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Original Article

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 wive. 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 successfuly 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

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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 lemgh 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 fromNCBI (http://www.ncbi.nlm.nih.gov/nuccore). The sequences were edited by MEGA 4 software (*MEGA* version 4, Tamura, Dudley, Nei, and Kumar 2007), and sujected to popular sequence alignment tool of ClustalW. The arranged sequences were then exported to DNASIS software (Hitachi DNASIS® 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 28ºC 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 ºC 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 94ºC for 6 minutes, then PCR performed by the following protocol: 35 cycles of 30 seconds at 94ºC, 30 seconds at 58ºC and 1 min at 72ºC; a final extension at 72ºC for 10 min and followed by cooling at 4ºC.

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 37ºC. 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.

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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 consences 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 *Mva*I 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 *Mva*I. 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 *Mva*I 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 *Mva*I (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 Mva*I-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.

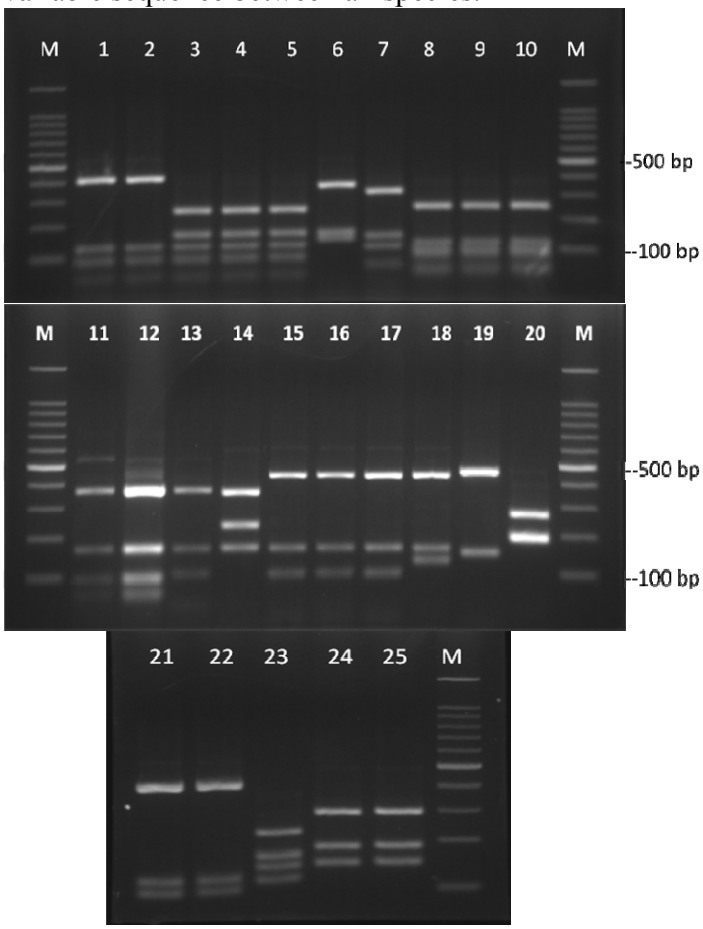
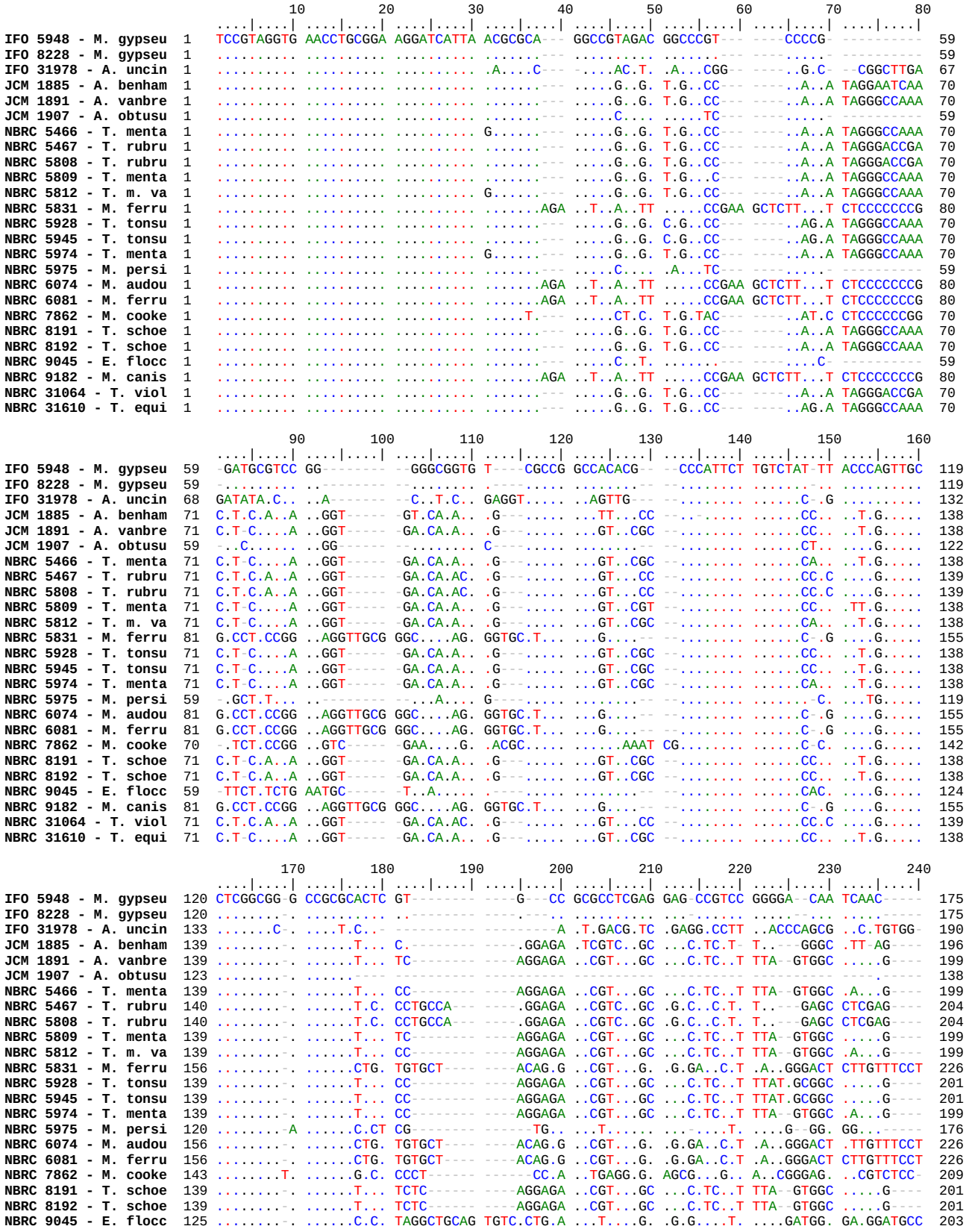


Fig. 1: Electrophoretic patterns of ITS-RFLP with *Mva*I for reference dermatophyte species.
Lanes 1, 3 and 5: *T. mentagrophytes* (NBRC 5809, NBRC 5974 and NBRC 5466), lane 2: *A. vanbreuseghemii* (JCM 1891), lane 4: *T. mentagrophytes* var. *interdigitale* (NBRC 5812), lane 6: *A. benhamiae* (JCM 1885), 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 *Mva*I

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 *Mva*I
A. vanbreuseghemii, T. interdigitale	AF170465, AJ270790	683	247, 159, 124, 89, 50, 14 (1st pattern)
A. vanbreuseghemii (*T. interdigitale*)	AB246678	683	406, 124, 89, 50, 14 (2nd pattern)
A. benhamiae	Z98015	679	360, 158, 141, 20
T. erinacei	Z97997	679	360, 157, 142, 20
T. simii	Z98017	685	372, 159, 90, 50, 14 (1st pattern)
T. simii	AJ000605	685	372, 159, 104, 50 (2nd pattern)
T. mentagrophytes (*T. m.* var *quinckeanum*)	Z97995	683	406, 124, 103, 50
T. schoenleinii	Z98011	685	405, 124, 104, 52
T. verrucosum	Z98003	678	517, 141, 20
M. persicolor	EU181457	670	323, 148, 112, 68, 19
T. rubrum	AF170471	692	368, 164, 95, 65
T. violaceum	AJ270796, EU590656	701, 711	369, 161(**164**), 106(**114**), 45, 20
T. gourvilii	EU181448	710	368, 164, 113, 45, 20
T. yaoundei	AJ270811, FJ479792	700, 710	368, 164, 103 (**113**), 45, 20
T. soudanense	EF631621, AF170473	690	368, 164, 93, 45, 20
T. tonsurans	EF043270	688	251, 124, 103, 90, 56, 50, 14
T. equinum	EF043274	688	251, 124, 103, 90, 56, 50, 14
E. floccosum	AY213646	780	361, 231, 169, 20
M. canis	EU200371	737	441, 165, 103, 28
M. ferrugineum	EF581133	737	441, 165, 103, 28
M. audouinii	AJ000625	734	441, 162, 131
M. nanum	AJ970149	628	459, 150, 19
M. gallinae (*M. vanbreuseghemii*)	AJ000620 (AJ970147)	614 (**617**)	319 (**323**), 150 (**149**), 126, 19
M. fulvum	AM850135	652	322, 147, 112, 52, 19
A. uncinatum (*T. ajelloi*)	EF568086	658	271, 195, 192 (1st pattern)
A. uncinatum (*T. ajelloi*)	AJ000608	657	465, 192 (2nd pattern)
A. gypseum (*M. gypseum*)	EF568061	666	289,179, 146, 33, 19 (1st pattern)
A. incurvatum (*M. gypseum*)	AJ000621	619	389, 147, 64, 19 (2nd pattern)
M. racemosum	AJ970146	701	225, 165, 144, 120, 47
M. cookei	AB193713	701	225, 166, 143, 120, 47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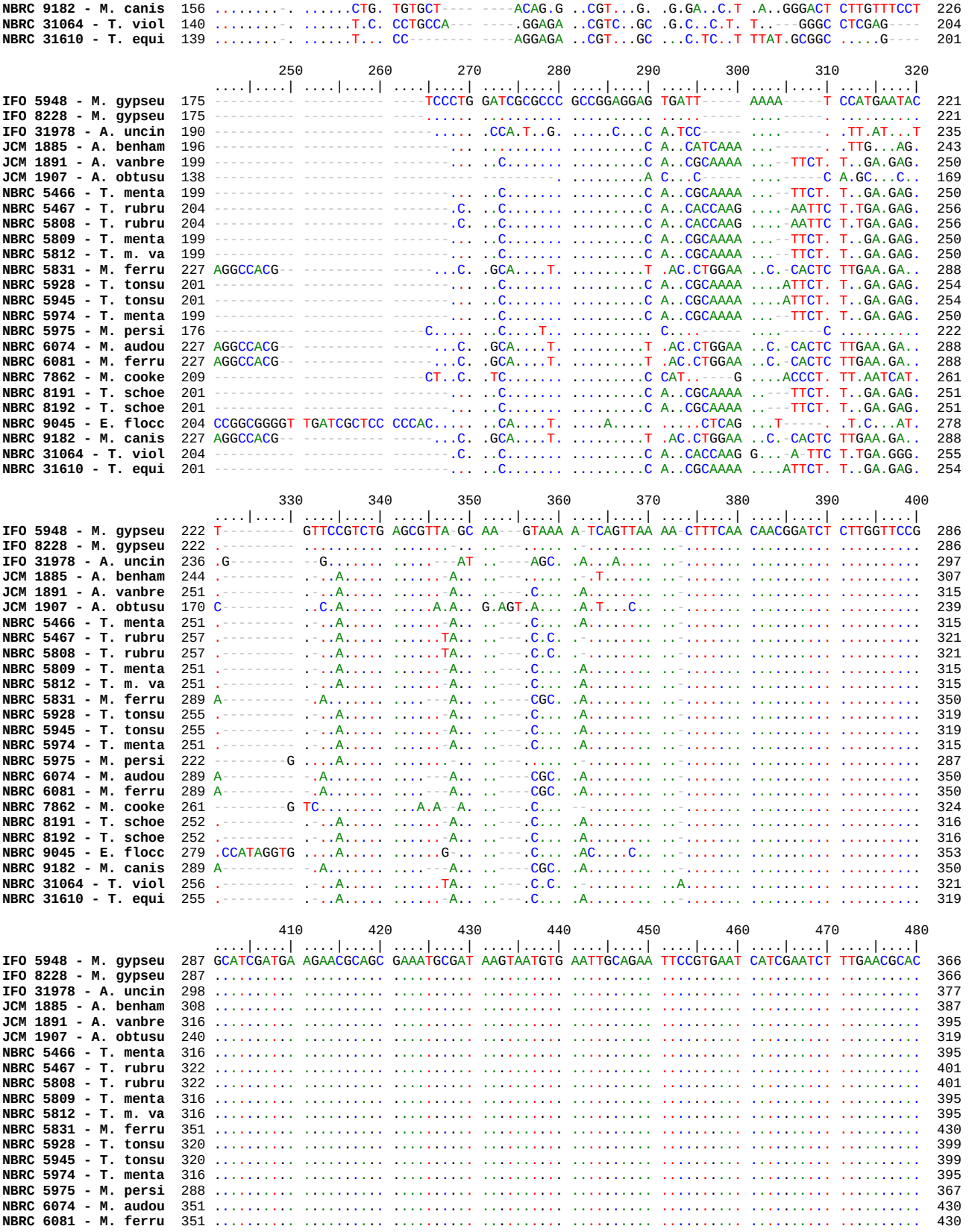
IFO 8228 - M. gypseu
IFO 31978 - A. uncin
JCM 1885 - A. benham
JCM 1891 - A. vanbre
JCM 1907 - A. obtusu
NBRC 5466 - T. menta
NBRC 5467 - T. rubru
NBRC 5808 - T. rubru
NBRC 5809 - T. menta
NBRC 5812 - T. m. va
NBRC 5831 - M. ferru
NBRC 5928 - T. tonsu
NBRC 5945 - T. tonsu
NBRC 5974 - T. menta
NBRC 5975 - M. persi
NBRC 6074 - M. audou
NBRC 6081 - M. ferru
NBRC 7862 - M. cooke
NBRC 8191 - T. schoe
NBRC 8192 - T. schoe
NBRC 9045 - E. flocc
NBRC 9182 - M. canis
NBRC 31064 - T. viol
NBRC 31610 - T. equi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IFO 8228 - M. gypseu 175 221
IFO 31978 - A. uncin 190 235
JCM 1885 - A. benham 196 243
JCM 1891 - A. vanbre 199 250
JCM 1907 - A. obtusu 138 169
NBRC 5466 - T. menta 199 250
NBRC 5467 - T. rubru 204 256
NBRC 5808 - T. rubru 204 256
NBRC 5809 - T. menta 199 250
NBRC 5812 - T. m. va 199 250
NBRC 5831 - M. ferru 227 288
NBRC 5928 - T. tonsu 201 254
NBRC 5945 - T. tonsu 201 254
NBRC 5974 - T. menta 199 250
NBRC 5975 - M. persi 176 222
NBRC 6074 - M. audou 227 288
NBRC 6081 - M. ferru 227 288
NBRC 7862 - M. cooke 209 261
NBRC 8191 - T. schoe 201 251
NBRC 8192 - T. schoe 201 251
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NBRC 9182 - M. canis 227 288
NBRC 31064 - T. viol 204 255
NBRC 31610 - T. equi 201 254
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NBRC 5928 - T. tonsu 255 319
NBRC 5945 - T. tonsu 255 319
NBRC 5974 - T. menta 251 315
NBRC 5975 - M. persi 222 287
NBRC 6074 - M. audou 289 350
NBRC 6081 - M. ferru 289 350
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NBRC 8191 - T. schoe 252 316
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NBRC 9182 - M. canis 289 350
NBRC 31064 - T. viol 256 321
NBRC 31610 - T. equi 255 319
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JCM 1891 - A. vanbre 316 395
JCM 1907 - A. obtusu 240 319
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NBRC 5809 - T. menta 316 395
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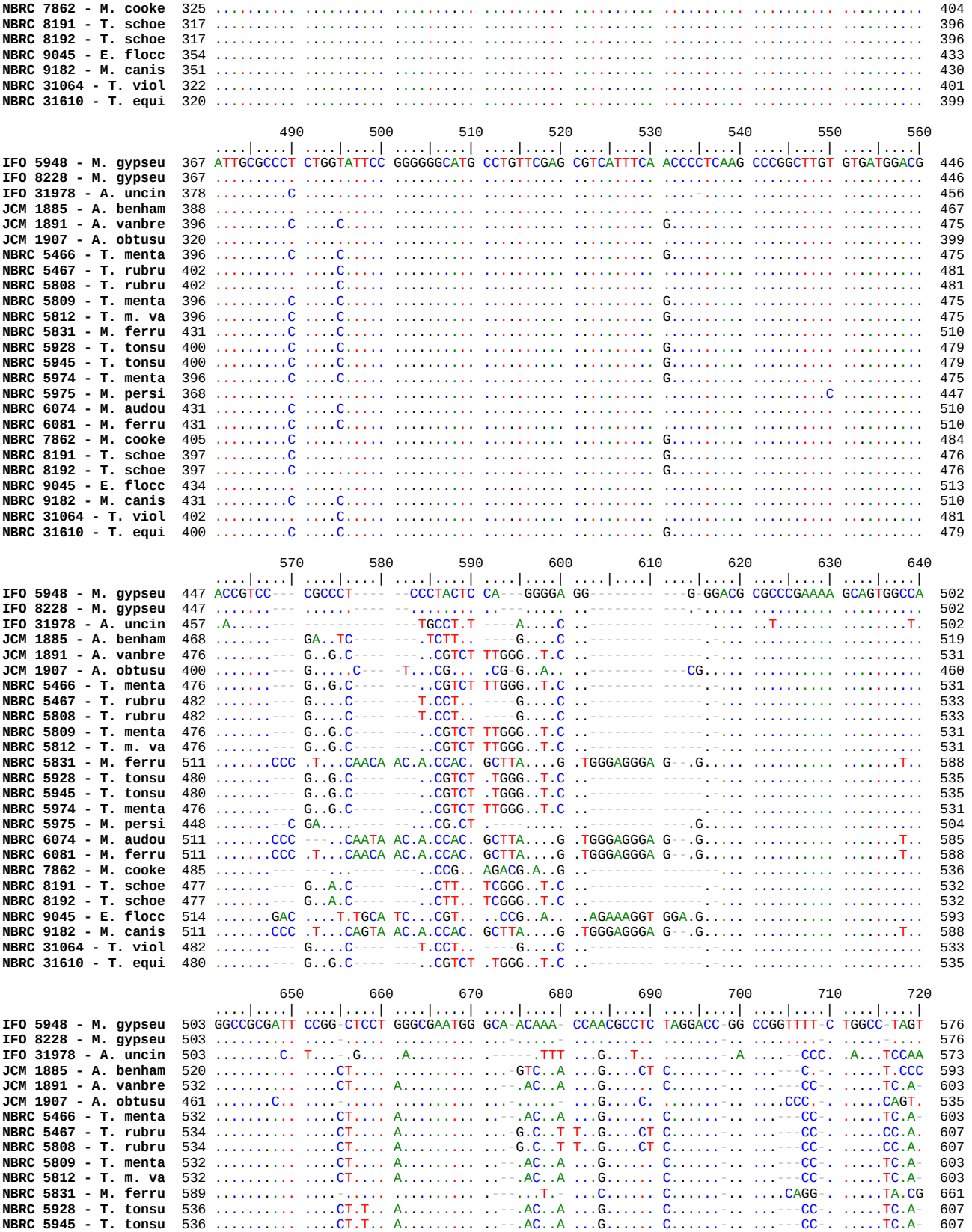
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IFO 8228 - M. gypseu 367 446
IFO 31978 - A. uncin 378 456
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JCM 1891 - A. vanbre 396 475
JCM 1907 - A. obtusu 320 399
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NBRC 5831 - M. ferru 431 510
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NBRC 5945 - T. tonsu 400 479
NBRC 5974 - T. menta 396 475
NBRC 5975 - M. persi 368 447
NBRC 6074 - M. audou 431 510
NBRC 6081 - M. ferru 431 510
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NBRC 8192 - T. schoe 397 476
NBRC 9045 - E. flocc 434 513
NBRC 9182 - M. canis 431 510
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IFO 8228 - M. gypseu 447 502
IFO 31978 - A. uncin 457 TGCCT.T A....C T 502
JCM 1885 - A. benham 468 GA..TC .TCTT.. G....C 519
JCM 1891 - A. vanbre 476 G..G.C ..CGTCT TTGGG..T.C 531
JCM 1907 - A. obtusu 400 G.....C T...CG... .CG G..A.. CG 460
NBRC 5466 - T. menta 476 G..G.C ..CGTCT TTGGG..T.C 531
NBRC 5467 - T. rubru 482 G....C T.CCT.. G....C 533
NBRC 5808 - T. rubru 482 G....C T.CCT.. G....C 533
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NBRC 7862 - M. cooke 485 ..CCG.. AGACG.A..G 536
NBRC 8191 - T. schoe 477 G..A.C ..CTT.. TCGGG..T.C 532
NBRC 8192 - T. schoe 477 G..A.C ..CTT.. TCGGG..T.C 532
NBRC 9045 - E. flocc 514 GAC ....T.TGCA TC...CGT.. ..CCG..A.. ..AGAAAGGT GGA.G 593
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NBRC 31064 - T. viol 482 G....C T.CCT.. G....C 533
NBRC 31610 - T. equi 480 G..G.C ..CGTCT .TGGG..T.C 535
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IFO 8228 - M. gypseu 503 576
IFO 31978 - A. uncin 503 .C. T...G... .A..... .TTT ...G...T.. .....A ....CCC. .A...TCCAA 573
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JCM 1907 - A. obtusu 461 .....C.. ....G....C. ....CCC. . .....CAGT. 535
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NBRC 5467 - T. rubru 534 ....CT.... A..... ...G.C..T T..G....CT C..... ....CC. . .....CC.A. 607
NBRC 5808 - T. rubru 534 ....CT.... A..... ...G.C..T T..G....CT C..... ....CC. . .....CC.A. 607
NBRC 5809 - T. menta 532 ....CT.... A..... ....AC..A ...G..... C..... ....CC. . .....TC.A 603
NBRC 5812 - T. m. va 532 ....CT.... A..... ....AC..A ...G..... C..... ....CC. . .....TC.A 603
NBRC 5831 - M. ferru 589 .....T. ...C..... C..... ....CAGG . .....TA.CG 661
NBRC 5928 - T. tonsu 536 ....CT.T.. A..... ....AC..A ...G..... C..... ....CC. . .....TC.A 607
NBRC 5945 - T. tonsu 536 ....CT.T.. A..... ....AC..A ...G..... C..... ....CC. . .....TC.A 607

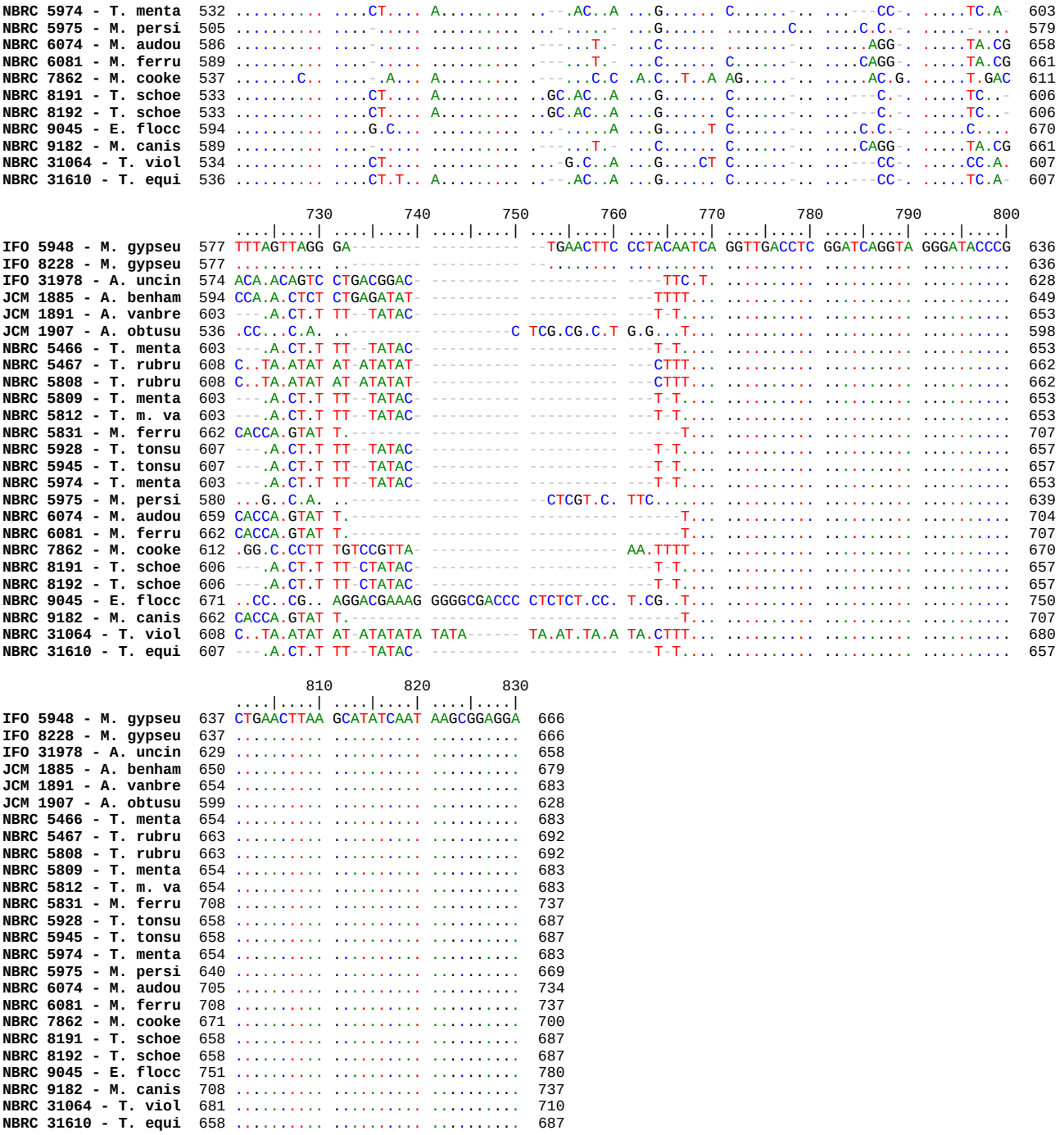

Fig. 2: Pair wise alignment of ITS1-5.8S-ITS2 sequence in reference strains of drmatophytes used in the study

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

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

[1] The exact size of the amplicons and restriction fragments was respectively determined after sequencing and *in-silico* RFLP of obtained sequences.

[2] The former name of the species (upon receipt from collection)

[3] 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 *Mva*I, 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* teleomorphic stage, had similar restriction pattern in virtual and practical restriction analysis (Table 1 and 2). The only type strain of *A. benhamiae* (JCM 1885) produced 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 *Mva*I (Table1 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 *Mva*I in ITS-RFLP of *M. audouinii*, our exploration showed not only this enzyme has cutting site for this *Microsporum* 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 *Mva*I 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* cmplex, vary entirely in two

base pair in ITS2 region (13) that is not the cutting site for *Mva*I 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 *Mva*I 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) 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 *Mva*I 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 *Mva*I 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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