Identification of dermatophytes by sequence analysis of the rRNA gene internal transcribed spacer regions

Hsin Chieh Li,¹ Jean-Philippe Bouchara,^{2,3} Mark Ming-Long Hsu,⁴ Richard Barton,⁵ Shuli Su¹ and Tsung Chain Chang¹

Correspondence

Tsung Chain Chang
tsungcha@mail.ncku.edu.tw

¹Department of Medical Laboratory Science and Biotechnology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

²Host-Pathogen-Interaction Study Group, UPRES-EA 3142, Angers University, Angers, France

³Laboratory of Parasitology and Mycology, University Hospital, Angers, France

⁴Department of Dermatology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, ROC

⁵Institute of Molecular and Cellular Biology, School of Biochemistry and Microbiology, University of Leeds, Leeds, UK

Identification of dermatophytes using the traditional method is sometimes problematic because of atypical microscopic or macroscopic morphology. The aim of this study was to evaluate the feasibility of using sequencing of the ribosomal internal transcribed spacer (ITS)1 and ITS2 regions for identification of 17 dermatophyte species. The ITS regions of 188 strains (62 reference strains and 126 clinical isolates) were amplified by PCR and sequenced. Species identification was made by sequence comparison with an in-house database comprising ITS sequences of type or neotype strains or by BLAST searches for homologous sequences in public databases. Strains producing discrepant results between conventional methods and ITS sequence analysis were analysed further by sequencing the D1–D2 domain of the large-subunit rRNA gene for species clarification. The identification rates by ITS1 and ITS2 sequencing were higher than 97 %. Based on reference sequences of type or neotype strains, it was noted that most strains of *Trichophyton mentagrophytes* were misidentifications of *Trichophyton interdigitale*. In addition, barcode sequences were present in species of the *Microsporum canis* complex and *Trichophyton rubrum* complex. These barcode sequences are useful for species delineation when the results of ITS sequencing are ambiguous. In conclusion, ITS sequencing provides a very accurate and useful method for the identification of dermatophytes.

Received 3 September 2007

Accepted 25 January 2008

INTRODUCTION

Dermatophytes are keratinophilic fungi capable of causing dermatophytosis and are among the most adaptable parasitic associates of humans. Dermatophytes are classified in three anamorphic genera: *Epidermophyton*, *Microsporum* and *Trichophyton* (Weitzman & Summerbell, 1995). Classical parameters for the identification of dermatophytes

include clinical features, culture characteristics, microscopic morphology and physiological test results. However, identification of dermatophytes sometimes remains difficult or uncertain due to their overlapping phenotypic characteristics, variability and pleomorphism (Gräser *et al.*, 1999b, 2000b; Weitzman & Summerbell, 1995).

Molecular approaches have been developed to provide more rapid and accurate alternatives for dermatophyte identification. These methods include gene-specific PCR (Kamiya *et al.*, 2004; Kanbe *et al.*, 2003a; Liu *et al.*, 2001; Yoshida *et al.*, 2006), RFLP analysis (Kamiya *et al.*, 2004; Kanbe *et al.*, 2003b; Leon-Mateos *et al.*, 2006; Mochizuki *et al.*, 2003), sequencing of the large subunit rRNA gene (Ninet *et al.*, 2003) or the chitin synthase-encoding gene (Kano *et al.*, 2000), PCR fingerprinting (Gräser *et al.*, 2000a, b) and DNA hybridization (El Fari *et al.*, 1999).

Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the ITS sequences of dermatophytes reported in this study are DQ860729, DQ860737, DQ860739, DQ860747, DQ860749, DQ860753, DQ860755, DQ860762, DQ860768, DQ860769, DQ860773, DQ860774, DQ860776, DQ860778, DQ860784, DQ860785, DQ860794, DQ860802, DQ860804, DQ860812, DQ860814, DQ860818, DQ860820, DQ860827, DQ860833, DQ860834, EF078476, EF078481 and EU362731–EU362734.

Sequencing of the internal transcribed spacer (ITS) regions (Gräser *et al.*, 1999a, b, 2000a, b; Kaszubiak *et al.*, 2004; Makimura *et al.*, 1998, 1999, 2001; Mochizuki *et al.*, 1999; Sharma *et al.*, 2006) has proved to be a useful method for phylogenetic analysis and identification of dermatophytes. However, several strongly ecologically and phenotypically separated *Trichophyton* species may have only a small number of nucleotide differences in the ITS regions (Summerbell *et al.*, 1999). The aim of this study was to evaluate the feasibility of sequencing the ITS1 and ITS2 regions for identification of 17 dermatophyte species, using a collection of reference strains and clinical isolates.

METHODS

Fungal strains. A total of 62 reference strains (17 species) and 126 clinical isolates of dermatophytes were analysed in this study (Table 1). Reference strains were obtained from the American Type Culture Collection (ATCC), USA, the Bioresources Collection and Research Center (BCRC), Taiwan, the Belgian Co-ordinated Collections of Micro-organisms (BCCM/IHEM), Belgium, and the Centraalbureau voor Schimmelfcultures (CBS), The Netherlands. Clinical isolates were obtained from the Laboratory of Parasitology and Mycology, University Hospital, Angers, the Mycology Reference Centre, University of Leeds, and the National Cheng Kung University Hospital, Tainan. Clinical isolates, which were recovered from a variety of specimens including skin, nail and hair, were identified by microscopic and macroscopic characteristics (De Hoog *et al.*, 2000; Larone, 2002). The species names used in this study were in agreement with current taxonomy proposed by Gräser *et al.* (2006), except *Trichophyton terrestre* and *Trichophyton soudanense* that were not considered as distinct species by them. The origin of the clinical isolates of the *Trichophyton mentagrophytes* complex is included in Table 1. Fungal strains were grown on Sabouraud dextrose agar (Difco) and incubated at 30 °C until there was evidence of hyphal growth.

Amplification and sequencing of the ITS regions. Fungal DNA extraction, ITS amplification using fungal universal primers ITS1 and ITS4, and sequencing of the PCR products were as described previously (Hsiao *et al.*, 2005). The sequences of the 18S, 5.8S and 28S rRNA gene regions were excluded to give the exact ITS1 and ITS2 sequences (Hsiao *et al.*, 2005).

Construction of an ITS sequence database. An in-house database comprising ITS sequences of type, neotype or syntype strains was constructed from the following strains (GenBank accession numbers for ITS1 and ITS2 sequences are given in parentheses): *Microsporum nanum* CBS 314.54 (DQ860729 and DQ860794), *Microsporum persicolor* CBS 871.70 (DQ860737 and DQ860802), *Trichophyton erinacei* CBS 511.73 (EF078476 and EF078481), *Trichophyton interdigitale* BCRC 31782=CBS 642.73=ATCC 28146 (DQ860739 and DQ860804), *T. mentagrophytes* CBS 318.56 (Z97995), *Trichophyton tonsurans* ATCC 56186 (DQ860762 and DQ860827), and *T. soudanense* ATCC 64654 (DQ860755 and DQ860820) and *Trichophyton violaceum* CBS 374.92 (AJ270810). The ITS sequences with a prefix of 'DQ' and 'EF' were sequenced in this study and submitted to GenBank (National Center for Biotechnology Information, USA), while other reference sequences were obtained from GenBank. The database was constructed using Vector NTI Advance 9 software (Invitrogen).

Identification of dermatophytes by ITS sequencing. Species identification was made by sequence comparison with reference

sequences in the constructed ITS database. Multiple ITS sequence alignment, and calculation of the similarity score between the query sequence and reference sequence, were performed by using the algorithms of 'Align' and 'Similarity', respectively, of the Vector NTI Advance 9 software. Species identification was determined from the best-scoring reference sequence of the similarity output that had an identity of $\geq 97\%$ with the query sequence. For species that do not have type or neotype strains, species identification was made by searching databases using the BLASTN algorithm of the BLAST sequence analysis tool (<http://www.ncbi.nlm.nih.gov/BLAST/>) from the National Center for Biotechnology Information. Strains producing discrepant results between morphological identification and ITS sequence analysis were further analysed by sequencing the D1–D2 region of the large-subunit rRNA gene (Hall *et al.*, 2004). Primers NL1 and NL4 (Kurtzman & Robnett, 1997) were used for amplification of the D1–D2 region, following the conditions described previously (Hsiao *et al.*, 2005). Species identification by D1–D2 sequencing was determined from the best-scoring reference sequence of the BLAST output that had an identity of $\geq 99\%$ with the query sequence.

RESULTS AND DISCUSSION

Amplification of the ITS regions

The ITS regions were successfully amplified from all the dermatophytes by the fungus-specific universal primers ITS1 and ITS4. The ITS fragments of the 17 species listed in Table 1 were less than 300 bp. The length of ITS1 ranged from 169 bp (*Microsporum gallinae*) to 293 bp (*Epidermophyton floccosum*), while the length of ITS2 ranged from 174 bp (*T. terrestre*) to 243 bp (*E. floccosum*) (data not shown). Among the 17 dermatophyte species, *E. floccosum* could be easily recognized since it possessed the longest ITS1 (293 bp) and ITS2 (243 bp) sequences.

Barcode sequences in the ITS regions

Multiple sequence alignment demonstrated that some dermatophyte species possessed barcode sequences (or species-specific sequences) in the ITS1 and/or ITS2 regions. For *T. soudanense*, two ITS1 genotypes were found in different strains, with one genotype having a deletion of 36 bases starting at position 122, e.g. ATCC 64654 (Fig. 1). Barcode sequences were found in *Trichophyton rubrum*, *T. soudanense* and *T. violaceum* in the ITS1 region at positions 167, 209 and 214 (Fig. 1). In the ITS2 region, barcode sequences also appeared in *T. rubrum* at positions 106 and 124 (Fig. 2). In addition, a variable number (ranging from 6 to 15) of AT repeats starting at position 164 was seen for strains of *T. soudanense* and *T. violaceum* (Fig. 2). In contrast, the number of AT repeats was relatively constant (six or seven) in strains of *T. rubrum*. These barcode sequences may be useful for differentiating species of *T. rubrum*, *T. soudanense* and *T. violaceum* when the results of conventional identification are ambiguous. Clinical isolates of the three *Trichophyton* species also displayed the same barcode sequences shown in Figs 1 and 2 (data not shown). Editing of the PCR product sequences to remove non-ITS sequences helps in the recognition of these barcode sequences at the indicated positions.

Table 1. Dermatophyte strains used in this study and strains producing discrepant identification by ITS sequencing

Species (teleomorph)	Reference strain*	Clinical isolate†	No. of discrepant strains/ total no. of strains
<i>E. floccosum</i>	ATCC 9646, ATCC 52066	NCKU 1862, NCKU 1973, NCKU 2827	0/5
<i>M. audouinii</i>	ATCC 10008, ATCC 10216, ATCC 42558	LMA 597, LMA 854, LMA 971, LMA 941276, LMA 50002007, LMA 50500271, LMA 50501240, LMA 50600031	0/11
<i>M. canis</i> (<i>Arthroderma otae</i>)‡	ATCC 10214, CBS 282.63, CBS 643.74	LM 40, LMA 720, LMA 921, LMA 922, LMA 94.595, LMA 94.604, LMA 5990586, LMA 50000068, LMA 50000070, LMA 50000071, LMA 50000091, LMA 50200087, LMA 50401206, NCKU 1200, NCKU 3401, NCKU 3404, NCKU 3486	1/20
<i>Microsporum cookei</i> (<i>Arthroderma cajetanum</i>)‡	CBS 129.67, CBS 202.66, CBS 227.58, CBS 228.58; IHEM 14178, IHEM 14180	LMA 94.548	0/7
<i>M. ferrugineum</i>	ATCC 11992, ATCC 42560, ATCC 52060	NCKU 1582, NCKU 1627, NCKU 2513, NCKU 3385	0/7
<i>M. gallinae</i> (<i>Arthroderma grubyi</i>)‡	CBS 133.89, CBS 215.38, CBS 221.55		0/3
<i>M. gypseum</i> (<i>Arthroderma gypseum</i>)‡	BCRC 30542, CBS 161.69, CBS 286.63, CBS 311.61	LMA 961, LMA 90.603, LMA 93.629, LMA 94.446, LMA 91.1379, LMA 98.1320, LMA 98.01190, LMA 50401101, NCKU 2001, NCKU 3386	1/14
<i>M. nanum</i> (<i>Arthroderma obtusum</i>)‡	CBS 321.61, CBS 569.80, CBS 632.82, IHEM 19092, IHEM 21111		0/5
<i>M. persicolor</i> (<i>Arthroderma persicolor</i>)‡	CBS 421.74, CBS 468.74	LMA 90.76, LMA 92.136, 95.974, LMA 95.1741, LMA 40401293, LMA 50401174	0/8
<i>T. erinacei</i>	IHEM 15931, IHEM 20118		0/2
<i>T. interdigitale</i> (<i>Arthroderma vanbreuseghemii</i>)‡	ATCC 200099, CBS 101.68, CBS 436.73	LMA 905.92, LMA 91.309, LMA 94.677, LMA 95.1682, LMA 50000604, LMA 50000617, LMA 50000627, LMA 50300859, LMA 50300894, LMA 50401331, LMA 50401377, LMA 59800858 (all from human tinea pedis, except LMA 50300894, LMA 50401331 and LMA 50401377 from tinea unguium)	2/15
<i>T. mentagrophytes</i>	BCRC 32066, CBS 160.66, CBS 361.62	LMA 90.791, LMA 95.1932, LMA 50100370, LMA 50400833, LMA 59801326, NCKU 3361, NCKU 3389, NCKU 3534 (all from human tinea corporis, except LMA 50100370 and LMA 50400833 from tinea manuum, NCKU 3534 from tinea faciei)	11/11
<i>T. rubrum</i>	ATCC 28188, BCRC 32805, BCRC 32806	LMA 559, LMA 569, LMA 585, LMA 590, LMA 606, LMA 610, LMA 679, LMA 685, LMA 9025, LMA 90.412, LMA 96.432, LMA 9.7858, LMA 95.00311; NCKU 1213, NCKU 3399, NCKU 3591	0/19
<i>Trichophyton schoenleinii</i>	ATCC 22775, ATCC 22776, CBS 174.40		0/3
<i>T. soudanense</i>	ATCC 24583, ATCC 52936, ATCC 64654	LMA 831, LMA 835, LMA 966, LMA 979, LMA 95.32, LMA 96.248, LMA 95.1336, LMA 50401328, LMA 50500572, LMA 50500769, LMA 50500944, LMA 50600067, LMA 59900985	6/16
<i>T. terrestre</i> (<i>Arthroderma quadrifidum</i>)‡	CBS 117.61, CBS 464.62, CBS 465.62, CBS 567.94	LMA 92.347, LMA 93.293, LMA 7971445, LMA 50200212, LMA 59901091	0/9

Table 1. cont.

Species (teleomorph)	Reference strain*	Clinical isolate†	No. of discrepant strains/ total no. of strains
<i>T. tonsurans</i>	ATCC 10217, ATCC 10220	LMA 856, LMA 857, LMA 50500106, LMA 50500764, LMA 50600039, NCKU 3059, NCKU 3156, NCKU 3166, NCKU 3364, NCKU 3383, NCKU 3549	1/13
<i>T. verrucosum</i>	ATCC 28203, ATCC 28943, ATCC 38485 IHEM 5480, IHEM 17755	LMA <u>40204990</u> , LMA <u>50200673</u>	2/7
<i>T. violaceum</i>	ATCC 24787, ATCC 28944, ATCC 62858	NCKU 3164, NCKU 3264, NCKU 3372, NCKU 3395, NCKU 3415	0/8
Total	62	126	24/188

*Strains were obtained from the following collections: ATCC, American Type Culture Collection; BCRC, Bioresources Collection and Research Center; CBS, Centraalbureau voor Schimmelcultures; IHEM, Belgian Co-ordinated Collections of Micro-organisms.

†Underlined strains produced discrepant results between conventional methods and ITS sequencing.

‡Only the teleomorph name was given on the CBS website.

The *Microsporum canis* complex consists of a cluster of phylogenetically closely related species (*Microsporum audouinii*, *M. canis* and *Microsporum ferrugineum*) (Kaszubiak *et al.*, 2004). High sequence similarities were observed in the ITS1 (99–100 % similarities) and ITS2 regions (94–100 % similarities) among members of the

complex (our unpublished data). Interestingly, barcode sequences were found in the ITS2 regions at nucleotide positions 45 to 47 (a 3 nt deletion in *M. audouinii*), 52 and 170 (Fig. 3). Clinical isolates of the three *Microsporum* species also displayed the same barcode sequences (data not shown).

Fig. 1. Partial sequence alignment and barcode sequences in the ITS1 regions in species of the *T. rubrum* complex. For *T. soudanense*, two ITS1 genotypes were found in different strains, with one genotype having a deletion of 36 bases starting at position 122. In *T. rubrum*, the base at position 167 was A, while the base was G in *T. soudanense* and *T. violaceum*. *T. violaceum* was differentiated from *T. rubrum* and *T. soudanense* by the base G at position 209; the base was A in *T. rubrum* and *T. soudanense*. A single base deletion at position 214 was found in *T. violaceum*. *T. raubitschekii* ATCC 42631 had an identical sequence to *T. rubrum*. *T. gourvilii* var. *intermedium* CBS 170.65, with a deletion of 36 bases starting at position 122, had an identical sequence with one genotype of *T. soudanense*. All the sequences were determined in this study and have been submitted to GenBank. The species and the corresponding GenBank accession number are indicated on the left of each sequence. Only one to two strains of each species are shown.

Fig. 2. Partial sequence alignment and barcode sequences in the ITS2 regions in species of the *T. rubrum* complex. In *T. rubrum*, the bases at positions 106 and 124 were A and T, respectively, while they were G and A, respectively, in *T. soudanense* and *T. violaceum*. *T. raubitschekii* ATCC 42631 had an identical sequence to *T. rubrum*, while *T. gourvilii* var. *intermedium* CBS 170.65 had an identical sequence to *T. soudanense* and *T. violaceum*, except for the number of AT repeats. The number of AT repeats at the 3' end was either 6 or 7 in *T. rubrum*, while it ranged from 6–15 in *T. soudanense* and *T. violaceum*. *T. gourvilii* var. *intermedium* had an extraordinarily high number (31) of AT repeats. All the sequences were determined in this study and have been submitted to GenBank. The species and the corresponding GenBank accession number are indicated on the left of each sequence. Only one to two strains of each species are shown.

Identification of the *T. mentagrophytes* complex by ITS sequencing

The two reference strains of *T. erinacei* (IHEM 15931 and IHEM 20118) were correctly identified based on the constructed in-house database (Table 1). All *T. interdigitale* strains, except LMA 95.1682 and LMA 59800858, were correctly identified by ITS sequence analysis. *T. interdigitale* LMA 95.1682 was not identified, since there was no matching sequence (identity <97%) in the current in-house ITS database and public databases (Table 2). At both

ITS regions, *T. interdigitale* LMA 59800858 (isolated from a woman with tinea pedis) displayed higher similarities with the type strain *T. erinacei* CBS 511.73 than with the neotype strain *T. interdigitale* BCRC 31782 (Table 2), but the strain had a similarity of 100% with the sequence of the *T. interdigitale* type strain BCRC 31782 (=CBS 642.73=ATCC 28146) at the D1–D2 region. Thus, the identity of *T. interdigitale* LMA 59800858 was not resolved.

Surprisingly, it was found that the three *T. mentagrophytes* reference strains (BCRC 32066, CBS 160.66 and CBS

Fig. 3. Partial sequence alignment and barcode sequences in the ITS2 regions in species of the *M. canis* complex. In *M. ferrugineum*, the base at position 52 was C, but it was T in *M. audouinii* and *M. canis*. The base at position 170 was T in *M. audouinii*, while it was C in *M. canis* and *M. ferrugineum*. There was a 3 base deletion at positions 45–47 in *M. audouinii*. All the sequences were determined in this study and have been submitted to GenBank. The species and the corresponding GenBank accession number are indicated on the left of each sequence. Only two strains of each species are shown.

Table 2. Summary of the analysis of clinical isolates that produced discrepant results between the conventional methods and ITS sequencing

Strain	Species received as:	Species identification (percentage identity with reference sequence in GenBank or database) by:			Final identification
		ITS1 sequencing	ITS2 sequencing	D1–D2 sequencing*	
LMA 922	<i>M. canis</i>	NI	NI	<i>Chrysosporium tropicum</i> (97.7)	NI
LMA 90.603	<i>M. gypseum</i>	<i>M. canis</i> (42) <i>M. fulvum</i> (99.5) <i>M. gypseum</i> (87.1)	<i>M. canis</i> (46) <i>M. fulvum</i> (99.5) <i>M. gypseum</i> (94.9)	<i>M. fulvum</i> (99.7) <i>M. gypseum</i> (99.7)	<i>M. fulvum</i>
LMA 95.1682	<i>T. interdigitale</i>	NI	NI	<i>Apinisia graminicola</i> (97.7)	NI
LMA 59800858	<i>T. interdigitale</i>	<i>T. mentagrophytes</i> (56) <i>T. erinacei</i> (97.2) <i>T. interdigitale</i> (87.5)	<i>T. mentagrophytes</i> (57) <i>T. erinacei</i> (97.9) <i>T. interdigitale</i> (85.9)	<i>T. interdigitale</i> (100) <i>T. erinacei</i> (97.5)	NI
BCRC 32066	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.0) <i>T. mentagrophytes</i> (96.5)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (99.8)	<i>T. interdigitale</i>
CBS 160.66	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.1) <i>T. mentagrophytes</i> (96.5)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (99.5)	<i>T. interdigitale</i>
CBS 361.62	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.8) <i>T. mentagrophytes</i> (97.3)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (99.5)	<i>T. interdigitale</i>
LMA 90.791	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (97.7) <i>T. mentagrophytes</i> (96.1)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (100)	<i>T. interdigitale</i>
95.1932	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (97.7) <i>T. mentagrophytes</i> (96.1)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (99.8)	<i>T. interdigitale</i>
50100370	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.1) <i>T. mentagrophytes</i> (96.5)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (99.5)	<i>T. interdigitale</i>
50400833	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.0) <i>T. mentagrophytes</i> (96.5)	<i>T. interdigitale</i> (100) <i>T. mentagrophytes</i> (94.1)	<i>T. interdigitale</i> (100)	<i>T. interdigitale</i>
59801326	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (97.3) <i>T. mentagrophytes</i> (95.7)	<i>T. interdigitale</i> (99.5) <i>T. mentagrophytes</i> (94.6)	<i>T. interdigitale</i> (99.8)	<i>T. interdigitale</i>
NCKU 3361	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.8) <i>T. mentagrophytes</i> (97.3)	<i>T. interdigitale</i> (100) <i>T. mentagrophytes</i> (94.1)	<i>T. interdigitale</i> (100)	<i>T. interdigitale</i>
NCKU 3389	<i>T. mentagrophytes</i>	<i>T. interdigitale</i> (98.8) <i>T. mentagrophytes</i> (97.3)	<i>T. interdigitale</i> (100) <i>T. mentagrophytes</i> (94.1)	<i>T. interdigitale</i> (100)	<i>T. interdigitale</i>
NCKU 3534	<i>T. mentagrophytes</i>	<i>T. erinacei</i> (98.0) <i>T. interdigitale</i> (87.5)	<i>T. erinacei</i> (97.3) <i>T. interdigitale</i> (85.9)	<i>T. erinacei</i> (99.6) <i>T. interdigitale</i> (97.5)	<i>T. erinacei</i>
LMA 831	<i>T. soudanense</i>	<i>T. violaceum</i> (97.7) <i>T. soudanense</i> (96.6)	<i>T. violaceum</i> (100) <i>T. soudanense</i> (100)	<i>T. violaceum</i> (99.8)	<i>T. violaceum</i>
LMA 835	<i>T. soudanense</i>	<i>T. violaceum</i> (97.7) <i>T. soudanense</i> (97.3)	<i>T. violaceum</i> (100) <i>T. soudanense</i> (100)	<i>T. violaceum</i> (99.8)	<i>T. violaceum</i>
LMA 979	<i>T. soudanense</i>	<i>T. violaceum</i> (100) <i>T. soudanense</i> (98.9)	<i>T. violaceum</i> (99.0) <i>T. soudanense</i> (99.0)	<i>T. violaceum</i> (99.7)	<i>T. violaceum</i>
LMA 95.1336	<i>T. soudanense</i>	<i>T. tonsurans</i> (99.2) <i>T. soudanense</i> (77)	<i>T. tonsurans</i> (100) <i>T. soudanense</i> (81)	<i>T. tonsurans</i> (100)	<i>T. tonsurans</i>
LMA 50500944	<i>T. soudanense</i>	<i>T. violaceum</i> (99.6) <i>T. soudanense</i> (96.2)	<i>T. violaceum</i> (100) <i>T. soudanense</i> (100)	<i>T. violaceum</i> (99.8)	<i>T. violaceum</i>
LMA 50600067	<i>T. soudanense</i>	<i>T. violaceum</i> (100) <i>T. soudanense</i> (98.9)	<i>T. violaceum</i> (100) <i>T. soudanense</i> (100)	<i>T. violaceum</i> (99.7)	<i>T. violaceum</i>
NCKU 3156	<i>T. tonsurans</i>	<i>T. rubrum</i> (100) <i>T. tonsurans</i> (79)	<i>T. rubrum</i> (100) <i>T. tonsurans</i> (80)	<i>T. rubrum</i> (100)	<i>T. rubrum</i>
LMA 40204990	<i>T. verrucosum</i>	<i>T. rubrum</i> (100) <i>T. verrucosum</i> (87.4)	<i>T. rubrum</i> (100) <i>T. verrucosum</i> (93.5)	<i>T. rubrum</i> (100)	<i>T. rubrum</i>
LMA 50200673	<i>T. verrucosum</i>	<i>T. rubrum</i> (100) <i>T. verrucosum</i> (87.4)	<i>T. rubrum</i> (100) <i>T. verrucosum</i> (93.5)	<i>T. rubrum</i> (100)	<i>T. rubrum</i>

NI, Not identified

*Results of BLAST search of GenBank.

361.62) and all clinical isolates of the species, except the strain NCKU 3534, were identified as *T. interdigitale* by ITS sequence analyses. Both ITS1 and ITS2 sequences of these *T. mentagrophytes* strains, except NCKU 3534, had higher similarities with the type strain *T. interdigitale* BCRC 31782 than with the neotype strain *T. mentagrophytes* CBS 318.56 (Table 2). *T. mentagrophytes* NCKU 3534 (isolated from a woman with tinea faciei) displayed high similarities with the type strain *T. erinacei* CBS 511.73 at both ITS and D1–D2 regions (Table 2). These results indicate that most human isolates of the *T. mentagrophytes* complex are *T. interdigitale*, with a few isolates, e.g. *T. mentagrophytes* NCKU 3534, being *T. erinacei*.

Identification of the other dermatophyte species by ITS sequencing

In addition to strains of the *T. mentagrophytes* complex, 11 isolates of other species produced discrepant identifications between the conventional identification methods and ITS sequencing (Table 2). *M. canis* LMA 922 was not identified by ITS sequence analysis, since there was no matching sequence (identity <97%) in the public databases. Sequencing of the D1–D2 domain also failed to identify the isolate to species level. The remaining 10 discordant clinical isolates were correctly identified to species level by ITS1 sequence analysis, as further confirmed by sequencing of the D1–D2 regions of these isolates (Table 2). Since the identity of two strains (LMA 922 and LMA 95.1682) was not resolved, they were excluded from the performance evaluation of ITS sequencing for species identification. In summary, all reference strains and clinical isolates were correctly identified by ITS1 sequencing, producing an identification rate of 100% (186/186). Conversely, sequence analysis of the ITS2 region revealed that five discrepant clinical isolates (*T. soudanense* LMA 831, LMA 835, LMA 979, LMA 50500944 and LMA 50600067) had identical similarities with reference sequences of *T. soudanense* and *T. violaceum* (Table 2). This was due to the fact that *T. soudanense* and *T. violaceum* have identical ITS2 sequences, although strains of these two species may differ in the number of AT repeats at the end of the ITS2 region (Fig. 2). Thus, five clinical isolates were not identified by ITS2 sequencing, and the identification rate by sequencing of this region was 97.3% (181/186).

Among the clinical isolates misidentified by conventional methods, five were *T. violaceum* (misidentified as *T. soudanense*) and three were *T. rubrum* (misidentified as *T. tonsurans* or *Trichophyton verrucosum*) (Table 2). These results reflect that phenotypic differentiation of these *Trichophyton* species may be ambiguous sometimes. They suggest that the morphological features reported for *T. soudanense*, e.g. the presence of reflexive hyphae (Larone, 2002), are not an exclusive characteristic for this species. *T. soudanense* was reduced to synonymy with *T. violaceum*, based on amplified fragment length polymorphism analysis, PCR fingerprinting and the ITS sequence (Gräser *et al.*

et al., 2000b). *T. soudanense* is also recognized as a synonym of *T. violaceum* (De Hoog *et al.*, 2000). But the unification of *T. soudanense* and *T. violaceum* may conceal possible evolutionary diversification, since both species have their unique geographical distributions (Larone 2002; Padhye & Summerbell, 2005). In contrast, analysis of microsatellite DNA markers demonstrated that *T. soudanense* is somewhat closer to *T. rubrum* than to *T. violaceum* (Ohst *et al.*, 2004). In this study, the three *Trichophyton* species were clearly differentiated by their barcode sequences in the ITS1 region (Fig. 1), a result that supports the idea that *T. rubrum*, *T. soudanense* and *T. violaceum* are distinct species. Very recently, Summerbell *et al.* (2007) reported the presence of ITS barcode sequences (single nucleotide polymorphism and repeat adenine bases) in *T. tonsurans* and *Trichophyton equinum*. They proposed that these discrete barcode sequences for each species will aid future identifications involving molecular methods.

Since *Trichophyton raubitschekii* and *Trichophyton gourvilii* are closely related to *T. rubrum* (Gräser *et al.*, 2000b, 2007), the ITS regions of *T. raubitschekii* ATCC 42631 and *T. gourvilii* var. *intermedium* CBS 170.65 were also sequenced and aligned in this study (Fig. 1). *T. raubitschekii* ATCC 42631 had identical sequences to *T. rubrum* in both ITS regions and presented, as did *T. rubrum*, a small number (seven) of AT repeats at the 3' end of the ITS2 region (Fig. 2). Therefore, it is most likely that *T. raubitschekii* is a variant of *T. rubrum* as suggested by Gräser *et al.* (2000b). In the ITS1 region, *T. gourvilii* var. *intermedium* CBS 170.65 had a deletion of 36 bases starting at position 122 as did one genotype of *T. soudanense* (Fig. 1). Analysis of the ITS2 region revealed that *T. gourvilii* var. *intermedium* CBS 170.65 had an identical sequence to reference sequences of *T. soudanense* and *T. violaceum*, but the strain had an extraordinary number (31) of AT repeats at the end of this region (Fig. 2). Based on these observations, it is proposed that *T. gourvilii* represents a distinct species from *T. rubrum* and it may not be conspecific as *T. violaceum*. *T. gourvilii* was considered to be a synonym of *T. violaceum* (Otčenášek & Dvůrák, 1975) and this was further supported by Gräser *et al.* (2000b).

The clinical isolate *Microsporium gypseum* LMA 90.603 was a misidentification of *Microsporium fulvum*, as revealed by its ITS1 and ITS2 sequences (Table 2). It is not easy to differentiate *M. gypseum* from *M. fulvum* on the basis of morphological criteria (De Hoog *et al.*, 2000). To the best of our knowledge, *M. fulvum* is not described as a human pathogen (De Hoog *et al.*, 2000), and the misidentification of *M. fulvum* as *M. gypseum* raises an important question about this. The isolate LMA 90.603 was recovered from a patient with an inflammatory lesion of the left thigh. So, it would be interesting to verify the morphologically based identification of *M. gypseum* isolates of human origin in order to determine the prevalence of *M. fulvum* in dermatophytosis.

The present method can be completed within approximately 24 h from isolated colonies. The results described

herein also reveal the importance of using type (or neotype) strains as a basis for sequence comparison, especially in identifying members of the *T. mentagrophytes* complex (Table 2). It is to our surprise that three reference strains of *T. mentagrophytes* (BCRC 32066, CBS 160.66 and CBS 361.62) had higher sequence similarities with *T. interdigitale* BCRC 31782 (type strain) than with *T. mentagrophytes* CBS 318.56 (neotype strain) in both ITS and D1–D2 regions. The species status of these three reference strains should be re-evaluated.

It was noted that the ITS1 region is a better target for differentiating *T. rubrum*, *T. soudanense* and *T. violaceum*, since the latter two species had identical sequence in the ITS2 region (Fig. 2). In contrast, the ITS2 region constitutes a better target for differentiating members of the *M. canis* complex (*M. audouinii*, *M. canis* and *M. ferrugineum*), since a few single nucleotide polymorphisms (barcode sequences) were found in the ITS2 region (Fig. 3). The reported method is straight forward, but the main issue that would detract from the application of this approach in routine clinical laboratories is the added cost. However, it is likely that many laboratories would find a use for this approach to identify a minority of atypical isolates or those leading to ambiguous results with conventional identification.

ACKNOWLEDGEMENTS

This project was supported by grants (96-2320-B-006-024-MY3 and 96-EC-17 A-10-S1-0013) from the National Science Council and Department of Economic Affairs, Taiwan, Republic of China.

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