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Rapid identification of fungi by sequencing the ITS1 and ITS2 regions using an automated capillary electrophoresis system

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We developed a standardized DNA sequence-based approach for the accurate and timely identification of medically important fungi by sequencing polymerase chain reaction (PCR) products with a rapid automated capillary electrophoresis system. A simple DNA extraction method and PCR amplification using universal fungal primers was used to amplify ribosomal DNA from a range of clinical isolates and reference strains. The entire internal transcribed spacer (ITS) 1-5.8S-ITS2 ribosomal DNA region was sequenced using automated dye termination sequencing for 89 clinical isolates. These had previously been identified by traditional methods and included 12 ascomycetous yeast species, three basidiomycetous yeast species, eight dermatophyte species and two thermally dimorphic fungi, *Scedosporium prolificans* and *S. apiospermum*. Furthermore, 21 reference strains representing 19 different *Candida* species, *Geotrichum candidum* and *Malassezia furfur* were also sequenced as part of this study and were used either as standards for sequence-based comparisons, or as assay controls. Sequence-based identification was compared to traditional identification in a blinded manner. Of the clinical isolates tested, 88/89 had DNA sequences that were highly homologous to those of reference strains accessioned in GenBank, and 87/89 gave a sequence-based identification result that correlated with the traditional identification. In contrast to relatively slow conventional methods of identification, a sequence-based identification from a pure culture can be obtained within 24 h of a DNA extraction carried out after a minimal period of culture growth. We conclude that this approach is rapid, and may be a more accurate cost-effective alternative than most phenotypic methods for identification of many medically important fungi frequently encountered in a routine diagnostic microbiology laboratory.

Keywords DNA sequencing, identification, ITS regions

Introduction

Fungi, all but a few species of which were once considered to be microbiological curiosities, have increasingly emerged as human pathogens capable of causing life-threatening disease, particularly in immunocompromised and other high-risk patient groups [1]. Rapid and accurate identification of fungi is essential for guiding early appropriate therapy. Many clinically important fungi, however, may take weeks to grow in the laboratory [2]. Identification of many fungi can be time consuming and complex, and can require the use of a wide range of specialized laboratory culture media [3,4]. Traditional identification methods for dermatophytes may take many weeks, are labour intensive, may lack specificity, and require experienced personnel to identify less commonly encountered pathogens or

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variant strains [5]. Traditional methods of reference yeast identification, based on carbohydrate assimilation and fermentation tests, are cumbersome and not suitable for the non-specialized clinical microbiology laboratories [6]. Rapid kits and automated identification systems have been developed but are often unreliable and may take up to 7 days for a final result [7,8].

With the emergence of fungi that are resistant to many of the antifungal drugs available, rapid species-level identification of significant clinical isolates is important, as delays in the initiation of appropriate therapy often correlate with poor outcomes [2,6,9]. It has therefore become essential to have rapid and accurate methods for identification of fungi that can easily be implemented in a routine diagnostic microbiology laboratory. Current diagnostic methods to identify many clinically important fungi combine morphological criteria using identification keys and physiological tests with molecular diagnostics [10]. Molecular methods are increasingly being utilized to aid traditional identification and to study the phylogeny of many clinically important fungi [11–20]. These studies have identified ribosomal DNA (rDNA) sequence information for a variety of fungal species and analysis of this region has been used as the basis for the organization of fungi into taxonomic groups. The intervening internal transcribed spacer (ITS) regions have become important molecular targets for taxonomy and identification [21]. Due to greater sequence variation, the ITS1/ITS2 domains are more suited for species and strain identification than the 18S region (small subunit), the 5.8S region and the 28S region (large subunit) [21]. Several groups have developed methods utilizing the ITS regions to identify species and strains of a range dermatophytes, yeasts and moulds [17,22–26]. Despite the variety of methods described, most have limited applicability for routine use in a clinical laboratory due to the use of tedious DNA extractions and labour-intensive blotting techniques using species-specific digoxigenin or radiolabelled probes. Useful sequence-based approaches have been reported to identify fungi rapidly [27,28] and show great promise. However, most reports identify a limited range of fungi within a genus, use GenBank ITS sequences that are incomplete or use GenBank records that have been derived from a non-referenced culture.

In this report, we describe the development of a fungal identification strategy based on direct sequence analysis of amplified rDNA using an automated capillary electrophoresis system. The ITS1-5.8S-ITS2 region from reference strains and clinical isolates from members of the genus *Candida*, members of the family Arthrodermataceae (dermatophytes) and other medically important fungi were amplified, sequenced and compared with reference strain sequences in GenBank. Sequence-based identification of 89 clinical isolates previously identified by traditional methods was evaluated in a blinded study. The validity of this approach for rapid and accurate identification of a variety of clinically important fungi in routine diagnostic microbiology is reported.

Materials and methods

Fungal strains

Referenced fungal organisms used as controls for this study were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA and the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Clinical isolates used in this study were obtained from the Royal Perth Hospital Mycology Culture Collection (RPMCC), Department of Microbiology and Infectious Diseases, Royal Perth Hospital, Perth, Australia. All clinical isolates and controls were previously identified by colony characteristics, microscopic morphology and physiological testing, using identification keys [10] and specialized handbooks [29–31]. Where possible, commercially available systems to identify yeasts were also used. All clinical isolates and controls are shown in Table 1. Furthermore, a variety of CBS type strains were sequenced for this study and used as standards for DNA sequence comparisons (Table 2).

Identification of ascomycetous and basidiomycetous yeasts

The germ tube broth was prepared and performed from direct culture for all presumptive yeast isolates. Typical *Candida albicans* isolates, identified by a positive germ tube test, were not evaluated in this study, because presumptive identification of *C. albicans* (in a manner not excluding the uncommonly encountered *C. dubliniensis*) using the germ-tube test is simple and cost-effective. However, germ-tube-positive yeasts isolated from the oral cavity of HIV-positive patients, from bloodstream infections of neutropenic patients and from patients with other immune disorders were further identified to exclude *C. dubliniensis*. A dark-green appearance on CHROMagar Candida (CHROMagar Company, Paris, France), inhibited growth at 45 °C, and the morphological characteristics seen on corn-meal/Tween 80 (CMAT) were used to identify *C. dubliniensis*. Common clinical ascomycetous yeasts (*Saccharomyces cerevisiae*, *Geotrichum candidum*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, *C. kefyr*, *C. famata*) that were germ

Table 1 Clinical isolates and controls analysed in the study

Organism and conventional identification*	Strain no.	Source of isolate	rDNA sequence-based identification results†		
			Sequence-based identification	GenBank accession no.	Reference source
Ascomycetous yeasts					
Candida species					
C. albicans	Control 14053	ATCC 14053	*C. albicans*	AF217609	ATCC 28516
C. albicans (germ tube negative)	RPMCC 2031	Clinical isolate	*C. albicans*	AF217609	ATCC 28516
C. dubliniensis	RPMCC 8357	Clinical isolate	*C. dubliniensis*	AB049123	CBS 7987
C. dubliniensis	RPMCC 1075	Clinical isolate	*C. dubliniensis*	AB049123	CBS 7987
C. famata (*Debaryomyces hansenii* var. *hansenii*)	Control 1962	CBS 1962	*D. hansenii*	AF210327	CBS 767
C. famata (*D. hansenii*)	RPMCC 8165	Clinical isolate	*D. hansenii*	AF210326	CBS 789
C. glabrata	Control 2238	ATCC 2238	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 9700	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 9703	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 9162	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 4635	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 6240	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 5779	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 4759	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 0935	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 9223	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 3217	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 1041	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 0130	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 8378	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. glabrata	RPMCC 5435	Clinical isolate	*C. glabrata*	AF167993	ATCC15545
C. guilliermondii (*Pichia guilliermondii*)	RPMCC 5150	Clinical isolate	*P. guilliermondii*	AB054109	JCM 10735
C. guilliermondii (*P. guilliermondii*)	RPMCC 9646	Clinical isolate	*P. guilliermondii*	AB054109	JCM 10735
C. intermedia	RPMCC 2222	Clinical isolate	*C. intermedia*	Authors' data	CBS 572
C. kefyr (*Kluyveromyces marxianus*)	RPMCC 2951	Clinical isolate	*K. marxianus*	AJ401699	CBS 4857
C. kefyr (*K. marxianus*)	RPMCC 0901	Clinical isolate	*K. marxianus*	AJ401699	CBS 4857
C. krusei (*Issatchenkia orientalis*)	Control 6528	ATCC 6528	*I. orientalis*	AF246989	ATCC 6258
C. krusei (*I. orientalis*)	RPMCC 1726	Clinical isolate	*I. orientalis*	AF246989	ATCC 6258
C. krusei (*I. orientalis*)	RPMCC 6136	Clinical isolate	*I. orientalis*	AF246989	ATCC 6258
C. krusei (*I. orientalis*)	RPMCC 2106	Clinical isolate	*I. orientalis*	AF246989	ATCC 6258
C. krusei (*I. orientalis*)	RPMCC 1609	Clinical isolate	*I. orientalis*	AF246989	ATCC 6258
C. krusei (*I. orientalis*)	RPMCC 8980	Clinical isolate	*I. orientalis*	AF246989	ATCC 6258
C. krusei (*I. orientalis*)	RPMCC 2409	Clinical isolate	*I. orientalis*	AF246989	ATCC 6258
C. parapsilosis	Control 22019	ATCC 22019	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 3185	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 2897	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 8359	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 7653	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 5108	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 8632	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 2150	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 9373	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 0770	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 0979	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. parapsilosis	RPMCC 1812	Clinical isolate	*C. parapsilosis*	AF287909	ATCC 22019
C. tropicalis	Control 750	ATCC 750	*C. tropicalis*	AF287910	ATCC 750
C. tropicalis	RPMCC 6961	Clinical isolate	*C. tropicalis*	AF287910	ATCC 750
C. tropicalis	RPMCC 1670	Clinical isolate	*C. tropicalis*	AF287910	ATCC 750
C. tropicalis	RPMCC 0998	Clinical isolate	*C. tropicalis*	AF287910	ATCC 750
C. tropicalis	RPMCC 4936	Clinical isolate	*C. tropicalis*	AF287910	ATCC 750
C. tropicalis	RPMCC 3412	Clinical isolate	*C. tropicalis*	AF287910	ATCC 750
Geotrichum species					
G. candidum (*Galactomyces geotrichum*)	Control 772.71	CBS 772.71	*G. geotrichum*	AJ279451	CBS 121.22
G. candidum (*G. geotrichum*)	RPMCC 5416	Clinical isolate	*G. geotrichum*	AJ279451	CBS 121.22

Table 1 (*Continued*)

Organism and conventional identification*	Strain no.	Source of isolate	rDNA sequence-based identification results†		
			Sequence-based identification	GenBank accession no.	Reference source
Saccharomyces species					
S. cerevisiae	Control 9763	RPMCC 9763	*S. cerevisiae*	Z95940	CBS 4903
S. cerevisiae	RPMCC 1490	Clinical isolate	*S. cerevisiae*	Z95940	CBS 4903
S. cerevisiae	RPMCC 5845	Clinical isolate	*S. cerevisiae*	Z95940	CBS 4903
Basidiomycetous yeasts					
Malassezia species					
M. furfur	Control 1878	CBS 1878	*M. furfur*	AF246896	ATCC 44344
Malassezia sp.	RPMCC 9603	Clinical isolate	*M. furfur*	AF246896	ATCC 44344
Malassezia sp.	RPMCC 4701	Clinical isolate	*M. furfur*	AF246896	ATCC 44344
Malassezia sp.	RPMCC 5576	Clinical isolate	*M. furfur*	AF246896	ATCC 44344
Malassezia sp.	RPMCC 5621	Clinical isolate	*M. furfur*	AF246896	ATCC 44344
Trichosporon species					
T. asahii	RPMCC 0065	Clinical isolate	*T. asahii*	AF444457	CBS 8520
T. asahii	RPMCC 2911	Clinical isolate	*T. asahii*	AF444457	CBS 8520
T. asahii	RPMCC 8399	Clinical isolate	*T. asahii*	AF444457	CBS 8520
T. inkin	RPMCC 3804	Clinical isolate	*T. inkin*	AF444420	CBS 5585
Dermatophytes					
Epidermophyton species					
E. floccosum	RPMCC 3409	Clinical isolate	*E. floccosum*	AJ000629	CBS 358.93
E. floccosum	RPMCC 6580	Clinical isolate	*E. floccosum*	AJ000629	CBS 358.93
E. floccosum	RPMCC 1320	Clinical isolate	*E. floccosum*	AJ000629	CBS 358.93
Microsporum species					
M. canis (*A. otae*)	RPMCC 3893	Clinical isolate	*A. otae*	AJ252339	CBS 495.86
M. gypseum (*A. gypseum*)	RPMCC 2631	Clinical isolate	*A. gypseum*	AF168128	CBS 170.64
Trichophyton species					
T. rubrum	RPMCC 5907	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 0263	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 6206	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 6691	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 6205	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 7086	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 4735	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 5918	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 3361	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. rubrum	RPMCC 1133	Clinical isolate	*T. rubrum*	AF170472	ATCC 28188
T. interdigitale (*A. vanbreuseghemii*)	RPMCC 4677.1	Clinical isolate	*A. vanbreuseghemii*	AF170466	UAMH 8544
T. interdigitale (*A. vanbreuseghemii*)	RPMCC 7122	Clinical isolate	*A. vanbreuseghemii*	AF170466	UAMH 8544
T. interdigitale (*A. vanbreuseghemii*)	RPMCC 6772	Clinical isolate	*A. vanbreuseghemii*	AF170466	UAMH 8544
T. interdigitale (*A. vanbreuseghemii*)	RPMCC 2939	Clinical isolate	*C. indicum*‡	AJ005369	CBS 117.63
T. interdigitale (*A. vanbreuseghemii*)	RPMCC 8529	Clinical isolate	*A. vanbreuseghemii*	AF170466	UAMH 8544
T. interdigitale (*A. vanbreuseghemii*)	RPMCC 5294	Clinical isolate	*A. vanbreuseghemii*	AF170466	UAMH 8544
T. mentagrophytes	RPMCC 4677.2	Clinical isolate	*A. vanbreuseghemii*	AF170466	UAMH 8544
T. tonsurans	RPMCC 0167	Clinical isolate	*T. tonsurans*	AF170479	UAMH 8552
T. violaceum	RPMCC 5519	Clinical isolate	*T. violaceum*	AJ270811	CBS 319.31
Dimorphic fungi					
Sporothrix species					
S. schenckii (*Ophiostoma*)	RPMCC 1254	Clinical isolate	*S. schenckii*	AF364061	ATCC 14284
S. schenckii (*Ophiostoma*)	RPMCC 0485	Clinical isolate	*S. schenckii*	AF364061	ATCC 14284
Histoplasma species					
H. capsulatum (*Ajellomyces capsulatus*)	RPMCC 2889	Clinical isolate	*A. capsulatus*	AF038353	UAMH 7141
H. capsulatum (*A. capsulatus*)	RPMCC 5400	Clinical isolate	*A. capsulatus*	AF038353	UAMH 7141
Other fungi					
Scedosporium species					
S. apiospermum (*Pseudallescheria boydii*)	RPMCC 3039	Clinical isolate	*P. boydii*	AF022486	CBS 101.22

Table 1 (*Continued*)

Organism and conventional identification*	Strain no.	Source of isolate	rDNA sequence-based identification results†		
			Sequence-based identification	GenBank accession no.	Reference source
S. apiospermum (*P. boydii*)	RPMCC 3293	Clinical isolate	*P. boydii*	AF022486	CBS 101.22
S. apiospermum (*P. boydii*)	RPMCC 6397	Clinical isolate	*P. boydii*	AF022486	CBS 101.22
S. prolificans	RPMCC 2811	Clinical isolate	*S. prolificans*	AF022484	CBS 114.90
S. prolificans	RPMCC 4297	Clinical isolate	*S. prolificans*	AF022484	CBS 114.90

ATCC, American Type Culture Collection, Manassas, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Japan; RPMCC, Royal Perth Hospital Mycology Culture Collection, Perth, Australia; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada.
*Teleomorph in parentheses if known.
†Based on the highest bit score (Materials and methods).
‡Confirmed as *Chrysosporium* species by traditional mycological techniques.

tube negative were identified by the Yeast Biochemical Card (YBC) system (BioMérieux Vitek, Inc., Hazelwood, MO, USA). The identification was confirmed by examination of conventional morphological characteristics using CMAT plate culture and CHROMagar. For common clinical yeasts, identification was achieved within 3–4 days of initial isolation. Yeasts not identified using the YBC system were identified within 48 h using the ID 32 C system (BioMérieux, Marcy l'Etoile, France). Clinical basidiomycetous yeasts such as *Trichosporon* species were also identified with the ID 32 C system. Primary isolation and subculture of *Malassezia* spp. was performed using Dixon's agar. These isolates were not identified to the species level due to the lack of reliable methods available in our laboratory.

Identification of dermatophytes

Microscopic morphology was studied on primary isolation media [Sabouraud's glucose agar with chloramphenicol, Casamino acids erythritol albumin agar, Mycosel agar (Difco, Detroit, MI, USA)]. Physiological testing included the following for suspected dermatophytes: growth at 27 °C and 37 °C on Sabouraud's glucose agar; dermatophyte test medium (DTM) was used according to Taplin *et al*. [32]; hydrolysis of urea by production of urease; growth characteristics on Littman oxgall agar, lactritmel agar, Sabouraud's glucose agar with 5% NaCl, 1% peptone agar, bromcresol purple–milk solids–glucose agar (BCP-MS-G) and *Trichophyton* agars (Difco); vitamin-free casamino acids agar (T1); vitamin-free casamino acid agar+inositol (T2); vitamin-free casamino acid agar+inositol+thiamine (T3); vitamin-free casamino acid agar+thiamine (T4); vitamin-free casamino acid agar+nicotinic acid (T5); vitamin-free ammonium nitrate agar (T6); vitamin-free ammonium nitrate agar+L-histidine (T7). Other tests that assisted in the identification of the dermatophytes tested included growth on boiled polished rice grains and examination of reverse pigment on potato glucose agar. Tests were incubated at 27 °C and incubated up to 4 weeks in an air incubator. For common clinical dermatophytes, identification was achieved within 2 weeks of the initial isolation.

Identification of other fungi

Other filamentous fungi in this study were identified using traditional mycological methods using specialized mycological techniques, handbooks and identification keys based on colony characteristics, microscopic morphology and physiological testing [10,29]. In most cases, identification was achieved within 2 weeks of the initial isolation.

Culture preparation and DNA isolation

A simple and universal DNA isolation method was used for all fungi tested. Fungi used in this study were subcultured onto routine culture media (Sabouraud's glucose agar or Dixon's agar) and incubated at 30 °C until the earliest visible signs of growth were noted. A small amount (approximately 1–2 mm^2) of fungal mycelial mass or yeast colony was removed and emulsified in 100 µl of extraction buffer (10 mm Tris–HCl [pH 8.0], 10 U of partially purified lyticase [Sigma, Castle Hill, NSW, Australia]) in a 1.5-ml microfuge tube. The tubes were incubated at 37 °C for 45 min then vortexed for 30 s. Chelex 100 (Bio-Rad Laboratories, Hercules, CA, USA) was added to each tube to a final concentration of 10% (w/v). All tubes were heated to 95 °C in a heating block for 10 min then allowed to cool before centrifugation for 2 min at $13000 \times g$. Samples were used immediately for polymerase chain reaction (PCR) or stored at −70 °C until use. *S.*

Table 2 Medically important fungi used as reference strains in this study

Organism	GenBank accession no.(s)*	Strain(s)†
Ascomycetous yeasts		
C. albicans	AF217609	ATCC 28516
C. catenulata	Authors' data	CBS 565
C. chiropterorum	Authors' data	CBS 6064
C. ciferrii (*Stephanoascus ciferrii*)	Authors' data	CBS 5295
C. dubliniensis	AB049123, AB035590	CBS 7987, CBS 7988
C. famata (*D. hansenii*)	AF210326, AF210327	CBS 789, CBS 767
C. glabrata	AF167993	ATCC 15545
C. guilliermondii (*P. guilliermondii*)	AB054109	JCM 10735
C. haemulonii	Authors' data	CBS 5149
C. intermedia	Authors' data	CBS 572
C. kefyr (*K. marxianus*)	AJ401699	CBS 4857
C. krusei (*I. orientalis*)	AF246989	ATCC 6258
C. lipolytica (*Yarrowia lipolytica*)	Authors' data	CBS 6124
C. lusitaniae (*Clavispora lusitaniae*)	Authors' data	CBS 1944
C. norvegensis (*Pichia norvegensis*)	Authors' data	CBS 6564
C. parapsilosis	AF287909	ATCC 22019
C. pelliculosa (*Pichia anomala*)	Authors' data	CBS 110
C. rugosa	Authors' data	CBS 613
C. tropicalis	AF287910	ATCC 750
C. utilis (*Pichia jadinii*)	Authors' data	CBS 621
C. viswanathii	Authors' data	CBS 4024
C. zeylanoides	Authors' data	CBS 619
G. candidum (*G. geotrichum*)	AJ279451	CBS 121.22
S. cerevisiae	Z95940	CBS 4903
Basidiomycetous yeasts		
M. furfur	AF246896	ATCC 44344
T. asahii	AF444457	CBS 8520
T. inkin	AF444420	CBS 5585
Dermatophytes		
E. floccosum	AJ000629	CBS 358.93
M. audouinii	AJ252333, AJ252332	CBS 344.50, CBS 317.51
M. canis (*A. otae*)	AJ252339	CBS 495.86
M. ferrugineum	AJ252335	CBS 457.80
M. gypseum (*A. gypseum*)	AF168128	CBS 170.64
T. concentricum	Z98012	CBS 196.26
T. erinacei (*Arthroderma benhamiae*)	Z97996	CBS 344.79
T. interdigitale (*A. vanbreuseghemii*)	Z98001, AF170466	CBS 558.66, UAMH 8544
T. mentagrophytes	Z97995	CBS 318.56
T. rubrum	Z97993, AF170472	CBS 392.58, ATCC 28188
T. schoenleinii	Z98011	CBS 855.71
T. simii (*Arthroderma simii*)	Z98017	CBS 417.65
T. tonsurans	Z98009	CBS 292.81
T. vanbreuseghemii (*Arthroderma gertleri*)	Z98013	CBS 598.66
T. verrucosum	Z98003	CBS 134.66
T. violaceum	AJ270811	CBS 319.31
Other fungi		
S. schenckii (*Ophiostoma*)	AF364061	ATCC 14284
H. capsulatum (*A. capsulatus*)	AF038353, AF038354	UAMH 7141, UAMH 3536
S. apiospermum (*P. boydii*)	AF022486	CBS 101.22
S. prolificans	AF022484	CBS 114.90
C. indicum	AJ005369	CBS 117.63

ATCC, American Type Culture Collection, Manassas, VA, USA; JCM, Japan Collection of Microorganisms, Institute of Physical and Chemical Research, Wako, Japan; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; WB, Laboratory for Mycology and Molecular Biology, ENT-University Hospital, Graz, Austria; UAMH, University of Alberta Microfungus Collection and Herbarium, Edmonton, AB, Canada.

Teleomorph in parentheses.

*GenBank accession numbers for rDNA sequences used as references.

†Culture collections and strain numbers of sequences reported in GenBank, respectively.

cerevisiae (RPMCC 9763) was used as a positive control for DNA isolation, PCR and DNA sequencing for each experiment.

Primers

The primers used for universal fungal DNA amplification from all isolates were V9D, 5′-TTA AGT CCC TGC CCT TTG TA-3′ [33], and LS266, 5′-GCA TTC CCA AAC AAC TCG ACT C-3′ [34]. These primers bind to conserved regions, with corresponding positions to *S. serevisiae* 18S (1609–1627) and 26S (287–266) rRNA genes, and amplify a product that encompasses a portion of the 18S and 26S rRNA gene and the entire intervening ITS1, 5.8S and ITS2 rRNA gene regions. The size of the product generated varies according to the organism tested. Primers used for direct sequencing were ITS1; 5′-TCC GTA GGT GAA CCT GCG G-3′ (position corresponding to *S. serevisiae* small subunit 1769–1787), or both ITS1 and ITS4; 5′-TCC TCC GCT TAT TGA TAT GC-3′ (position corresponding to *S. cerevisiae* large subunit 41–60) [20]. All primers were synthesized by Gibco BRL, Life Technologies, Melbourne, Australia.

PCR amplification

The PCR assay was performed with 5 μl of DNA template in a total reaction volume of 50 μl. The PCR reaction mixture contained 5 μl of 10 × reaction buffer (Applied Biosystems, Foster City, CA, USA); 3 μl of 25 mm $MgCl_2$; 1.5 μl of 20 μm of each oligonucleotide; 200 μm of each deoxynucleoside triphosphate; dATP, dGTP, dCTP, dTTP; 2.25 U of AmpliTaq Gold (Applied Biosystems); and 33 μl of sterile distilled H_2O. The PCR was performed in a DNA Engine, PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA) with the following program; 95 °C for 9 min initial inactivation step followed by 95 °C for 30 s, 62 °C for 60 s, 72 for 2 min for 33 cycles, and then the mixture was incubated at 72 °C for 5 min for final extension.

Agarose gel electrophoresis

Detection of PCR-amplified product was performed by electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide. A volume of 5 μl of PCR-amplified product and 1 μl of Gel Loading Solution (Sigma) was loaded into each lane. A volume of 2 μl of a 50–2000-bp molecular weight marker (AmpliSize Molecular Ruler, Bio-Rad) was run in parallel to approximate PCR-amplified product size. Amplified DNA from *S. cerevisiae* (RPMCC 9763) was used as a control.

DNA sequencing and editing

All PCR-amplified products were sequenced at the West Australian Genome Resource Centre at Royal Perth Hospital by automated dye termination sequencing. Each PCR-amplified product was purified with UltraClean PCR Clean-up Kit (MO BIO Laboratories, Carlsbad, CA, USA) and sequenced using a 16-capillary 3100 Genetic Analyzer (Applied Biosystems). The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3 (Applied Biosystems) was used with protocols supplied by the manufacturer. PCR-amplified products from clinical isolates were directly sequenced using the ITS1 primer. PCR-amplified products from referenced cultures were sequenced in a forward and reverse direction using ITS1 primer and the ITS4 primer respectively. Sequences were visualized and edited using Chromas Version 1.45; Technelysium Pty. Ltd. [http://www.technelysium.com.au/chromas.html]) or SEQSCAPE Version 1.1 (Applied Biosystems).

GenBank search

Sequence search was performed using the BLAST standard nucleotide-nucleotide basic local alignment search tool [National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA (http://www.ncbi.nlm.nih.gov/BLAST/)]. All GenBank, EMBL, DDBJ, PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences) were searched with the expectation frequency minimized at 0.0001. Sequences were filtered for low complexity.

Clinical isolate identification study

Eighty-nine clinical isolates previously identified by traditional mycological techniques were assigned unique laboratory identification numbers prior to DNA extraction (Table 1). The universal DNA extraction was performed as described earlier, and the PCR products were sequenced by personnel from a remote laboratory in a blinded manner. Sequence editing and analysis to determine the sequence-based identification was performed by a second person blinded to the traditional identification results. Where possible, sequence-based identification was determined from the entry with the highest bit score listed in the BLAST search with an expect threshold closest to zero that fulfilled the following additional criteria: (i) the sequence included the entire ITS1-5.8S-ITS2 region; (ii) the sequence was derived from a referenced culture; (ii) the nomenclature ascribed to the referenced culture was valid. The

GenBank records of referenced cultures obtained in this manner are shown in Table 2. A few clinical isolates could not be identified using this approach because the organism identification obtained from a BLAST search did not meet all the criteria described. These identifications were considered a preliminary result until confirmed by the following process. A well characterized reference culture of current nomenclature representing the preliminary species identification was sequenced using ITS1 and ITS4 primers and an accurate consensus sequence was generated using SEQSCAPE. Alignment of this sequence with the test sequence was performed using the BLAST 2 alignment tool (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) using identical parameters as for the initial BLAST search. Greater than 98% homology with the referenced culture was required to confirm the preliminary identification of the test sequence. A more detailed alignment to confirm this result was performed using the GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.6.001, Pittsburgh Supercomputing Center (PSC), Carnegie Mellon University, University of Pittsburgh, Pittsburgh, PA, USA [35]. Sequences from other fungi were also included to cover a range of medically important fungi that may be encountered in a clinical microbiology laboratory (Table 2). Furthermore, entire target sequences from certain medically important fungal reference strains could not be found in GenBank prior to this study. Hence, to improve the range of fungi able to be identified using this approach, sequences from 13 reference strains of medically important fungi not reported in GenBank at the time of this study were sequenced (Table 2).

Results

Sequence-based identification and correlation with phenotypic identification

Among the 89 clinical isolates, 87 had a sequence-based identification result that correlated with conventional phenotypic identification (Table 1). Furthermore, nine control strains sequenced in this study also had a sequence-based identification that correlated with their known identification (Table 1). Forty-eight clinical ascomycetous yeasts identified phenotypically as germ-tube-negative *C. albicans* ($n = 1$), *C. dubliniensis* ($n = 2$), *C. famata* ($n = 1$), *C. glabrata* ($n = 14$), *C. guilliermondii* ($n = 2$), *C. intermedia* ($n = 1$), *C. kefyr* ($n = 2$), *C. krusei* ($n = 6$), *C. parapsilosis* ($n = 11$), *C. tropicalis* ($n = 5$), *S. cerevisiae* ($n = 2$), and *G. candidum* ($n = 1$) were reliably identified to the species level using sequence-based identification. The sequence-based identification also correlated with routine identification for the basidiomycetous yeasts identified as *Trichosporon asahii* ($n = 3$) and *T. inkin* ($n = 1$). *Malassezia* spp. ($n = 4$) were not identified to the species level using recently developed physiological methods; all had sequenced-based identifications of *M. furfur*. Twenty-two clinical dermatophyte isolates previously identified as *Epidermophyton floccosum* ($n = 3$), *Microsporum canis* ($n = 1$), *M. gypseum* ($n = 1$), *Trichophyton rubrum* ($n = 10$), *T. interdigitale* ($n = 5$), *T. tonsurans* ($n = 1$) and *T. violaceum* ($n = 1$) were also reliably identified to the species level using sequence-based identification, as were dimorphic fungi identified by routine methods as *Sporothrix schenckii* ($n = 2$), *Histoplasma capsulatum* ($n = 2$). *Scedosporium apiospermum* ($n = 3$) and *S. prolificans* ($n = 2$) were also recognized by sequencing.

Two discordant identification results were observed between the phenotypic identification and the sequence-based identification. One isolate identified phenotypically as *T. interdigitale* had a 99% homology (591/592 bp) with a GenBank sequence from a referenced *Chrysosporium indicum* culture, CBS 117.63. In contrast, no significant alignments were possible ($< 50\%$ homology) when compared to the reference sequence from *T. interdigitale* strain CBS 558.66 using BLAST 2. The isolate produced a pH change on DTM, demonstrated a moderate alkaline change on BCP-MS-G, and grew well at 37 °C. Small, slender, clavate, smooth-walled conidia were seen; however, the presence of small numbers of cymbiform (boat-shaped) conidia were initially overlooked, and the absence of small numbers of macroconidia after 7 days of incubation was not considered unusual for some strains of *T. interdigitale*. Repeat microscopy and culture for 14 days revealed moderate numbers of cymbiform conidia and the absence of macroconidia, as well as a stronger alkaline reaction on BCP-MS-G agar. This isolate was subsequently confirmed as belonging to the genus *Chrysosporium* and the initial identification of *T. interdigitale* was amended to the sequence-based identification of *C. indicum*. The second discordant result was an isolate identified phenotypically as *T. mentagrophytes* (RPMCC 4677.2). This isolate shared a 99% sequence homology (669/676 bp) with the reference sequence from the teleomorph *Arthroderma vanbreuseghemii* UAMH 8544 (University of Alberta Microfungus Collection, Edmonton, AB, Canada), and a 99% sequence homology (657/664 bp) with the reference sequence from the anamorph *T. interdigitale* CBS 558.66. In both cases, a 7-bp insertion in the test sequence was responsible for the difference. In contrast, the same clinical isolate

shared a 96% sequence homology (641/667 bp) with the reference sequence from *T. mentagrophytes sensu stricto*, CBS 318.56. This included a 6-bp insertion and an additional 20 base changes comprising of 14 substitutions, three insertions and three deletions (sequence data not shown). The original laboratory phenotypic identification was reviewed and the organism was found to be granular in appearance and the microscopy was consistent with the recently refined *sensu stricto* concept of *T. mentagrophytes* except for the absence of favic chandelier-like structures. However, these structures are only found in some strains. There were no additional definitive phenotypic characteristics to aid identification and the initial identification of *T. mentagrophytes* had been made on the basis of colony morphology and microscopy. However, this isolate was co-isolated with a downy *T. interdigitale* also identified in this study (RPMCC 4677.1) from a chronic low-grade tinea pedis infection from a city-dweller. Sequence alignment and comparison of ITS sequences from the two isolates showed that they were identical. Furthermore, there was no supporting clinical evidence of animal contact. Based on the clinical evidence, co-isolation with a typical *T. interdigitale* and the sequence-based result, we conclude that the isolate is most likely to be a granular *T. interdigitale*.

Cost and time comparisons

For yeasts other than *C. albicans*, the average total cost in Australian dollars of performing conventional identification was $20.07 per isolate. This comprised reagents ($13.75) and labour ($6.32). Time taken for a final identification was 3–4 days for common yeasts and up to 11 days for less commonly encountered yeasts. The average total cost of identification of dermatophytes, in Australian dollars was $25.01 per isolate. This comprised reagents ($10.25) and labour ($14.76). Time taken for a final identification of these organisms ranged from 14 to 21 days. Cost and time comparisons for the identification of other filamentous fungi have not been reported, as labour and consumable costs vary significantly depending on the organism.

Based on identifying a batch of six isolates, the total cost in Australian dollars of sequence-based identification, which includes the initial amplification step plus the sequencing reaction, averaged $16.66 per isolate. This comprised reagents ($10.12) and labour ($6.54). A final identification was usually obtained within 48 h of obtaining a pure isolate, but could be available within 24 h if required.

Discussion

This present work is based on the premise that more rapid methods of identifying fungi are required, particularly with the increase in morbidity and mortality associated with fungal infections caused by resistant fungi [9]. Many traditional methods of fungal identification are slow, based on colony characteristics, microscopic morphology and physiological tests. In most cases, confident identification of fungi to the species level requires specialized staff trained in the use of mycological identification keys and culture techniques.

Commercial yeast identification systems have provided laboratories with a reasonably accurate and cost-effective means to identify clinical isolates. However, some systems have proven to be unreliable and they may be restricted to common clinical isolates [7,8]. Some may lack the ability to identify a number of species altogether due to incomplete biochemical databases [36]. The performance of commercial systems may also vary according to inoculum and geographic isolate source [6].

Classical parameters for the identification of dermatophytes and other filamentous fungi are broad, and include conidial morphology, cultural characteristics, physiological tests and clinical features. Identification of dermatophytes is time-consuming and requires extensive familiarity with the microscopic and cultural characteristics of these taxa [5]. Furthermore, dermatophyte identification can be difficult due the variety of species names ascribed for the same organism. In contrast to the rapid commercial systems available for yeasts, identification of dermatophytes and other filamentous fungi are often based solely on phenotypic methods. Reliable identification to the species level may often take many weeks. As a strategy to overcome these limitations, we have developed a sequenced-based approach for the identification of slow-growing fungi from the early stages of growth.

Common molecular targets for rapid methods identification include the ribosomal small subunit (SSU) and large subunit (LSU) rDNA regions. Traditionally, these regions have been used to study phylogenetic relationships, because they evolve slowly and are relatively conserved among fungi [20]. However, these regions subtend relatively large DNA sequences and may lack sufficient heterogeneity for species identification. This has led to the increased use of the ITS regions as targets for identifying fungi to the species level. Recent molecular methods using the ITS regions as targets to separate and identify fungal species have been reviewed, demonstrating that the ITS regions offer

a powerful tool for the identification and typing of fungi [21]. Most studies have shown that sufficient variation exists within the ITS regions to allow for species identification [21]. The ITS regions have been used as targets to investigate the validity of dermatophyte taxonomy [14,15,18], and to determine the phylogenetic relationships among other fungal species [11,16,19,22]. Some of the cited studies (and others) have provided ITS sequence information for a variety of fungi and are available in GenBank for analysis. However, until recently the use of direct sequence analysis for diagnostic purposes has been limited due to the lack of automated high-throughput DNA sequencing instruments and high running costs.

Current molecular methods utilizing the ITS regions for the identification of fungi include the use of genus- or species-specific primers and probes, restriction fragment length polymorphism of amplified DNA or direct sequence analysis of amplified DNA. These methods are commonly used to identify and type many fungi [21]. Molecular methods using species-specific primers have been used to rapidly identify *T. asahii* and *Paracoccidioides brasiliensis* [26,37,38]. The major disadvantage with species-specific PCR assays is that multiple assays would need to be implemented to cover a range of medically important fungi encountered in a clinical microbiology laboratory. In contrast, methods used for the rapid identification of one or more fungal pathogens often involve the use of universal primers combined with genus- or species-specific probes to identify or detect a range of fungal pathogens in cultures or clinical samples [24,25,39,40]. Although these approaches have the ability to differentiate fungal species, some probes may lack specificity [24]. These approaches are more suited for diagnostic use, particularly in clinical samples, because more than one pathogen may be detected. However, the use of probes to identify a range of fungi is problematic due to the need to develop species-specific probes that have similar properties for DNA hybridization. That is, probes used for species identification must be free of secondary structure, must have appropriate melting temperatures and must not cross-react with other species. To develop a panel of probes with these characteristics to accurately identify many different fungal species is challenging and would involve considerable development cost. Recently, a PCR-enzyme immunoassay (EIA) technique was developed that used streptavidin-coated 96-well microtitre plates, a universal capture probe and eight species-specific digoxigenin probes to identify yeast-like fungal pathogens [25]. Such methods are well suited to clinical laboratory use because of the rapid and convenient EIA detection format. However, probe design, high development costs and a small number of species identified are still the limiting factors for the development and implementation of routine PCR-EIA systems to identify a broad-range of fungal pathogens in a clinical microbiology laboratory.

Another disadvantage of some molecular approaches to identify fungi is the use of tedious methods of DNA isolation. Methods include freezing and grinding mycelium, detergent lysis, extraction with solvents and precipitation with alcohol [17]. Others use commercial kits [25] or a combination of detergent lysis with a commercial kit [27]. Overall, commercial methods of DNA extraction decrease the time required for DNA isolation and are well suited for diagnostic use. However, commercial methods have a relatively high unit cost per purification. Overall, the rapid cost-effective DNA extraction method (< 1 h) described in this study performed well, with sufficient DNA present in each tube to amplify a single PCR product from all clinical isolates and controls. The band intensity of each amplification was relatively uniform and slight variations in PCR product concentrations had minimal or no effect on the DNA sequencing result. Furthermore, non-specific PCR products were not amplified from any media or the lyticase lysing enzyme (data not shown).

In the methodology described in this study, we refer to sequences from referenced cultures in GenBank as standards for identification of fungi. In a similar approach, medically important *Aspergillus* species were identified to the species level when sequences from test isolates were compared to sequences in GenBank using BLAST [27]. An evaluation of a blind clinical study was performed and 11 clinical isolates could be correctly identified to the species level. The evaluators concluded that both the ITS1 and ITS2, combined with the highest BLAST bit score, were required for the accurate identification of *Aspergillus* species. The method showed promise for rapid identification and earlier initiation of appropriate therapy for the treatment of invasive aspergillosis.

In our approach, a sequence with the highest bit score and with the expectation frequency value closest to zero that was derived from a referenced culture was considered a match. The selection of DNA sequences (GenBank records or a reference cultures sequenced as part of this study) to be used as standards for sequence-based identification was based on the following criteria: (i) the sequence should be complete, i.e. it should represent the whole region of interest and not just a part thereof; (ii) all sequences used should be derived from cultures obtained from reference collections,

preferably cultures nomenclaturally designated as ex-type (the fungal equivalent to bacteriological-type strains) or, failing that, cultures identified by use of the most stringent level of traditional mycological techniques based on authoritative monographs and up-to-date literature in an upper level reference laboratory and; (iii) to the best of the investigator's knowledge, each sequence should have been designated with a species name that was nomenclaturally valid and currently recognized as correct at the time of this study. Following these criteria we were able to overcome some of the major limitations concerning the use of sequences in GenBank for organism identification. These include the use of incomplete sequences or sequences derived from non-referenced [27] or even misidentified cultures. The development of a reliable database of entire ITS1-5.8S-ITS2 sequences from a collection of reference cultures may further overcome these limitations. Such databases are currently being developed for the identification of bacteria, fungi and mycobacteria [41].

Using a list of selected GenBank records and sequences derived from referenced cultures, each of the fungi tested in this study could be identified to the species level. We observed two discordant results between the phenotypic identification and the sequenced-based identification, as detailed above. Our discovery that a granular-textured dermatophyte isolate most closely resembling *T. mentagrophytes ss. str.* phenotypically had a sequence-based identification of *T. interdigitale* (*A. vanbreuseghemii*) was of particular interest. It is well known that these two species are physiologically indistinguishable and demonstrate variable cultural and morphological differences. Furthermore, granular strains corresponding to the current molecular concept of *T. interdigitale* are relatively well known, e.g. as mating type strains of *A. vanbreuseghemii* [R.C. Summerbell, personal communication]. Clinical differences do occur, and *T. mentagrophytes ss. str.* is predominantly a zoophilic species capable of causing human infection, whereas *T. interdigitale*, as seen in the clinical but not the veterinary laboratory, is predominantly anthrophilic. In this particular case, animal contact was unlikely and the clinical presentation was more supportive of a chronic low-grade tinea pedis infection. In each case, the discordant identification was resolved in favour of the sequencing result, highlighting the usefulness of this approach, particularly in cases when the phenotypic characteristics show variability and are not sufficiently conclusive to allow a definitive identification.

Our approach has a relatively high set-up cost; however, significant savings in variable costs have been achieved. Although the long-term financial advantages are yet to be assessed, the impact of an earlier and more definitive diagnosis may improve the clinical outcome for some patients. For example, a more rapid result may lead to the earlier initiation of therapy, or the sequence-based identification may alter the therapeutic management. This may occur when the clinical diagnosis remains uncertain, or when the laboratory has isolated a fungus of an unknown clinical significance. Additionally, the time taken for a final result ranged from 3–21 days for phenotypic identification, compared to 24–48 h for sequence-based identification from the first visible signs of growth. Carry-over of small amounts of growth medium does not affect the final result. Colonies of this size are usually sterile and, in microscopy, can only be used to make elementary distinctions such as aseptate mucoralean zygomycetous vs. septate hyphae. Morphological features may take a further 3–5 days to develop before a preliminary identification is possible. Final species identification may take an additional 7 days using traditional mycological techniques. Rapid sequence-based identification from single colony at the earliest signs of growth may be useful for guiding early appropriate therapy. However, several issues may effect the time required to obtain a result, including the availability of a DNA sequencer and repeat sequencing due to poor signal strengths. Repeating the DNA extraction and sequencing for a second time delays the final result only by 48 h. This differs from repeating or adding confirmatory phenotypic tests, a procedure that may require an additional 7 days. Finally, the total cost of a sequence-based identification and the time taken for a final identification remained constant and did not vary from one organism to the next, compared to significant variations in cost and time for phenotypic methods.

At the present time, direct DNA sequencing of ITS1-5.8S-ITS2 rDNA to identify fungi may only be suitable for larger reference laboratories. However, the development of compact DNA sequencers, or the use of commercial DNA sequencing services, may allow other laboratories with access to a thermal-cycler, to utilize this approach. Additional isolates representing the other fungal species not encountered in this study are needed to further evaluate this approach, as relatively few species have as yet had their ITS regions evaluated and some fungi may not show sufficient ITS sequence variation for reliable species separation. In some cases, re-evaluation of the phenotypic positioning or the proposed revision of morphological-based taxonomic schemes of some fungal species may be required. Whether or not ITS sequences will provide the ultimate sequence-based reference method needs to be estab-

lished [21]. More reference sequences are being added constantly and will eventually constitute a very large collection. We are currently evaluating an in-house sequence database for future implementation as a diagnostic tool for rapid fungal identification. A large blinded clinical evaluation (> 1000 isolates) is currently under investigation to assess more accurately the utility of DNA sequencing to identify a broader range of medically important fungi in a diagnostic clinical microbiology laboratory.

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