



## Use of Single-enzyme PCR-restriction Digestion Barcode Targeting the Internal Transcribed Spacers (ITS rDNA) to Identify Dermatophyte Species

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### Abstract

**Background:** Dermatophytes are the most common causative agents of superficial mycoses. Species identification of these fungi is important from therapeutic and epidemiological point of view. Traditional approaches for identification of dermatophytes at the species level, relying on macroscopic and microscopic features of the colonies, usually are time-consuming and unreliable in many circumstances. Recently a broad varieties of rapid and accurate DNA-based techniques were successfully utilized for species delineation of dermatophytes.

**Methods:** The ITS1-5.8S-ITS2 region of rDNA from various reference strains of dermatophyte species were amplified using the universal fungal primers ITS1 and ITS4. The PCR products were digested by a single restriction enzyme, *Mva*I. The enzyme was evaluated in both *in silico* and practical PCR-RFLP assay to find the exact differentiating restriction profiles for each species. To validate the standardized PCR-RFLP system, all tested strains were subjected to sequencing and sequence analysis.

**Results:** The obtained RFLP patterns were specific for many species including *T. interdigitale*, *T. rubrum*, *T. violaceum*, *M. persicolor*, *M. audouinii*, *M. nanum* (*A. obtusum*) and *E. floccosum* but were similar for some closely related species such as *M. canis* / *M. ferrugineum*. Sequencing of the ITS1-5.8S-ITS2 fragment from all type strains affirmed the RFLP findings.

**Conclusion:** It was practically revealed that the ITS-PCR followed by *Mva*I-RFLP is a useful and reliable schema for identification and differentiation of several pathogenic species and can be used for rapid screening of even closely related species of dermatophytes in clinical and epidemiological settings.

**Keywords:** Dermatophytes, Identification, ITS, PCR-RFLP

### Introduction

Dermatophytes are a group of specialized molds, affecting the superficial keratinized structures (skin, hair and nails) of human and animal hosts, producing dermatophytosis, commonly referred to as 'ringworm' or tinea. They are classified in three genera, *Epidermophyton*, *Microsporum*, and *Trichophyton* containing three ecological

groups of anthropophilic, zoophilic and geophilic species (1, 2). Dermatophytes are the most common agents of cutaneous fungal infections worldwide (3, 4). Infections are contagious and represent a significant public health problem in many parts of the world. Dermatophytosis is not a reportable disease but

is a matter of concern because of its contagiousness nature (5).

Correct identification of dermatophytes at the species level is useful for differentiating between dermatophytosis and dermatomycosis (6), to control of environmental and animal sources of infection and help for developing the preventive strategies (7). From clinical point of view, for definition of species and performance of an epidemiologic study it is important to have a reliable method for identification of dermatophytes (8). Species-level identification of these fungi classically relies on macro and micro morphological features of the colonies on general and specific culture media and on some biochemical and physiological complementary tests (1). However, in many circumstances phenotypic characteristics overlap between species, and many isolates have atypical nature in primary isolation thus attempt for final identification is time consuming and requires expertised personel on microscopical properties (2, 9-11). By development of PCR technology, a wide variety of molecular techniques such as RAPD-PCR, Nested-PCR, PCR-RFLP, PCR-EIA, Real-time PCR and microarray technology were employed as possible alternatives for routine identification of fungi including dermatophytes (2).

At the present study, the ITS1-5.8S-ITS2 fragment of ribosomal DNA gene (rDNA) in the dermatophyte species were used as a reliable marker for species identification. We retrieved the reliable sequences of internal transcribed spacers (ITS) regions from GenBank, then computationally (*in-silico*) and practically subjected them to a polymerase chain reaction-restriction enzyme (PCR-RE) assay for identifying nearly all pathogenic dermatophyte species. Additionally, we amplified and digested the DNA target in some reference dermatophyte strains to confirm the method. We prepared a relatively perfect restriction fragment length polymorphism (RFLP) barcode by using only a single enzyme and believe that it could be useful for clinical and epidemiological aims.

## Material and Methods

### Virtual restriction enzyme digestion

The complete sequence of internal transcribed spacers 1 and 2 regions and the 5.8S ribosomal DNA subunit flanked these regions were retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/nucore>). The sequences were edited by MEGA 4 software (MEGA version 4, Tamura, Dudley, Nei, and Kumar 2007), and subjected to popular sequence alignment tool of ClustalW. The arranged sequences were then exported to DNASIS software (Hitachi DNASIS<sup>®</sup> MAX v3.0) and subjected to digital digestion with 610 restriction enzymes included in the software. The enzymes with the best discriminatory power were selected and species-specific RFLP profiles were determined.

### Reference strains

To assess the actual feasibility and applicability of the *in silico* PCR-RFLP findings, twenty five reference strains of different dermatophyte species were prepared from two reference collections: NBRC (NITE Biological Resource Center) and JCM (Japan Collection of Microorganisms) and used in the study. The strains were *T. mentagrophytes* (NBRC 5974, NBRC 5809, NBRC 5466), *T. mentagrophytes* var. *interdigitale* (NBRC 5812), *Arthroderma vanbreuseghemii* (JCM 1891), *A. benhamiae* (JCM 1885), *M. persicolor* (NBRC 5975), *T. tonsurans* (NBRC 5928, NBRC 5945), *T. equinum* (NBRC 31610), *T. rubrum* (NBRC 5808, NBRC 5467), *T. violaceum* (NBRC 31064), *E. floccosum* (NBRC 9045), *M. canis* (NBRC 9182), *M. ferrugineum* (NBRC 6081, NBRC 5831), *M. audouinii* (NBRC 6074), *A. obtusum* (JCM 1907), *A. uncinatum* (NBRC 31978), *T. schoenleinii* (NBRC 8192, NBRC 8191), *M. cookei* (NBRC 7862), and *M. gypseum* (NBRC 8228, NBRC 5948). Strains inoculated into the plates containing Potato Dextrose Agar (PDA) medium (Difco, USA) and incubated at 28°C for 2-3 weeks till the colonies came up.

### DNA extraction

DNA was extracted from the strains using the method described by Makimura et al. (12). Briefly, amount (approximately 5 cubic millimeter) of a fresh colony was placed in lysis buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 0.5% w/v SDS, 250 mM NaCl), and crushed with a conical grinder. Samples were incubated for 20 min at 100°C and mixed with 150 µl of 3.0 M sodium acetate, kept at -20°C for 10 min and centrifuged at 12,000 g for 10 min. The supernatants were extracted once with phenol chloroform iso-amyl alcohol (25:24:1), and subsequently extracted once again with chloroform. DNA was precipitated with an equal volume of iso-propanol, washed with 300 µl of 70% ethanol, dried and suspended in 50 µl of ultrapure water. The final solution was kept at -20 °C until using as template for PCR.

### Primers and PCR condition

The universal fungal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (11) were used to amplify the entire ITS rDNA region in the standard strains. Amplification was carried out by a PCR mixture contained 2.5 µl of 10X reaction buffer, 200 µM of dNTPs mixture, 0.125 µl of *Taq* polymerase (5 U/µl), 30 pmol of each forward and reverse primers, 1 µl of DNA template solution and enough ultrapure water up to a final volume of 25 µl. Each reaction mixture was preheated to 94°C for 6 minutes, then PCR performed by the following protocol: 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 1 min at 72°C; a final extension at 72°C for 10 min and followed by cooling at 4°C.

### Restriction digestion of the PCR products

The amplified products were subjected to digestion with *Mva*I Fast digest (Fermentas Life Sciences, Lithuania) for 10 min at 37°C. The reaction mixture contained 10 µl of PCR amplicons, 0.5 µl of the enzyme, 1.5 µl of 10X buffer and 3 µl of water to a final volume of 15 µl.

### Detection of amplified products and restriction digestion

PCR amplicons were separated by running the 5 µl of products in a 1.5% (w/v) agarose gel incorporated with 2 µl ethidium bromide and electrophoresed in TBE (90mM Tris, 90 mM boric acid, 2 mM EDTA) at 100V for 60 min. A 10 µl aliquot of restriction digestion products were separated by running in a 2% agarose gel. A 100 base pair (bp) ladder was used as DNA molecular weight marker in each run. The gels were visualized using gel documentation system and recorded photographically, then were compared with the profiles obtained by *in silico* analysis.

### Sequencing and multiple alignments

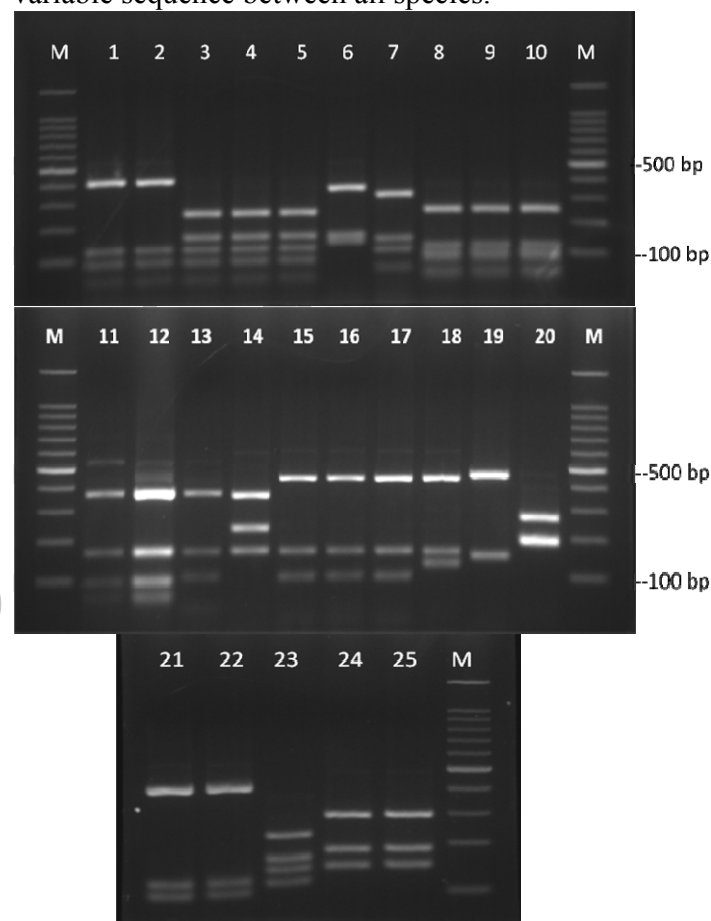
All reference strains which preliminarily identified by PCR-RFLP, were sequenced by both ITS1 and ITS4 primers using an automated DNA Sequencer (ABI PRISM™ ABI-3730 Genetic Analyzer, PE Applied Biosystem). For final identification, the obtained consensus sequences were compared with the Dermatophytes ITS DNA barcode database (<http://www.cbs.knaw.nl/dermatophytes/BioLoMICSID.aspx>). Alignment of the obtained edited forward and reverse sequences was conducted using BioEdit software: (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>)

## Results

Alignments of consensus sequences by MEGA software showed that almost all dermatophyte species have expectedly similar sequence in 5.8S subunit but are different in ITS1 and ITS2 non-coding regions of rDNA complex. The size of entire ITS1-5.8S-ITS2 fragment (including primers) ranked between 614 bp for *M. gallinae* to 780 bp for *E. floccosum* (Table 1). The dissimilarities seemed to be enough to select the enzymes for distinguishing between the species in a PCR-RE system. *In silico* analysis of the sequences by DNASIS software revealed that

many restriction enzymes can digest the intended sequences. Some enzymes had no cutting site in all or some species. Some others had many cutting sites; however, the sites were not sufficiently divergent between different species and could not meet our purpose (data not shown). Finally *MvaI* was considered as the enzyme with the most discriminatory power for differentiation of many species. Table 1 shows the cutting sites and produced fragments from ITS regions after digestion with *MvaI*. The ITS1 region was successfully amplified in all tested strains using the ITS1/ITS4 primers. The obtained bands were variable in size among different species as the biggest size for *E. floccosum* and the smallest one for *A. obtusum* (data not shown). In actual restriction digestion of the amplified products by *MvaI* all achieved electrophoretic patterns were congruent with those findings in *in-silico* analysis (Fig. 1). Some species including *T. interdigitale*, *T. rubrum*, *T. violaceum*, *M. persicolor*, *M. audouinii*, *M. nanum* (*A. obtusum*) and *E. floccosum* produced specific profile in both virtual and actual ITS-RFLP with *MvaI* (Table 1 and 2, Fig. 1), however some closely related species like *T. equinum* / *T. tonsurans*, *M. canis* / *M. ferrugineum* and *M. cookei* / *M. racemosum* had the same profiles. We did not use *T. simii*, *T. verrucosum*, *M. fulvum* and *M. gallinae* in our experiment, however, *T. simii* produced two patterns and three other species also produced unique pattern in computational PCR-RFLP (Table 1). In RFLP analysis, two different patterns were distinguished for *M. gypseum* and *T. ajelloi* (*A. uncinatum*) (Table 1); nonetheless, it was observed only one electrophoretic profile for each species (Table 2, Fig. 1; lanes 20, 24-25). The *in silico* *MvaI*-restriction profiles for *T. gourvilii*, *T. soudanense* and *T. yaoundei* resembled to the *T. violaceum*. Comparing the obtained sequences of all reference strains with the open access validated CBS-database confirmed their species identification by established RFLP system. The comparison of sequencing data and PCR-RFLP profiles for identification of the

tested strains were outlined in Table 2. The overall alignment of ITS1-5.8S-ITS2 sequences for all 25 standard strains was illustrated in Figure 2. As it observed, the sequence of 5.8S subunit is relatively similar among all type strains species but both ITS1 and ITS2 regions have variable sequence between all species.



**Fig. 1:** Electrophoretic patterns of ITS-RFLP with *MvaI* for reference dermatophyte species.

Lanes 1, 3 and 5: *T. mentagrophytes* (NBRC 5809, NBRC 5974 and NBRC 5466), lane 2: *A. vanbreuseghemii* (JCM 1891), lane 4: *T. mentagrophytes* var. *interdigitale* (NBRC 5812), lane 6: *A. benhamiae* (JCM 1885), lane 7: *M. persicolor* (NBRC 5975), lane 8-9: *T. tonsurans* (NBRC 5928, NBRC 5945), lane 10: *T. equinum* (NBRC 31610), lane 11-12: *T. rubrum* (NBRC 5808, NBRC 5467), lane 13: *T. violaceum* (NBRC 31064), lane 14: *E. floccosum* (NBRC 9045), lane 15: *M. canis* (NBRC 9182), lane 16 and 17: *M. ferrugineum* (NBRC 6081, NBRC 5831), lane 18: *M. audouinii* (NBRC 6074), lane 19: *A. obtusum* (JCM 1907), lane 20: *A. uncinatum* (NBRC 31978), lane 21 and 22: *T. schoenleinii* (NBRC 8192, NBRC 8191), lane 23: *M. cookei* (NBRC 7862), lane 24 and 25: *M. gypseum* (NBRC 8228, NBRC 5948), lanes M: 100 bp Ladder

**Table 1:** Fragment size of the ITS1-5.8S-ITS2 regions in tested species of dermatophytes before and after *in silico* digestion with *MvaI*

Species of dermatophyte	GenBank accession no. for ITS1-5.8S-ITS2 regions	Size of the ITS1-5.8S-ITS2 (bp)	Size of fragments after digestion with <i>MvaI</i>
<i>A. vanbreuseghemii</i> , <i>T. interdigitale</i>	AF170465, AJ270790	683	247, 159, 124, 89, 50, 14 (1 <sup>st</sup> pattern)
<i>A. vanbreuseghemii</i> ( <i>T. interdigitale</i> )	AB246678	683	406, 124, 89, 50, 14 (2 <sup>nd</sup> pattern)
<i>A. benhamiae</i>	Z98015	679	360, 158, 141, 20
<i>T. erinacei</i>	Z97997	679	360, 157, 142, 20
<i>T. simii</i>	Z98017	685	372, 159, 90, 50, 14 (1 <sup>st</sup> pattern)
<i>T. simii</i>	AJ000605	685	372, 159, 104, 50 (2 <sup>nd</sup> pattern)
<i>T. mentagrophytes</i> ( <i>T. m.</i> var <i>quinc-keanum</i> )	Z97995	683	406, 124, 103, 50
<i>T. schoenleinii</i>	Z98011	685	405, 124, 104, 52
<i>T. verrucosum</i>	Z98003	678	517, 141, 20
<i>M. persicolor</i>	EU181457	670	323, 148, 112, 68, 19
<i>T. rubrum</i>	AF170471	692	368, 164, 95, 65
<i>T. violaceum</i>	AJ270796, EU590656	701, 711	369, 161( <b>164</b> ), 106( <b>114</b> ), 45, 20
<i>T. gourvilii</i>	EU181448	710	368, 164, 113, 45, 20
<i>T. yaoundei</i>	AJ270811, FJ479792	700, 710	368, 164, 103 ( <b>113</b> ), 45, 20
<i>T. soudanense</i>	EF631621, AF170473	690	368, 164, 93, 45, 20
<i>T. tonsurans</i>	EF043270	688	251, 124, 103, 90, 56, 50, 14
<i>T. equinum</i>	EF043274	688	251, 124, 103, 90, 56, 50, 14
<i>E. floccosum</i>	AY213646	780	361, 231, 169, 20
<i>M. canis</i>	EU200371	737	441, 165, 103, 28
<i>M. ferrugineum</i>	EF581133	737	441, 165, 103, 28
<i>M. audouinii</i>	AJ000625	734	441, 162, 131
<i>M. nanum</i>	AJ970149	628	459, 150, 19
<i>M. gallinae</i> ( <i>M. vanbreuseghemii</i> )	AJ000620 (AJ970147)	614 ( <b>617</b> )	319 ( <b>323</b> ), 150 ( <b>149</b> ), 126, 19
<i>M. fulvum</i>	AM850135	652	322, 147, 112, 52, 19
<i>A. uncinatum</i> ( <i>T. ajelloi</i> )	EF568086	658	271, 195, 192 (1 <sup>st</sup> pattern)
<i>A. uncinatum</i> ( <i>T. ajelloi</i> )	AJ000608	657	465, 192 (2 <sup>nd</sup> pattern)
<i>A. gypseum</i> ( <i>M. gypseum</i> )	EF568061	666	289, 179, 146, 33, 19 (1 <sup>st</sup> pattern)
<i>A. incurvatum</i> ( <i>M. gypseum</i> )	AJ000621	619	389, 147, 64, 19 (2 <sup>nd</sup> pattern)
<i>M. racemosum</i>	AJ970146	701	225, 165, 144, 120, 47
<i>M. cookei</i>	AB193713	701	225, 166, 143, 120, 47

		10	20	30	40	50	60	70	80									
IFO 5948 - M. gypseu	1	TC	CCGTAGGTG	AACCTGCGGA	AGGATCATT	ACGCGCA	GGCCGTAGAC	GGCCCCG	CCCCG	59								
IFO 8228 - M. gypseu	1	.....	.....	.....	.....	.....	.....	.....	.....	59								
IFO 31978 - A. uncin	1	.....	.....	.....	A...C	...AC.T	...A...CGG	...G.C	...CGGTTGA	67								
JCM 1885 - A. benham	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGAATCAA	70								
JCM 1891 - A. vanbre	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
JCM 1907 - A. obtusu	1	.....	.....	.....	.....	...C.....	...TC.....	.....	.....	59								
NBRC 5466 - T. menta	1	.....	.....	G.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 5467 - T. rubru	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 5808 - T. rubru	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 5809 - T. menta	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 5812 - T. m. va	1	.....	.....	G.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 5831 - M. ferru	1	.....	.....	.....	...AGA	...T..A..TT	...CCGAA	...GCTCTT	...TCTCCCCCG	80								
NBRC 5928 - T. tonsu	1	.....	.....	.....	.....	...G..G	...C.G..CC	...AG..A	...TAGGGCCAAA	70								
NBRC 5945 - T. tonsu	1	.....	.....	.....	.....	...G..G	...C.G..CC	...AG..A	...TAGGGCCAAA	70								
NBRC 5974 - T. menta	1	.....	.....	G.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 5975 - M. persi	1	.....	.....	.....	.....	...C.....	...A...TC	.....	.....	59								
NBRC 6074 - M. audou	1	.....	.....	.....	...AGA	...T..A..TT	...CCGAA	...GCTCTT	...TCTCCCCCG	80								
NBRC 6081 - M. ferru	1	.....	.....	.....	...AGA	...T..A..TT	...CCGAA	...GCTCTT	...TCTCCCCCG	80								
NBRC 7862 - M. cooke	1	.....	.....	.....	...T.....	...CT.C	...T.G.TAC	...AT.C	...TCTCCCCCG	70								
NBRC 8191 - T. schoe	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 8192 - T. schoe	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 9045 - E. flocc	1	.....	.....	.....	.....	...C..T.....	...C.....	.....	.....	59								
NBRC 9182 - M. canis	1	.....	.....	.....	...AGA	...T..A..TT	...CCGAA	...GCTCTT	...TCTCCCCCG	80								
NBRC 31064 - T. viol	1	.....	.....	.....	.....	...G..G	...T.G..CC	...A..A	...TAGGGCCAAA	70								
NBRC 31610 - T. equi	1	.....	.....	.....	.....	...G..G	...T.G..CC	...AG..A	...TAGGGCCAAA	70								
IFO 5948 - M. gypseu	59	.....	.....	.....	.....	.....	.....	.....	.....	119								
IFO 8228 - M. gypseu	59	GATGGTCC	GG	GGGCGGTG	T	CGCCG	GCCACACG	CCCATCT	TGTCTAT	TT	ACCCAGTTGC	119						
IFO 31978 - A. uncin	68	GATATA.C	..A	..C..T.C	GAGGT	..AGTTG	.....	.....	.....	.....	.....	132						
JCM 1885 - A. benham	71	C.T.C.A.A	..GGT	GT.CA.A	..G	..TT..CC	.....	.....	.....	.....	.....	138						
JCM 1891 - A. vanbre	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
JCM 1907 - A. obtusu	59	..C.....	..GG	..C.....	.....	.....	.....	.....	.....	.....	.....	122						
NBRC 5466 - T. menta	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CCG	.....	.....	.....	.....	.....	138						
NBRC 5467 - T. rubru	71	C.T.C.A.A	..GGT	GA.CA.AC	..G	..GT..CC	.....	.....	.....	.....	.....	139						
NBRC 5808 - T. rubru	71	C.T.C.A.A	..GGT	GA.CA.AC	..G	..GT..CC	.....	.....	.....	.....	.....	139						
NBRC 5809 - T. menta	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGT	.....	.....	.....	.....	.....	138						
NBRC 5812 - T. m. va	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
NBRC 5831 - M. ferru	81	G.CCT.CCGG	..AGGTTGCG	GGC...AG	GGTGC.T	..G.....	.....	.....	.....	.....	.....	155						
NBRC 5928 - T. tonsu	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
NBRC 5945 - T. tonsu	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
NBRC 5974 - T. menta	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
NBRC 5975 - M. persi	59	..GCT.T.....	.....	..A.....	G.....	.....	.....	.....	.....	.....	.....	119						
NBRC 6074 - M. audou	81	G.CCT.CCGG	..AGGTTGCG	GGC...AG	GGTGC.T	..G.....	.....	.....	.....	.....	.....	155						
NBRC 6081 - M. ferru	81	G.CCT.CCGG	..AGGTTGCG	GGC...AG	GGTGC.T	..G.....	.....	.....	.....	.....	.....	155						
NBRC 7862 - M. cooke	70	..TCT.CCGG	..GTC	..GAA...G	..ACGC.....	..AAAT	CG	.....	.....	.....	.....	142						
NBRC 8191 - T. schoe	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
NBRC 8192 - T. schoe	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CGC	.....	.....	.....	.....	.....	138						
NBRC 9045 - E. flocc	59	..TCT.TCTG	AATGC	..T.A.....	.....	.....	.....	.....	.....	.....	.....	124						
NBRC 9182 - M. canis	81	G.CCT.CCGG	..AGGTTGCG	GGC...AG	GGTGC.T	..G.....	.....	.....	.....	.....	.....	155						
NBRC 31064 - T. viol	71	C.T.C.A.A	..GGT	GA.CA.AC	..G	..GT..CC	.....	.....	.....	.....	.....	139						
NBRC 31610 - T. equi	71	C.T.C.A.A	..GGT	GA.CA.A	..G	..GT..CC	.....	.....	.....	.....	.....	138						
IFO 5948 - M. gypseu	120	CTGGCGG	G	CCGCGCACTC	GT	G	CC	GCGCCTCGAG	GAG	CCGTCC	GGGGA	CAA	TCAAC	175				
IFO 8228 - M. gypseu	120	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	175				
IFO 31978 - A. uncin	133	.....	.....	.....	.....	A	T.GACG	TC	GAGG	CCTT	..ACCCAGCG	..C	TGTGG	190				
JCM 1885 - A. benham	139	.....	.....	.....	.....	..GGAGA	TCGT	..GC	..C.TC	..T	..GGGC	..TT	AG	196				
JCM 1891 - A. vanbre	139	.....	.....	.....	.....	..AGGAGA	..CGT	..GC	..C.TC	..T	TTA	..GTGGC	.....	199				
JCM 1907 - A. obtusu	123	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	138				
NBRC 5466 - T. menta	139	.....	.....	.....	.....	..AGGAGA	..CGT	..GC	..C.TC	..T	TTA	..GTGGC	..A..G	199				
NBRC 5467 - T. rubru	140	.....	.....	.....	.....	..GGAGA	..CGTC	..GC	..G.C	..C.T	..T	..GAGC	CTCGAG	204				
NBRC 5808 - T. rubru	140	.....	.....	.....	.....	..GGAGA	..CGTC	..GC	..G.C	..C.T	..T	..GAGC	CTCGAG	204				
NBRC 5809 - T. menta	139	.....	.....	.....	.....	..AGGAGA	..CGT	..GC	..C.TC	..T	TTA	..GTGGC	.....	199				
NBRC 5812 - T. m. va	139	.....	.....	.....	.....	..AGGAGA	..CGT	..GC	..C.TC	..T	TTA	..GTGGC	..A..G	199				
NBRC 5831 - M. ferru	156	.....	.....	.....	.....	..CTG	TGTGCT	..ACAG	..G	..CGT	..G	..G.GA	..C.T	..A	GGGACT	CTTGTTCCT	226	
NBRC 5928 - T. tonsu	139	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	201	
NBRC 5945 - T. tonsu	139	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	201	
NBRC 5974 - T. menta	139	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	199	
NBRC 5975 - M. persi	120	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	176	
NBRC 6074 - M. audou	156	.....	.....	.....	.....	..CTG	TGTGCT	..ACAG	..G	..CGT	..G	..G.GA	..C.T	..A	GGGACT	CTTGTTCCT	226	
NBRC 6081 - M. ferru	156	.....	.....	.....	.....	..CTG	TGTGCT	..ACAG	..G	..CGT	..G	..G.GA	..C.T	..A	GGGACT	CTTGTTCCT	226	
NBRC 7862 - M. cooke	143	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	209	
NBRC 8191 - T. schoe	139	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	201	
NBRC 8192 - T. schoe	139	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	201	
NBRC 9045 - E. flocc	125	.....	.....	.....	.....	..C.C	TAGGCTGCAG	TGTC	CTG	A	..T	..G	..G.G	..T	..GATGG	GA	GGATGCC	203







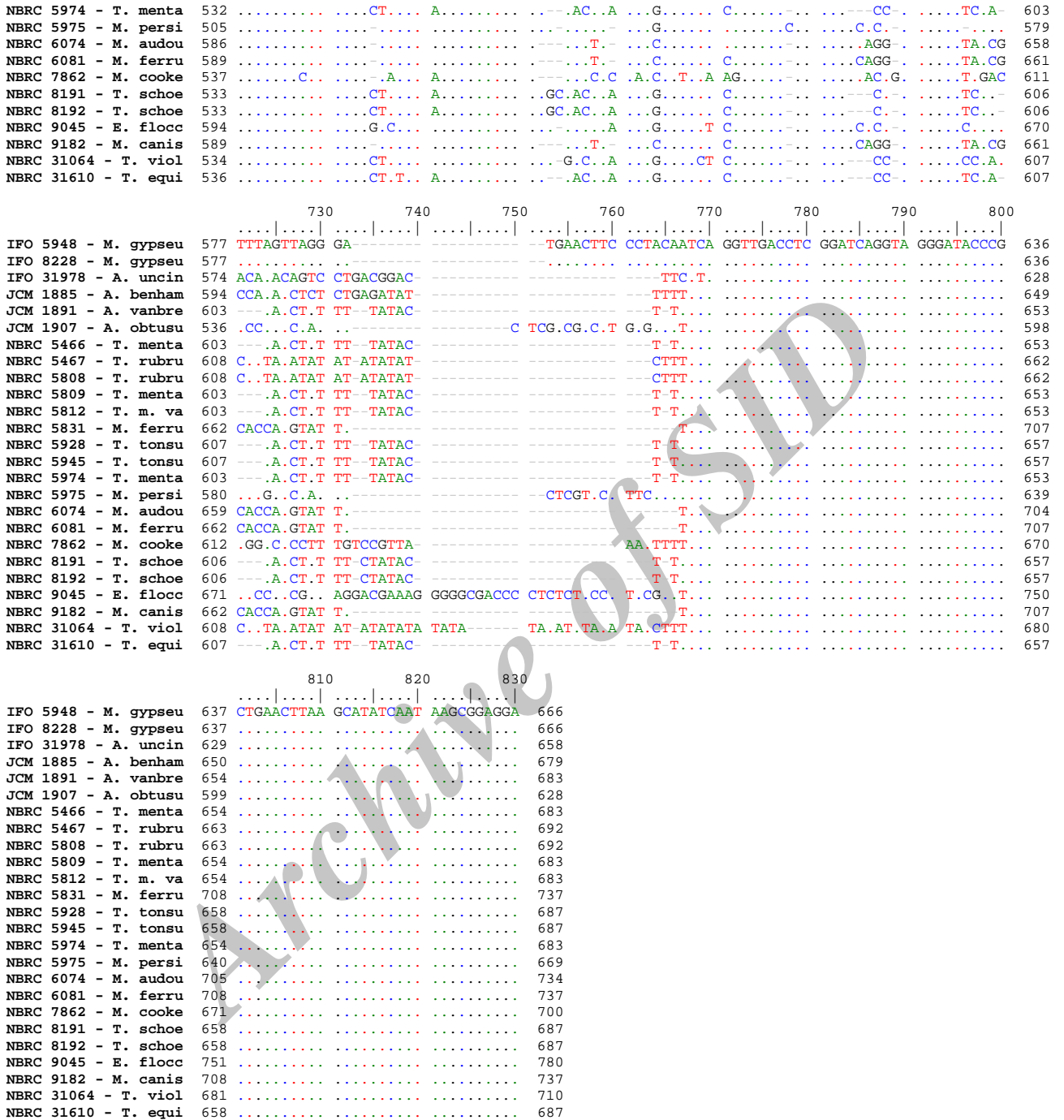


Fig. 2: Pair wise alignment of ITS1-5.8S-ITS2 sequence in reference strains of dermatophytes used in the study

**Table 2:** Results of ITS-RFLP with *MvaI* and Sequencing for identification of reference dermatophyte strains

Strain (upon receipt)	Size of the ITS amplicon (bp) <sup>1</sup>	ITS-RFLP profile after digestion with <i>MvaI</i> (bp) <sup>1</sup>	Species identification by ITS-RFLP profile	Species identification by ITS sequencing
<i>T. mentagrophytes</i> <sup>2</sup> (NBRC 5974, NBRC 5466)	683	247, 159, 124, 89, 50, 14	<i>T. interdigitale</i> <sup>3</sup>	<i>T. interdigitale</i> <sup>3</sup>
<i>T. mentagrophytes</i> var. <i>Interdigitale</i> <sup>2</sup> (NBRC 5812)	683	247, 159, 124, 89, 50, 14	<i>T. interdigitale</i> <sup>3</sup>	<i>T. interdigitale</i> <sup>3</sup>
<i>T. mentagrophytes</i> <sup>2</sup> (NBRC 5809)	683	406, 124, 89, 50, 14	<i>T. interdigitale</i> <sup>3</sup>	<i>T. interdigitale</i> <sup>3</sup>
<i>A. vanbreuseghemii</i> (JCM 1891)	683	406, 124, 89, 50, 14	<i>T. interdigitale</i>	<i>T. interdigitale</i>
<i>A. benhamiae</i> (JCM 1885)	679	360, 158, 142, 20	<i>A. benhamiae</i>	<i>A. benhamiae</i>
<i>T. schoenleinii</i> (NBRC 8192, NBRC 8191)	687	407, 124, 104, 52	<i>T. schoenleinii</i> & <i>T. mentagrophytes</i> ( <i>T. m.</i> var <i>quinckeanum</i> )	<i>T. schoenleinii</i>
<i>T. rubrum</i> (NBRC 5808, NBRC 5467)	692	368, 164, 95, 65	<i>T. rubrum</i>	<i>T. rubrum</i>
<i>T. violaceum</i> (NBRC 31064)	710	368, 164, 114, 45, 20	<i>T. violaceum</i>	<i>T. violaceum</i>
<i>E. floccosum</i> (NBRC 9045)	780	361, 231, 168, 20	<i>E. floccosum</i>	<i>E. floccosum</i>
<i>T. tonsurans</i> (NBRC 5928, NBRC 5945)	686	251, 124, 103, 89, 56, 50, 14	<i>T. tonsurans</i> & <i>T. equinum</i>	<i>T. tonsurans</i>
<i>T. equinum</i> (NBRC 31610)	686	251, 124, 103, 89, 56, 50, 14	<i>T. tonsurans</i> & <i>T. equinum</i>	<i>T. equinum</i>
<i>M. canis</i> (NBRC 9182)	737	441, 165, 103, 28	<i>M. canis</i> & <i>M. ferrugineum</i>	<i>M. canis</i>
<i>M. ferrugineum</i> (NBRC 6081, NBRC 5831)	737	441, 165, 103, 28	<i>M. ferrugineum</i> & <i>M. canis</i>	<i>M. ferrugineum</i>
<i>M. audouinii</i> (NBRC 6074)	734	441, 162, 131	<i>M. audouinii</i>	<i>M. audouinii</i>
<i>A. obtusum</i> (JCM 1907)	628	459, 150, 19	<i>A. obtusum</i> ( <i>M. nanum</i> )	<i>A. obtusum</i> ( <i>M. nanum</i> )
<i>M. persicolor</i> (NBRC 5975)	669	323, 147, 112, 68, 19	<i>M. persicolor</i>	<i>M. persicolor</i>
<i>M. cookei</i> (NBRC 7862)	700	225, 165, 143, 120, 47	<i>M. cookei</i> & <i>M. racemosum</i>	<i>M. cookei</i>
<i>M. gypseum</i> (NBRC 8228, NBRC 5948)	666	289, 179, 146, 33, 19	<i>M. gypseum</i>	<i>A. gypseum</i> ( <i>M. gypseum</i> )
<i>A. uncinatum</i> (NBRC 31978)	658	270, 195, 193	<i>A. uncinatum</i> ( <i>T. ajelloi</i> )	<i>A. uncinatum</i> ( <i>T. ajelloi</i> )

<sup>1</sup> The exact size of the amplicons and restriction fragments was respectively determined after sequencing and *in-silico* RFLP of obtained sequences.

<sup>2</sup> The former name of the species (upon receipt from collection)

<sup>3</sup> The current name for the species

## Discussion

Identification of dermatophytes at the species level is essential because of the therapeutic and epidemiological importance. Identification process for this closely related group of fungi classically is based on phenotypic and physiological criteria (1, 10, 13).

Therefore, due to the high degree of phenotypic similarity between these relative species identification problems are unavoidable. Furthermore, traditional methods are time-consuming, laborious and many isolates reveal unusual characteristics (2, 10).

To overcome these limitations, recently PCR-based appliances relying on genetic makeup have been developed. At present, sequencing of ITS rDNA region is the golden standard for delineation of dermatophyte species (13-18). In this study we presented a virtual and practical PCR-RFLP assay, targeting the ITS-rDNA complex, for identification/differentiation of common pathogenic dermatophyte species. For the first time Jackson et al. (11) introduced a PCR-RFLP assay targeting the ITS regions for identification of 17 dermatophyte species and after that this method was used by some researchers (8, 10, 19). However, our study is the first quest that completely was performed based on sequence analysis and outlined the details of RFLP pattern representative for nearly all pathogenic dermatophytes by both computational and experimental digestion of the ITS regions. Likewise, our PCR-RE findings were compatible with the latest suggested changes in the classification of dermatophytes (13). For instance, based on the ITS sequence phylogeny, recently four new species have been created in the species formerly known as *Trichophyton mentagrophytes* complex (13, 20): the zoophilic *T. mentagrophytes sensu stricto* that previously was known as *T. mentagrophytes* var. *quinckeanum* (its teleomorph is related to *A. simii*), the zoophilic and anthropophilic *T. interdigitale sensu stricto* (related to *A. vanbreuseghemii* teleomorph), the zoophilic *T. erinacei* (related to *A. benhamiae*) and the zoophilic *T. anamorph* of *A. benhamiae*. All of these new species had ITS restriction profiles related to their teleomorphs in *in silico* ITS-RFLP analysis (Table 1). We found two ITS-RFLP profiles by *MvaI*, specific for *T. interdigitale* (*A. vanbreuseghemii*), in both *in silico* and experimental practice (Tables 1 and 2, Fig. 1; lanes 1-5) while, Jackson et al. described only one profile for this species. Although, we did not use any standard strain of *T. mentagrophytes sensu stricto*, however like the study of Jackson et al. the *in silico* restriction profile obtained for this species was the same as *T. schoenleinii*, *T. erinacei* and *T. anamorph* of *A. benhamiae*, both related to *A. benhamiae* teleomorph stage, had similar restriction pattern in virtual and practical restriction analysis (Table 1 and 2). The only type strain of *A. benhamiae* (JCM 1885) pro-

duced expected restriction pattern in RFLP (Table 2 and Fig. 1). As mentioned in the results, *T. interdigitale*, *T. rubrum*, *T. violaceum*, *M. persicolor*, *M. audouinii*, *M. nanum* (*A. obtusum*) and *E. floccosum* were distinctively identifiable in both virtual and actual digestion of the ITS regions with *MvaI* (Table 1 and 2, Fig. 1). The ITS restriction profiles for *T. gourvilii*, *T. soudanense* and *T. yaoundei* were similar to those of *T. violaceum* (Table 1) and this is congruent with the recent conclusion of Graser et al. (21) that reduced *T. gourvilii*, *T. soudanense* and *T. yaoundei* as synonyms for *T. violaceum* based on the ITS sequence, PCR fingerprinting, and AFLP analysis. Comparison with previous study (11) that found no cutting site for *MvaI* in ITS-RFLP of *M. audouinii*, our exploration showed not only this enzyme has cutting site for this *Microsporium* species (Table 1) but also the obtained restriction profile is characteristic (Table 2, Fig. 1; lane 18). Jackson et al. (11) did not included the species of *M. ferrugineum*, *M. nanum*, *M. fulvum*, *M. gallinae*, *T. ajelloi*, *M. racemosum*, *M. cookei* and *T. simii* in their study, while we computationally depicted the exact ITS-RFLP diagram by *MvaI* for the mentioned species (Table 1) as well as the actual profiles at least for *M. ferrugineum*, *M. nanum*, *T. ajelloi* and *M. cookei* in electrophoretic assessment (Table 2, Fig. 1). We observed no pattern differences in the *in silico* RFLP between *T. equinum* / *T. tonsurans*, *M. canis* / *M. ferrugineum* and *M. cookei* / *M. racemosum* species (Table 1). We also observed such a result in experimental ITS-RFLP of *T. equinum* / *T. tonsurans* and *M. canis* / *M. ferrugineum* reference strains (Table 2 and Figure 1). *T. equinum* and *T. tonsurans* are two closely related *Trichophyton* species that differ just in a single base pair on ITS1 region (15, 22, 23) and in the study of Jackson and Mochizuki et al. (11, 19) also both species had the same RFLP pattern, however these species ecologically are different because *T. tonsurans* is an anthropophilic species that isolated only from human infections while *T. equinum* is a zoophilic (horse associated) *Trichophyton* that rarely causes infection in human and almost all infections acquired by direct contact with a horse or its fomites (24). *M. ferrugineum* and *M. canis*, two members of the *A. otae* complex, vary entirely in two

base pair in ITS2 region (13) that is not the cutting site for *MvaI* therefore differentiation among them may need to more restriction analysis. At present study we included a type strain of *M. cookei* but not any of *M. racemosum* and the electrophoretic restriction profile that we attained for the *cookei* species was as the same as *in silico* estimated one (Table 2, Fig. 1; lane 23). *M. cookei* and *M. racemosum* are two geophilic species that have more than 97% similarity in ITS regions sequence (25). Our sequence analysis indicated that two species differed in 6 bp of entire ITS sequence (data not shown) and these differences were not placed in *MvaI* cutting sites. That's why both species had the same restriction profile, however as two species rarely isolated from human infections this similarity is insignificant. All of our practical PCR-RE findings were confirmed by sequencing and comparison of the obtained sequences by the Dermatophytes ITS DNA barcode database. As there are many wrong sequences in GenBank, the reliable identification of dermatophytes cannot be performed by BLAST analysis (22). Contrariwise, there are many reliable ITS sequences from all species of dermatophyte in CBS-database ([www.cbs.knaw.nl/dermatophytes](http://www.cbs.knaw.nl/dermatophytes)) that some belong to the CBS collection strains while the remaining sequences have been selected from GenBank in view of covering the extant biodiversity in this database. We even compared the sequences used in virtual analysis with this database and retrieved the reliable sequences for our study. Alignment of the obtained sequences for standard stains used in this project (Fig. 2) plus the reliable GenBank sequences confirmed this fact that almost all dermatophyte species have different ITS1 and ITS2 sequences, make the ITS region as good targets for post PCR maneuvers such our PCR-RFLP assay.

In conclusion, it should be emphasized even though using of *MvaI* for differentiation of dermatophytes species in a PCR-RFLP system was not new, however the data of such PCR-RE schema in this study had novelty about many species. Despite the similarity of restriction profile obtained for some closely related species, ITS-RFLP by *MvaI* is a powerful tool for both identification and preliminary screening of many

dermatophyte species, especially in large scale epidemiological studies.

## Ethical considerations

Ethical issues (Including plagiarism, Informed Consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc) have been completely observed by the authors.

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