azoles and echinocandins[40,41]. Similarly, *M. caribbica* and *M. guilliermondii* show comparable

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susceptibility patterns[42]. No inter-complex misidentifications that could lead to inappropriate antifungal selection were observed in our cohort. However, accurate species-level identification within complexes remains important for epidemiological tracking and understanding virulence differences. For instance, *Candida orthopsilosis* and *C. metapsilosis* differ from *C. parapsilosis* in biofilm formation capacity and tissue tropism[43]. Such information is valuable for infection control and outbreak investigation, even when immediate therapeutic decisions are unaffected.

In our findings, when focusing on the most common pathogenic *Candida* species present in the database, MALDI-TOF MS showed almost perfect agreement with ITS barcoding (κ = 0.81), achieving 100% identification rate and accuracy in *C. albicans* and *C. tropicalis*. Furthermore, the MS proved highly reliable in identifying *Nakaseomyces glabratus* (syn. *Candida glabrata*) with 100% accuracy. To improve the identification capability and accuracy of MALDI-TOF MS, expanding the database to include a broader range of species associated with human and animal infections, such as *Meyerozyma caribbica*[42,44], *Diutina mesorugosa*[45], and *Debaryomyces nepalensis*[46], should be considered a critical priority.

Additionally, our findings revealed that members of the *C. parapsilosis* complex, viz., *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*, were isolated from various environmental surfaces, including floors, electric fans, air conditioners, air purifiers, trash bins, and refrigerators. These observations are consistent with their established environmental persistence and hand-mediated transmission pathways[47]. These results highlight the critical role of environmental surveillance in monitoring the dissemination of azole-resistant strains[48] or potential sources of infection[49].

In conclusion, both MALDI-TOF MS and ITS barcoding have distinct roles in clinical mycology laboratories. For cost-effective laboratory practice, we recommend MALDI-TOF MS as the first-line method for rapid identification of common yeast pathogens. ITS barcoding should be reserved for unidentified isolates, species complexes, rare species, and high-impact clinical cases requiring definitive identification[39,50-52]. Continued expansion of MALDI-TOF reference databases, particularly to include closely related or cryptic species as well as newly described species or discovered pathogens, will further enhance its utility.

ACKNOWLEDGEMENT

This study was granted by the Faculty of Medicine, Khon Kaen University, Thailand (Grant Number IN67052).

CONFLICT OF INTEREST

In relation to this article, we declare that there is no conflict of interest.

DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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ETHICAL APPROVAL STATEMENT

This study, involving fungal strains, complied with all relevant national regulations and institutional policies and was conducted in accordance with the tenets of the Declaration of Helsinki. It was also approved by the Khon Kaen University Ethics Committee (KKUEC), with approval IRB No. HE671084.

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