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Identification of Medically Important Yeast Species by Sequence Analysis of the Internal Transcribed Spacer Regions

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Infections caused by yeasts have increased in previous decades due primarily to the increasing population of immunocompromised patients. In addition, infections caused by less common species such as *Pichia*, *Rhodotorula*, *Trichosporon*, and *Saccharomyces* spp. have been widely reported. This study extensively evaluated the feasibility of sequence analysis of the rRNA gene internal transcribed spacer (ITS) regions for the identification of yeasts of clinical relevance. Both the ITS1 and ITS2 regions of 373 strains (86 species), including 299 reference strains and 74 clinical isolates, were amplified by PCR and sequenced. The sequences were compared to reference data available at the GenBank database by using BLAST (basic local alignment search tool) to determine if species identification was possible by ITS sequencing. Since the GenBank database currently lacks ITS sequence entries for some yeasts, the ITS sequences of type (or reference) strains of 15 species were submitted to GenBank to facilitate identification of these species. Strains producing discrepant identifications between the conventional methods and ITS sequence analysis were further analyzed by sequencing of the D1–D2 domain of the large-subunit rRNA gene for species clarification. The rates of correct identification by ITS1 and ITS2 sequence analysis were 96.8% (361/373) and 99.7% (372/373), respectively. Of the 373 strains tested, only 1 strain (*Rhodotorula glutinis* BCRC 20576) could not be identified by ITS2 sequence analysis. In conclusion, identification of medically important yeasts by ITS sequencing, especially using the ITS2 region, is reliable and can be used as an accurate alternative to conventional identification methods.

The incidence of fungal infections has increased in the past few decades. Invasive infections caused by yeasts have become a major cause of morbidity and mortality in patients receiving immunosuppressive chemotherapy for cancer or organ transplantation or in immunodeficient patients, such as individuals with untreated AIDS (4, 13, 49). *Candida albicans* is the most common species causing a variety of infections. However, the incidence rates of non-*C. albicans Candida* infections have been increasing in recent years (46, 47). Moreover, outbreaks of systemic infections caused by yeasts in neonatal intensive care units have been described (6, 12, 20, 27, 36, 38). Recently, infections caused by less common yeast species such as *Pichia*, *Rhodotorula*, *Trichosporon*, and *Saccharomyces* spp. and other rarely encountered species have been reported (2, 16, 31, 44, 49, 51). More than 100 yeast species have been identified as human pathogens and have been isolated from virtually all body sites (14). Identification of the increasing diversity of pathogens by conventional methods may be difficult and sometimes inconclusive (34), especially for unusual yeast species.

The susceptibilities of different species to antifungal agents may be different (32, 52), and reliable identification of yeasts is helpful for treatment with appropriate antifungal agents. Commercially available biochemical and enzymatic panels, such as API ID32C (bioMérieux, Marcy-l'Etoile, France) and VITEK ID-YST (bioMérieux Vitek, Hazelwood, Mo.), are convenient for use. However, the disadvantages of limited databases (33) and misidentification using these kits (7, 24, 30) have been reported.

Molecular approaches have been developed to provide more rapid and accurate identification of fungi compared to traditional phenotypic methods. The internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions of the rRNA gene operon have been used extensively for PCR-based systems for detection and identification of fungal pathogens in a variety of formats. These methods include PCR (24, 26), ITS fragment length polymorphism (3, 4, 46), restriction fragment length polymorphism (15, 28, 45), DNA probe hybridization (7, 10, 25, 29, 48), and DNA sequencing. Among these molecular methods, ITS sequence analysis has been proven to be an accurate method for species delineation (4, 5, 18, 19, 21, 39, 40). However, until now only a limited number of species or just a specific genus has been evaluated for species identification by ITS sequence analysis (4, 19, 39, 40).

For ITS sequence analysis, several questions remain to be answered: (i) is only the ITS1 or ITS2 sequence enough for yeast identification? (ii) is the ITS2 sequence more species specific than ITS1 or vice versa? (iii) can this approach be applied to most species of clinical importance? The aim of this study was to clarify these questions by testing 373 yeast strains from 86 species including type strains, reference strains, and clinical isolates.

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TABLE 1. ITS sequences of 15 yeast species submitted to GenBank

Species	Strain no.[a]	GenBank accession no.	
		ITS1	ITS2
Arthroascus schoenii	BCRC 22503^T	AY936497	AY936498
Candida boidinii	BCRC 20464^T	AY936499	AY936500
Candida cantarellii	CBS 5383	AY936505	AY936506
Candida ciferrii	BCRC 22168^T	AY936511	AY936512
Candida globosa	CBS 162^T	AY936513	AY936514
Candida inconspicua	BCRC 21658^T	AY936515	AY936516
Candida kruisii	BCRC 21573^T	AY936517	AY936518
Candida maltosa	BCRC 21614^T	AY936521	AY936522
Candida melibiosica	CBS 5814^T	AY936523	AY936524
Candida norvegica	BCRC 21616^T	AY936525	AY936526
Candida santamariae	BCRC 21617^T	DQ066653	DQ066654
Candida silvicola	CBS 4140^T	AY936529	AY936530
Candida steatolytica	BCRC 21746^T	AY936531	AY936532
Candida utilis	BCRC 20928^T	AY936535	AY936536
Kloeckera japonica	CBS 2590	AY936537	AY936538

[a] T, type strain.

MATERIALS AND METHODS

Yeast strains. A total of 373 strains from 86 species, including 41 *Candida* spp. (207 strains) and 45 non-*Candida* spp. (166 strains), were used in this study (Table 1). Among these yeasts, 299 were reference (or type) strains and 74 were clinical isolates. All reference strains were obtained from the Bioresources Collection and Research Center (BCRC; Hsinchu, Taiwan) and Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands). Clinical isolates were obtained from the Mycology Reference Centre, Department of Microbiology, University of Leeds (Leeds, United Kingdom), the Laboratory of Parasitology and Mycology of Angers University Hospital (Angers, France), and the National Cheng Kung University Medical Center (Tainan, Taiwan). Isolates were identified to the species level based on traditional criteria (17) with the API ID32C system (bioMérieux Vitek).

DNA preparation. Yeasts were subcultured on Sabouraud dextrose agar (Difco, Detroit, Mich.) and incubated at 28°C for 24 to 48 h. Colonies of these strains were suspended in saline to obtain a turbidity of 0.5 McFarland standard at a 530-nm wavelength. One milliliter of the cell suspension was centrifuged at 5,000 × *g* for 3 min in a microcentrifuge. The genomic DNA was extracted by using the Blood and Tissue Genomic DNA Extraction Miniprep system (Viogene, Taipei, Taiwan) in accordance with the manufacturer's instructions, except that the step of lyticase digestion of yeast cells was omitted. The extracted DNA was stored at −20°C for further use.

Amplification and sequencing of the ITS regions. The fungus-specific universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS2 (5′-GCATCGATGAAGAACGCAGC-3′) were used to amplify the ITS1 region, while universal primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-GCATATCAATAAGCGGAGGA-3′) were used to amplify the ITS2 region (50). PCR was performed in a total reaction volume of 50 μl consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphates (0.2 mM each), 1.2 U of *Taq* DNA polymerase, 0.4 μM (each) of the ITS1 region primers (ITS1/ITS2) or the ITS2 region primers (ITS3/ITS4), 2 μl (1 to 5 ng) of DNA template, and 50 μl of a mineral oil overlay. PCR was carried out using the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min), and extension (72°C for 1 min); and a final extension step at 72°C for 3 min. A negative control was performed with each run by replacing the template DNA with sterile water in the PCR mixture.

All amplicons were purified using the PCR-M Clean Up System (Viogene, Taipei, Taiwan). The DNA fragments were sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Taipei, Taiwan) with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems). All amplicons were sequenced on both strands using primers ITS1 and ITS2 for the ITS1 region and primers ITS3 and ITS4 for the ITS2 region. After sequencing, portions of the 18S, 5.8S, and 26S rRNA gene sequences of the PCR products were removed to obtain the exact ITS1 and ITS2 sequences. For all yeasts, the sequences of the 3′ ends of the 18S and 5.8S rRNA genes were GCGGAAGGATCATTA and GTTTGAGCGTCATTT, respectively, and the sequences of the 5′ ends of the 5.8S and 26S rRNA genes were AAACTTTCAACAA and GACCTCAAATCAG, respectively. Since the ITS sequences of 15 of the yeast species examined in this study are not currently available in the GenBank database, the ITS sequences of the type strain (or a reference strain) of each of these yeasts were submitted to GenBank (Table 1) to facilitate sequence comparison of strains belonging to these species.

Identification of yeast by ITS sequencing. A total of 373 strains (86 species) including 41 *Candida* species (207 strains) and 45 non-*Candida* species (166 strains) were examined. Species were identified by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/). The ITS1 or ITS2 sequence was compared using nucleotide-nucleotide BLAST (blastn) with default settings except that sequences were not filtered for low complexity. Species identification was determined from the lowest expect value of the BLAST output. Occasionally, the BLAST search with the query sequence hit sequences from two different species with 100% identity. Under these conditions, the lengths of ITS1 and ITS2 were taken into consideration for species identification, since the lengths of both the ITS1 and ITS2 fragments are important characteristics of a fungal species (3, 4, 30, 46).

For strains producing discrepant identification between the methods based on phenotypic characteristics and ITS sequence analysis, the D1–D2 region of the large-subunit RNA gene was sequenced for species clarification. Primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) (22) were used to amplify this region. The procedures for PCR amplification, PCR product purification, and sequencing of the PCR products were the same as those described for the ITS regions.

Nucleotide sequence accession numbers. The GenBank accession numbers of the ITS1 and ITS2 regions of type (or reference) strains of 15 species sequenced in this study are given in Table 1.

RESULTS

Amplification of ITS regions. Both the ITS1 and ITS2 regions were successfully amplified from DNA from all strains by the fungus-specific universal primer pairs ITS1–ITS2 and ITS3–ITS4, respectively. The lengths of ITS1 ranged from 59 bp (*Candida lipolytica*) to 402 bp (*C. glabrata*), while the lengths of ITS2 ranged from 69 bp (*C. haemulonii*) to 261 bp (*C. colliculosa*) (data not shown). The ITS fragments of all species studied were less than 300 bp. However, the ITS1 fragments of *C. glabrata* (402 bp) and *Saccharomyces cerevisiae* (366 bp) were longer than 300 bp.

Identification of reference strains by ITS sequence analysis. A total of 299 reference strains including 41 *Candida* species (146 strains) and 45 non-*Candida* species (153 strains) were analyzed (Table 2). By ITS1 sequence analysis, 290 strains (97%) were correctly identified to species level. Reference strains producing discrepant identification by phenotypic characteristics and ITS sequence analysis are listed in Table 3. Nine strains (*C. intermedia* BCRC 22567, *C. melibiosica* CBS 6211, *C. silvicola* [CBS 4069 and 4141], *Pichia ohmeri* [BCRC 21592, 22556, and 22557], *Cryptococcus albidus* CBS 969, and *Rhodotorula glutinis* BCRC 20576) were not identified by ITS1 sequencing. In the ITS1 regions, *C. intermedia* BCRC 22567, *C. melibiosica* CBS 6211, and *Pichia ohmeri* (BCRC 21592, 22556, and 22557) had sequence similarities of only 0.78, 0.65, and 0.76 to 0.89, respectively, with their corresponding type strains *C. intermedia* BCRC 21250, *C. melibiosica* CBS 5814, and *Pichia ohmeri* BCRC 22178 (listed in Tables 1 and 2). However, other strains of *C. intermedia* (BCRC 20863, 21250, and 21604) and *Pichia ohmeri* (BCRC 21349 and 22178) (Table 1) were correctly identified by ITS1 sequence analysis.

A BLAST search revealed that *C. silvicola* (CBS 4069 and 4141), in addition to its own species, had an ITS1 sequence and length (199 bp) identical with those of the type strain of *C. ernobii* CBS 1737 (GenBank accession no. AY585212) (Table 3). The output of the BLAST search of the ITS1 sequence of

TABLE 2. Yeast species and strains used in this study

Microorganism	Reference strain(s)	Clinical isolate(s)	Total no. of strains
Ascomycetous yeast			
Arthroascus schoenii	BCRC 21401, 22503^T, 22504, CBS 2556		4
Candida albicans	BCRC 20511, 20512^T, 20513, 21538, 22063	LMA 938838, 962507, 499010350, ATTC 66390, RB 1325, 1326, 1331	12
C. boidinii[a]	BCRC 20472, 21432, 21483, 21757		4
C. cantarellii	CBS 5445, 5654		2
C. catenulata	BCRC 21507, 22316^T, CBS 564, 565, 1904	LMA 954976	6
C. ciferrii	BCRC 22408		1
C. colliculosa	BCRC 21429, 22074^T, CBS 158, 6991		4
C. dattila	BCRC 22043, CBS 1877, 2803, 2860, 2907		5
C. dubliniensis	CBS 2747, 7987, 7988, 8500, 8501	RB 1168, 1271, 1306	8
C. famata	BCRC 22304, 22712, CBS 1791, 1792, 1795^T		5
C. freyschussii	BCRC 21555^T, CBS 2161		2
C. glabrata	BCRC 20586^T, CBS 860, 861, 2175, 7307	LMA 901085, 905756, 945574, RB 1284, 1295, 1324	11
C. globosa	CBS 864		1
C. guilliermondii	BCRC 20862, 21500^T, 21549, 21559	RB 1012, 1055, 1216	7
C. haemulonii	BCRC 21572^T, CBS 6590, 7801, 7802		4
C. holmii	BCRC 21524^T, 21999, 22000		3
C. inconspicua	CBS 990, 1735, 2833	LMA 90289, RB 1226	5
C. intermedia	BCRC 20863, 21250^T, 21604, 22567		4
C. kefyr	BCRC 20516, 20517, 21269, 21355, 22057^T	LMA 911323, 938657, 938779, 944459, 947644, RB 1227	11
C. krusei	BCRC 20514^T, 21321, 21720, 21796, 22342	LMA 911256, 945615, 948501, RB 1222, 1237, 1317	11
C. lambica	BCRC 21347, 22067^T, 22068, 22071, 22090	LMA 937990	6
C. lipolytica	BCRC 20864, 21541, 21542, 21596		4
C. lusitaniae	BCRC 20326, 21387^T, 21740, CBS 7270	LMA 932648, 947060, 947315, 948764, RB 1283, 1288, 1294	11
C. maltosa	BCRC 21327, 21482		2
C. melibiosica	CBS 6211		1
C. membranaefaciens	BCRC 21563, 22398^T, 22399		3
C. norvegensis	BCRC 21851, 22096^T, 22097, CBS 1911		4
C. norvegica	CBS 2670, 4737		2
C. parapsilosis	BCRC 20515^T, 20865, 21253, 21544	43, 770, 8053, C4-2, LMA 938558, 961299, RB 1318, 1320	12
C. pelliculosa	BCRC 20857, 20858, 21359, 21741, 22583^T	LMA 892971, RB 766, 778, 913	9
C. pintolopesii	BCRC 21439, 22002, 22003, 22239		4
C. rugosa	BCRC 21356, 21709^T	RB 1158	3
C. sake	BCRC 21621^T, CBS 5690, 5740		3
C. santamariae	BCRC 21562, CBS 4261, 4515^T		3
C. silvicola	CBS 4069, 4141		2
C. sphaerica	BCRC 21716, 22153, 22154, 22055, 22604		5
C. steatolytica	BCRC 22232, CBS 7652		2
C. tropicalis	BCRC 20520^T, 20521, 21436, 21437, 21560	LMA 9077, 921810, 945762, RB 1298, 1330	10
C. utilis	BCRC 20260, 20325, 20334, 20860, 21357		5
C. valida	BCRC 22069^T, 21399, 21441		3
C. viswanathii	BCRC 21330^T, 22554		2
C. zeylanoides	BCRC 21743^T, 21749, 22396, CBS 947	LMA 91304	5
Debaryomyces marama	BCRC 21526, CBS 1958^T		2
Debaryomyces polymorphus	BCRC 21413		1
Dekkera bruxellensis	BCRC 20932, 21414^T, 21440, 21517, 21518, 21519		6
Endomyces fibuligera	BCRC 20455, 21379, 21380, 21449, 21465, 21511^T		6
Hansenula saturnus	BCRC 20463, 21360, 21659, 21692, 21765		5
Kloeckera apiculata	BCRC 20539, 21362, CBS 312, 314, 2582		5
Kloeckera apis	BCRC 22105, 22106, 22112^T, CBS 4378		4
Kloeckera japonica	CBS 281, 479^T		2
Kluyveromyces delphensis	BCRC 22017, CBS 2170		2
Kluyveromyces yarrowii	BCRC 21747, 22822, CBS 2684, 8242		4
Lodderomyces elongisporus	BCRC 21390^T, CBS 2606, 5912		3
Pichia carsonii	BCRC 21529, 22098^T, CBS 4409, 5254		4
Pichia etchellsii	BCRC 21479^T, CBS 2012, 5519, 5603		4
Pichia farinosa	BCRC 21368^T, 21682, 21881		3
Pichia ohmeri	BCRC 21349, 21592, 22178^T, 22556, 22557		5
Pichia spartinae	BCRC 22766^T, CBS 6059, 6077, 6661		4
Saccharomyces cerevisiae	BCRC 20263, 20270, 20271, 20405, 20490, 21447^T	RB 1254, 1299	8

Continued on following page

TABLE 2—*Continued*

Microorganism	Reference strain(s)	Clinical isolate(s)	Total no. of strains
Saccharomyces kluyveri	BCRC 21498^T, 21977, 22001, CBS 2861		4
Zygosaccharomyces bisporus	BCRC 21505^T, 21725, 21726		3
Zygosaccharomyces cidri	BCRC 21728^T, CBS 2950, 5666		3
Zygosaccharomyces fermentati	BCRC 21433, 21760^T, 22453		3
Zygosaccharomyces florentinus	BCRC 21648^T, CBS 748, 6078, 6703		4
Basidiomycetous yeast			
Cryptococcus albidus	BCRC 20526, 21672^T, 21860, CBS 969	LMA 935479	5
Cryptococcus curvatus	BCRC 21735, CBS 570, 2744, 8770		4
Cryptococcus humicolus	CBS 5123		1
Cryptococcus daszewskae	BCRC 21639^T		1
Cryptococcus laurentii	BCRC 20527^T, 21997		2
Cryptococcus luteolus	BCRC 22372^T		1
Cryptococcus neoformans	BCRC 20528^T, 20532, 22241, 22873, 22874, 22875, CBS 883, 919, 1622, 6955, 6997	LMA 94277, 925461, 935479, 957786, 959159, 49800123	17
Cryptococcus uniguttulatus	CBS 1727, 1730, 2770, 2994, 4257		5
Rhodotorula glutinis	BCRC 20576, 21418^T		2
Rhodotorula minuta	BCRC 22482, 22483, 22484, 22485		4
Rhodotorula rubra	BCRC 21442, 21667^T, 21712, 21713, 21770		5
Sporobolomyces roseus	BCRC 22375		1
Sporobolomyces salmonicolor	CBS 490, 4474		2
Trichosporon aquatile	BCRC 22271^T, 22272, CBS 5973, 5988		4
Trichosporon asahii	CBS 2479, 2936, 4829, 7631		4
Trichosporon cutaneum	BCRC 21675^T, 22273	LMA 94117, 94256, 931422, 931440	6
Trichosporon debeurmannianum	CBS 1896		1
Trichosporon dermatis	CBS 2043		1
Trichosporon inkin	BCRC 21503^T, CBS 7613, 7629, 7655		4
Trichosporon jirovecii	CBS 6864, 6950		2
Trichosporon mucoides	CBS 7625^T		1
Trichosporon pullulans	BCRC 22275, 22313, 22318^T, CBS 2543		4
Total no. of strains	299	74	373

[a] Three strains of *Candida boidinii* (BCRC 20472, 21432, and 21483) required assessment of the ITS1 and ITS2 lengths for accurate identification.

Cryptococcus albidus CBS 969 also showed 100% sequence identity with *Cryptococcus albidus* CBS 1925 (GenBank accession no. AB051041) and *Cryptococcus adeliensis* CBS 8351 (GenBank accession no. AF145328). ITS1 sequencing revealed that *Rhodotorula glutinis* BCRC 20576, in addition to its own species, shared an identical sequence with *Rhodosporidium babjevae* CBS 7808 (GenBank accession no. AF444542). Therefore, these four strains (*C. silvicola* CBS 4069 and 4141, *Cryptococcus albidus* CBS 969, and *Rhodotorula glutinis* BCRC 20576) could not be unambiguously identified by ITS1 sequence analysis. However, other strains of *Cryptococcus albidus* (BCRC 20527, 21672, and 21860) and *Rhodotorula glutinis* (BCRC 21418) were correctly identified by ITS1 sequence analysis.

By ITS2 sequencing, 99.7% (298/299) of reference strains were correctly identified. In contrast to the ITS1 region, a BLAST search of the ITS2 sequences of *Cryptococcus albidus* CBS 969 matched only the ITS2 sequence (100% identity) of *Cryptococcus adeliensis* CBS 8351 (GenBank accession no. AF145328). Sequence comparison of the D1–D2 region also revealed that *Cryptococcus albidus* CBS 969 had 100% sequence identify with *Cryptococcus adeliensis* (GenBank accession no. AF137603). Since *Cryptococcus albidus* CBS 969 had identical sequences with *Cryptococcus adeliensis* CBS 8351 at the ITS1, ITS2, and D1–D2 regions, CBS 969 might be a misidentification of *Cryptococcus adeliensis*.

In the ITS2 region, sequence analysis of *Rhodotorula glutinis* BCRC 20576, in addition to its own species, revealed 100% identity with *Rhodosporidium babjevae* JCM 9283 (GenBank accession no. AB073235). Strain BCRC 20576 also had 100% sequence identity with *Rhodosporidium babjevae* A130 (GenBank accession no. AF485991) and *Rhodotorula graminis* KCTC 17088 (GenBank accession no. AF459705) in the D1–D2 domain. Thus, *Rhodotorula glutinis* BCRC 20576 could not be differentiated from *Rhodosporidium babjevae* in both the ITS1 and ITS2 regions and could not be differentiated from *Rhodosporidium babjevae* and *Rhodotorula graminis* in the D1–D2 domain. In summary, of the 299 reference strains tested, 290 (97%) and 298 (99.7%) strains were correctly identified by sequence analysis of the ITS1 and ITS2 regions, respectively. No strain was misidentified by sequence analysis of either the ITS1 or the ITS2 region.

Identification of clinical isolates by ITS sequence analysis. A total of 74 clinical isolates, including 15 *Candida* species (61 strains) and 4 non-*Candida* species (13 strains), were analyzed. Three isolates (*Candida lusitaniae* LMA 948764, RB 1283, and *C. pelliculosa* LMA 892971) were not identified by ITS1 sequencing, since there were no matching sequences in the GenBank database. The identification of *Candida lusitania*e LMA 948764 and RB 1283 was correct, as evidenced by sequencing of the ITS2 and D1–D2 regions. *C. pelliculosa* LMA 892971 was a misidentification of *Pichia fabianii* (GenBank accession no. AF335967), as confirmed by sequences of the ITS2 and D1–D2 regions (Table 3).

C. dubliniensis RB 1168, *C. guilliermondii* RB 1055, *C. in-*

TABLE 3. List of reference strains and clinical isolates that produced discrepant identification by phenotypic characteristics and ITS sequence analysis

Strain no.	Species received as	Species identification (% identity with sequences in GenBank) by:		
		ITS1 sequence	ITS2 sequence	D1–D2 sequence
Reference strains				
BCRC 22567	*C. intermedia*	NI[a]	*C. intermedia* (100)	*C. intermedia* (99)
CBS 6211	*C. melibiosica*	NI	*C. melibiosica* (100)	*C. melibiosica* (100)
CBS 4069	*C. silvicola*	*C. silvicola* (100) *C. ernobii* (100)	*C. silvicola* (100)	*C. silvicola* (99)
CBS 4141	*C. silvicola*	*C. silvicola* (100) *C. ernobii* (100)	*C. silvicola* (100)	*C. silvicola* (99)
BCRC 21592	*Pichia ohmeri*	NI	*P. ohmeri* (100)	*P. ohmeri* (100)
BCRC 22556	*Pichia ohmeri*	NI	*P. ohmeri* (100)	*P. ohmeri* (100)
BCRC 22557	*Pichia ohmeri*	NI	*Pichia ohmeri* (100)	*Pichia ohmeri* (100)
CBS 969	*Cryptococcus albidus*	*Cryptococcus adeliensis* (100) *Cryptococcus albidus* (100)	*Cryptococcus adeliensis* (100)	*Cryptococcus adeliensis* (100)
BCRC 20576	*Rhodotorula glutinis*	*Rhodotorula glutinis* (100) *Rhodosporidium babjevae* (100)	*Rhodosporidium glutinis* (100) *Rhodosporidium babjevae* (100)	*Rhodotorula glutinis* (100) *Rhodosporidium babjevae* (100) *Rhodotorula graminis* (100)
Clinical isolates				
RB 1168	*C. dubliniensis*	*C. albicans* (100)	*C. albicans* (100)	*C. albicans* (100)
RB 1055	*C. guilliermondii*	*C. parapsilosis* (100)	*C. parapsilosis* (100)	*C. parapsilosis* (100)
LMA 90289	*C. inconspicua*	*C. krusei* (100)	*C. krusei* (99)	*C. krusei* (99)
RB 1226	*C. inconspicua*	*C. glabrata* (100)	*C. glabrata* (100)	*C. glabrata* (100)
RB 1237	*C. krusei*	*Pichia norvegensis* (99)	*P. norvegensis* (99)	*P. norvegensis* (99)
LMA 948764	*C. lusitaniae*	NI	*C. lusitaniae* (100)	*C. lusitaniae* (99)
RB 1283	*C. lusitaniae*	NI	*C. lusitaniae* (100)	*C. lusitaniae* (99)
LMA 892971	*C. pelliculosa*	NI	*Pichia fabianii* (100)	*Pichia fabianii* (100)
RB 1158	*C. rugosa*	*Saccharomyces cerevisiae* (99)	*S. cerevisiae* (99)	*S. cerevisiae* (100)
LMA 935479	*Cryptococcus albidus*	*Cryptococcus neoformans* (100)	*Cryptococcus neoformans* (100)	*Cryptococcus neoformans* (100)
LMA 94117	*Trichosporon cutaneum*	*Trichosporon dermatis* (100)	*T. dermatis* (100)	*T. dermatis* (100) *T. mucoides* (100)
LMA 94256	*Trichosporon cutaneum*	*Trichosporon dermatis* (100)	*T. dermatis* (100)	*T. dermatis* (100) *T. mucoides* (100)
LMA 931422	*Trichosporon cutaneum*	*Trichosporon dermatis* (100)	*T. dermatis* (100)	*T. dermatis* (100) *T. mucoides* (100)
LMA 931440	*Trichosporon cutaneum*	*Trichosporon dermatis* (100)	*T. dermatis* (100)	*T. dermatis* (100) *T. mucoides* (100)

[a] NI, not identified.

conspicua LMA 90289, *C. inconspicua* RB 1226, *C. krusei* RB 1237, *C. rugosa* RB 1158, *Cryptococcus albidus* LMA 935479, and *Trichosporon cutaneum* (LMA 94117, 94256, 931422, and 931440) were misidentifications of *C. albicans*, *C. parapsilosis*, *C. krusei*, *C. glabrata*, *Pichia norvegensis*, *Saccharomyces cerevisiae*, *Cryptococcus neoformans*, and *Trichosporon dermatis*, respectively, as revealed by sequence analysis of the ITS1, ITS2, and D1–D2 regions. However, in addition to *Trichosporon dermatis* (GenBank accesssion no. AY143555), a BLAST search of the D1–D2 sequences of *Trichosporon cutaneum* (LMA 94117, 94256, 931422, and 931440) revealed 100% identity with an additional sequence of *Trichosporon mucoides* CBS 7625 (GenBank accession no. AF075515). If the species names of those unidentified or misidentified clinical isolates were corrected according to their D1–D2 sequences, the identification rates of clinical isolates by ITS1 and ITS2 sequencing were 95.9% (71/74) and 100% (74/74), respectively. No misidentification of clinical isolates was caused by sequence analysis of either the ITS1 or the ITS2 region. If reference strains and clinical isolates were taken together, the identification rates were 96.8% (361/373) and 99.7% (372/373), respectively, by sequence analysis of the ITS1 and ITS2 regions.

DISCUSSION

In this study, the feasibility of using ITS sequencing for identification of clinically important yeasts was demonstrated. The whole procedure could be completed within 24 h from isolated colonies. With identification rates of 96.8% (ITS1) and 99.7% (ITS2), the present approach provides an accurate alternative for species delineation of clinically important yeasts. An important finding of this study was that the ITS2 sequence seems to be more species specific than the ITS1 sequence, and almost all clinically relevant species could be identified by using the ITS2 region alone, providing the ITS2 sequences corresponding to the unknown species are in the GenBank database.

In this study, it was found that the intraspecies sequence divergence of ITS1 is higher than that of ITS2. For example, of the four reference strains of *C. intermedia* tested, three were correctly identified while the remaining strain (BCRC 22567) was not identified by ITS1 sequencing (Tables 2 and 3). In addition, three reference strains of *Pichia ohmeri* (BCRC 21592, 22556, and 22557) not identified by ITS1 sequencing were accurately identified by their ITS2 sequences. Furthermore, although all seven clinical isolates of *C. lusitaniae* were unambiguously identified by ITS2 sequencing, two isolates (LMA 948764 and RB 1283) were not identified by ITS1 sequence analysis (Tables 2 and 3). It should be noted that the ITS1 sequence may not be sufficiently sensitive for identifying some species.

Cryptococcus albidus CBS 969 might be a misidentification of *Cryptococcus adeliensis*, since *Cryptococcus albidus* CBS 969 had 100% sequence identity with *Cryptococcus adeliensis* CBS 8351 in the ITS1, ITS2, and D1–D2 regions (Table 3). Multiple sequence alignment demonstrated that the nucleotides at positions 203 and 204 in the ITS2 region of *Cryptococcus albidus*

are C and G (GenBank accession no. AB051026, AB051037, AB051040, and AB051042 to AB051044), respectively, whereas the two nucleotides are A and C, respectively, in strains of *Cryptococcus adeliensis* (AF145328, AY733078, and AY733079). Therefore, these two positions in the ITS2 regions could be used as a signature sequence to differentiate the two species. Rimek et al. (35) recently published an account of the first case of meningitis caused by *Cryptococcus adeliensis* in a patient with acute myeloid leukemia. *Cryptococcus adeliensis* can be misidentified as *Cryptococcus albidus* due to the high variability of phenotypic markers of the latter. Tintelnot and Losert (43) reexamined six isolates from their collection originally identified as *Cryptococcus albidus* and found that three of the six strains in fact turned out to be *Cryptococcus adeliensis*.

Molecular approaches are now being developed to provide a more rapid and objective identification of yeasts compared to traditional phenotypic methods. Ribosomal targets, especially the ITS1, ITS2, and D1–D2 domains of the RNA operon, have shown particular promise for molecular identification. At present, the sequences of the D1–D2 regions of almost all yeasts, including nonpathogenic species, have been determined (11, 22, 23). Analysis of ITS sequences has been carried out mainly for pathogenic yeast species (7, 8, 15, 24, 30, 39). It should be noted that four clinical isolates of *Trichosporon cutaneum* (LMA 94117, 94256, 931440, and 931442) were clearly identified as *Trichosporon dermatis* by either ITS1 or ITS2 sequencing (Table 3); however, the four isolates could not be identified as *Trichosporon dermatis* by sequence analysis in the D1–D2 domain. A BLAST search revealed that the four *Trichosporon cutaneum* isolates had 100% sequence identity with *Trichosporon mucoides* (GenBank accession no. AF075515) and *Trichosporon dermatis* in the D1–D2 domain. In this special case, sequence analysis of both ITS1 and ITS2 is more specific for identification of *Trichosporon dermatis* than sequence analysis of the D1–D2 region.

An additional advantage of using ITS sequences for yeast identification is length polymorphisms among different species (3, 4, 30, 46). In this study, an interesting example was the fact that a BLAST search revealed that three *C. boidinii* strains (BCRC 20472, 21432, and 21483) had 100% sequence identities with *Pichia norvegensis* ATCC 22977 (GenBank accession no. AF333096) (data not shown). However, the ITS1 and ITS2 lengths of *C. boidinii* were 268 and 189 bp, respectively, while the ITS1 and ITS2 lengths of *Pichia norvegensis* were 108 and 142 bp, respectively. For this reason, *C. boidinii* could be easily differentiated from *Pichia norvegensis*.

The GenBank database currently lacks ITS sequence entries for some yeast species. However, the number of ITS sequences available in public databases has increased rapidly in recent years, and the expanding database may improve the quality and accuracy of fungal identification (19). In this study, the ITS sequences (30 entries) of type (or reference) strains of 15 species were submitted to GenBank (Table 1) to facilitate sequence comparison of these species. As mentioned above, the ITS1 sequence has higher intraspecies divergence than the ITS2 sequence for some species. We also found that different groups of *C. parapsilosis* had divergent ITS1 sequences (data not shown) as previously reported (37), and this may explain why a relatively lower identification rate was obtained by ITS1 sequence analysis. However, through a multilocus (*COX3*, *SADH*, and *SYA1*) sequence typing scheme (42) and the fact that DNA sequence similarities were <90% in the ITS1 sequence, *C. orthopsilosis* and *C. metapsilosis* were proposed to replace the existing designations of *C. parapsilosis* groups II and III, respectively. The species *C. parapsilosis* is retained for group I isolates. It is anticipated that as more entries of ITS1 sequences become available in public databases, the accuracy of yeast identification based on ITS1 sequences will increase.

Phylogenetically closely related species sometimes cannot be identified by sequence analysis of both the ITS and D1–D2 regions (Table 3). In this study, it was found that *Rhodotorula glutinis* BCRC 20576 could not be differentiated from *Rhodosporidium babjevae* in the ITS1, ITS2, and D1–D2 regions. Recently, the intergenic spacer (IGS) region of the fungal rRNA operon was found to have high intraspecies sequence divergence, which might be useful for genotyping of *Malassezia globosa* (41) and *Trichosporon asahii* (40) and for differentiation of varieties of *Cryptococcus neoformans* (9). However, due to the high intraspecies divergence in the IGS region, this region may not be suitable for yeast identification.

Many uncommon yeasts are emerging as human pathogens, and their identification may pose a challenge. A rapid and reliable identification method is urgently needed as the incidence of fungal infections increases (49). The API ID32C strips or Vitek YBC cards are commonly used for yeast identification. The VITEK system has the advantage of speed (a 15-h incubation) compared to ID32C (a 48- to 72-h incubation). The ID32C kit has a relatively large database (1) for 69 species; however, additional tests are needed for species confirmation of 11 of the 69 species. With the advanced technology available for sequence analysis and with open access to sequence databases, DNA sequence analysis for microorganism identification is expected to become more popular in the future. In conclusion, the present results clearly demonstrate that sequence analysis of the ITS regions (especially ITS2) can reliably identify yeasts of clinical importance. The method is straightforward and can be completed within 24 h from isolated colonies.

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